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The Diazo Reaction of Bilirubins and Phycorubins: A Quantitative Study*

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Photosynthesis, Phycobiliproteins, Antenna, Bile Pigments, Biliverdin

A quantitative study of the diazo reaction has been made with several bilirubins including a 2,3-dihydrobilirubin and several phycorubins. The latter have been prepared from the integral phycocyanin of two cyanobacteria (*Mastigocladus laminosus* and *Spirulina platensis*), and from the phycocyanin subunits. These phycorubins have been subjected to the diazo reaction in order to test for the presence of a second covalent chromophore protein bond in the latter.

1) The diazo reaction of unsymmetrically substituted bilirubins, mesobilirubin (**3**) and bilirubin IXα (**13**) in aqueous solution yields four products, two isomeric 9-azo-dipyrromethenones, and two isomeric 9-hydroxymethyl-dipyrromethenones. The maximum total yield is ≥ 97% with diazotized sulfanilic acid, and ≤ 60% with diazotized ethylanthranilate.

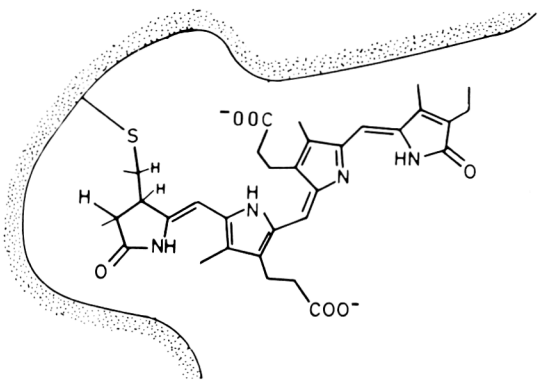
2) The diazo reaction of the 2,3-dihydrobilirubin (**16**) yields likewise four products, two of them containing the 2,3-dihydrogenated ring A/B fragment. The attack is regioselective at C-11 (6:4).

3) The diazo reaction of the phycorubins with ethylanthranilate yields two peptide bound products, containing the ring A/B fragment, and two low molecular weight products. The latter correspond to the C/D fragment and are identical with the respective products derived from mesobilirubin **3**. The attack is preferential at C-9 (≥ 4:1 with ≤ 1 mole reagent added). These results show, that there is no second covalent chromophore peptide bond in the PC investigated at the ring C/D fragment.

Introduction

More than a century after its discovery, Ehrlich's diazoreaction [2, 3] is still the most important analytical method for bilirubins. It is used for the qualitative and quantitative determination of bilirubin in body fluids, in metabolic studies of the different bilirubins, and also for mechanistic studies with oligopyrroles [4–7]. The diazo reaction involves the cleavage of the linear tetrapyrrole skeleton at the central methylene bridge with an aromatic diazonium salt to yield two dipyrrolic azopigments [4–7]. A second class of products are dipyrromethenones [8], formed by addition of a solvent molecule to the intermediate azafulveniumion [9]. Those products are stabilized against further attack by excess diazonium salt at least in some reaction media [8].

Bile pigments without a central methylene bridge are not directly amenable to this very mild and selective degradation method [4, 5], but may be rendered so, if they can be converted first to bilirubin type pigments. Examples for such priming reactions are the reduction of biliverdins to the respective bilirubins [6], or the denaturation – reduction sequence applied to the chromophores of plant biliproteins like phycocyanin (**1**) and phytochrome [10]. These plant biliproteins contain chromophores of the A-dihydrobiliverdin conjugation type, which are covalently bound to the apoprotein via a thioether bond



1: Phycocyanin (native).

* Studies on Plant Bile Pigments part 15. No. 14 (see [1]).

Abbreviations: TLC, thin layer chromatography; EDTA, ethylene diamin tetraacetic acid; PC, C-phycocyanin; Tris, tris-hydroxymethyl-aminoethane.

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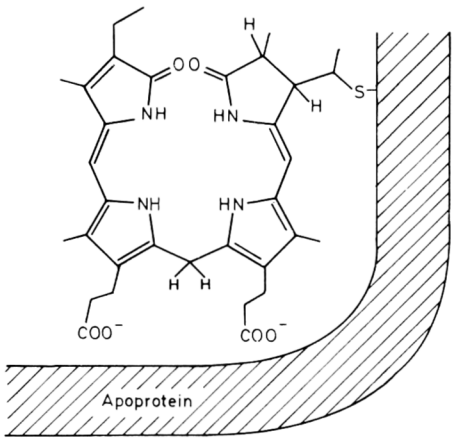
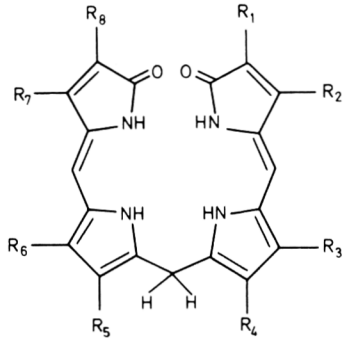
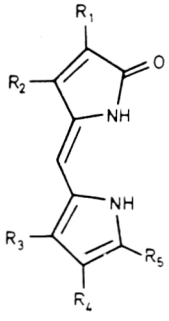


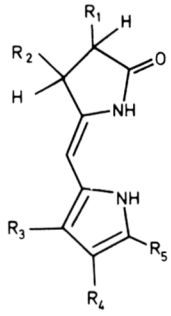
2: Phycorubin (denatured with 8 M urea).



	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8
3	Me	Et	Me	Pr	Pr	Me	Me	Et
10	Et	Et	Et	Et	Et	Et	Et	Et
13	Me	Vi	Me	Pr	Pr	Me	Me	Vi
14	Vi	Me	Me	Pr	Pr	Me	Me	Vi
15	Me	Vi	Me	Pr	Pr	Me	Vi	Me

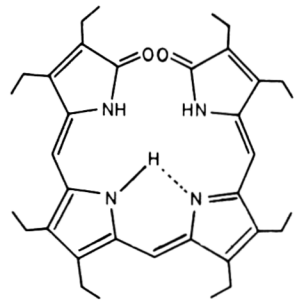


	R_1	R_2	R_3	R_4	R_5
5a	Et	Me	Me	Pr	AEA
5b	Et	Me	Me	Pr	ASA
7	Et	Me	Me	Pr	CH_2OH
11	Et	Et	Et	Et	AEA
12	Et	Et	Et	Et	CH_2OEt
19	Vi	Me	Me	Pr	ASA
20	Me	Vi	Me	Pr	ASA
21	Vi	Me	Me	Pr	CH_2OH
22	Me	Vi	Me	Pr	CH_2OH
23	Me	Et	Me	Pr	ASA
24	Me	Et	Me	Pr	CH_2OH

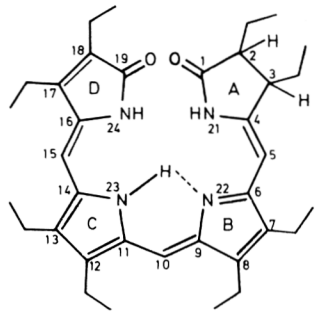


	R_1	R_2	R_3	R_4	R_5
4a	Me	EP	Me	Pr	AEA
4b	Me	EP	Me	Pr	ASA
6	Me	EP	Me	Pr	CH_2OH
25	Et	Et	Et	Et	AEA

EP* : CH(-S-Protein)-CH_3
ASA : Azosulfanilate (**17**)
AEA : Azoethylanthranilate (**18**)
Me : Methyl
Et : Ethyl
Pr : Propionicacid
Vi : Vinyl

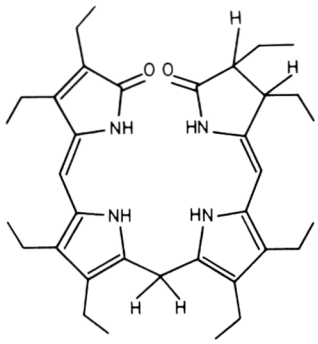


8: Octaethylbiliverdin (OEBV).

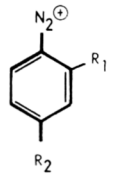


9: Dihydrooctaethylbiliverdin (DHOEBV).

to ring A [11–27]. Additional, but less stable covalent bonds have been implicated by several authors [24]. These include in particular an ester- (?) bond to ring C of the tetrapyrrole, which has been discussed on the basis of chromic acid degradation [11, 26] and proteolytic digestion [14, 15] of several phycobiliproteins. The controversial findings may be related to the fact, that both, the artefactual cleavage and formation of such bonds, is possible under the experimental conditions.



16: Dihydrooctaethylbilirubin (DHOEBR).



17: $R_1 = H$ $R_2 = SO_2^-$

18: $R_1 = COOC_2H_5$ $R_2 = H$

A search for milder and less extensive degradation methods has led us to a reaction sequence, which takes advantage of the diazo reaction. It can principally provide valuable information which is lost during more extensive degradation methods like chromic acid oxidation [11, 26]. The method involves the reduction of the chromophores in the denatured biliproteins, followed by the diazo reaction of the phycorubins thus obtained [28].

Phycorubin (**2**) contains two different π-systems. That of rings C and D is identical to that of mesobilirubin (**3**) and leads to its absorption in the visible spectral range (λ_{max} = 418 nm). That of rings A and B is one double bond shorter and does no longer absorb in the visible (λ_{max} = 320 nm) [10]. The diazo reaction of **2** (using **18**) should accordingly yield two different azopigments (*e.g.* **4a** and **5a**), which has been demonstrated in a previous work [28]. The finding of only one free (= organic solvent soluble) azopigment (**5a**), derived of the rings C and D after reaction of C-phycocyanin (PC) from *Spirulina platensis,* was in agreement with the presence of a stable bond to the apoprotein in the A/B fragment of the chromophore [11–23] and its absence in the C/D fragment.

A quantitative analysis became necessary, however, because PC carries three chromophores at distinct peptide sites, which could at least principally be attached differently. Such an analysis was difficult due to several facts: 1) The absorption band of the two azopigments **4a** and **5a** interfere. This made it difficult to quantify the percentage of **5a** extractable into organic solvent and hence unbound to the protein. 2) It has been assumed generally, that the diazoreaction of bilirubins yields exclusively azopigments in a 1:2 molar ratio [4–7]. The finding of an additional type of product, recently identified as dipyrromethenone [8] in the reaction mixture of phycorubin and other bilirubins [28], also prohibited a quantitative analysis on a basis taking only into account the azopigments. 3) The presence of two different conjugation systems in **2** [10] raises the question of the regioselectivity of the attack of the diazonium ion. An uneven distribution of the products azopigment (**4**) and dipyrromethenone (**7**) arising from attack at C-9, and the products **5** and **6** arising from attack at C-11, had thus to be considered. 4) Finally, the stability of the four products **4**–**7** towards excess diazo reagent was not clear, as well as the effect of an aqueous solvent system, which had to be used with the biliproteins.

These questions were investigated here in a quantitative study with the biliprotein PC, derived from two different cyanobacteria, but also with a series of model pigments, involving fully unsaturated bilirubins as well as a 2,3-dihydro-bilirubin.

Materials and Methods

General methods

Uv-vis absorption spectra were recorded on a model PE 320 (Perkin-Elmer, Germany) spectrophotometer providing digital baseline correction, or on a model DB-GT (Beckman, Germany). Since the compounds described were obtained in mg-amounts only, gravimetric determination of their extinction coefficients seemed unreliable and a spectrophotometric method was prefered. The reactions were followed at selected wavelengths, and ε of the products then determined relative to the known ε of the educts: (ε^{655}) = 15600 for compound **8**, ε^{595} = 17000 for **9** [29], ε^{470} = 60000 for **10**. The 1 to 1 conversion without side or subsequent reactions was ensured by TLC analysis, isosbestic points and by the linear extinction difference diagram [30].

An equimolar product distribution was assumed for the reaction of **10** to **11** and **12** during the initial phase.

Thin layer chromatography (TLC) was done on precoated HPTLC-plates (Merck, Germany) for analytical applications, or on homecoated preparative plates (20 × 20 cm, 0.75 mm silica H, Merck, Germany).

Preparation of bilirubins

Commercially available bilirubin IXα (**13**) (biochemical grade, Merck, FRG, or analytical grade, Fluka, Switzerland) was purified by extraction of the chloroform solution with aqueous $NaHCO_3$ (0.1 M) and subsequent crystallization from chloroform/methanol [6]. Analytical amounts of the bilirubins IIIα (**14**) and XIIIα (**15**) were obtained from "scrambled" **13** [6, 31] after isomer separation by preparative TLC on silica with chloroform/acetic acid = 97:3 as eluent [32]. Mesobilirubin IXα (**3**) was prepared by catalytic (10% Pd on charcoal) hydrogenation of bilirubin IXα [33, 34]. The reaction was followed spectrophotometrically as described previously [10] and the product crystallized from chloroform/methanol.

Octaethylbilirubin (**10**)

Octaethylbiliverdin (**8**) was obtained by coupled oxidation of octaethylhemin by a modification (50 °C reaction temperature) of the method of [29]. It was reduced to **10** with sodium borohydride. A solution of **8** (5 mg = 9 μmol) in methanol (10 ml) was treated at 30 °C under a stream of nitrogen with solid sodium borohydride (45 mg = 1.2 mmol). The reaction was followed spectrophotometrically (300–800 nm). In a typical experiment, 90% of **8** (λ_{max} = 650 nm) were converted within 20 min to the corresponding rubin **10** (λ_{max} = 430/395 nm). The reaction product was partitioned between water (10 ml) and chloroform (20 ml) by mixing the phases with a vigorous stream of nitrogen, and the organic phase washed neutral with water in the same way. After drying over NaCl and evaporation to dryness, the crude product was chromatographed (4 × 2 cm i.d. silica 60, Merck, FRG; eluation with chloroform/acetic acid = 99:1, saturated with nitrogen). The yellow fraction containing the product **10** was washed under nitrogen with a 1% $NaHCO_3$ solution and then with water, dried and evaporated to yield 2,3,7,8,12,13,17,18-octaethyl bilirubin (**10**) (4 mg = 80% of theory). λ_{max} (ε) in methanol: 430 nm (60000), 395 nm (58200); in chloroform: 395, 278 nm. TLC: R_f = 0.4 (silica, chloroform/acetic acid = 99:1), single yellow spot with normal and reverse phase adsorbents and several neutral and acidic solvent. ^{1}H NMR: 10.34, 10.29 (s, NH); 5.93 (s, 5, 15-H); 4.11 (s, 10-H_2); 2.2–2.67 (m, CH_2–CH_3); 0.39–1.33 (m, CH_2–CH_3).

Unreacted **8** adsorbed on the silica can be eluated with acetone and recycled after TLC (silica, carbon tetrachloride/acetone = 95:5).

2,3-dihydro-2,3,7,8,12,13,17,18-octaethylbilirubin (**16**)

16 was obtained similar to **10** starting from the dihydrobiliverdin **9** (5 mg = 9 μmol [29]). The reaction required, however, an elevated temperature (70 °C) and a larger excess of the reductant (75 mg = 2 mmol $NaBH_4$). Yield: 1.4 mg = 28% of theory. λ_{max} (ε) in methanol: 400 (30300), 425 (sh), 275 nm; in chloroform: 392, 300 nm. TLC: R_f = 0.45 (silica, chloroform/acetic acid = 99:1); single yellow spot with different TLC systems. ^{1}H NMR: 10.10 (s, NH); 6.0 (s, 15-H); 5.2 (s, 5-H); 3.99 (s, 10-H_2); 2.2–2.6 (m, CH_2CH_3); 0.4–1.3 (m, CH_2 CH_3).

Isolation of phycocyanin and preparation of phycorubin (**2**)

C-PC from *Spirulina platensis* (frozen algae from pond culture) was isolated by the method described previously [35], except that the gel filtration step was substituted by chromatography over a brushite column, to remove residual APC. Subunits of PC were prepared using the method of [36], renatured on a desalting column and concentrated 5-fold in a dialysis bag covered with aquazide I-A (Calbiochem-Behring, La Jolla, USA).

Mastigocladus laminosus was grown at 51 °C under fluorescent light in the medium of Castenholz [37]. C-PC was isolated and purified by DEAE-cellulose chromatography as described [35].

Phycorubin solutions were prepared from phycocyanins, denatured with 8 M urea, and reduced with sodium borohydride as described previously [10, 28].

Diazonium salt solutions

Diazotized sulfanilic acid (**17**) and diazotized ethyl anthranilate (**18**), 20 mM in water, were prepared by

standard procedures [38] from the respective aromatic amines.

Diazo reaction of bilirubins and isolation of products

Reaction of bilirubin IXα (**13**) with diazotized sulfanilic acid (**17**) in aqueous 8 M urea buffer

13 (10.4 mg = 17.8 μmol) was dissolved in thoroughly nitrogen saturated aqueous NaOH (10 ml, 100 mM), containing Na_4EDTA (5 mM) and added immediately to sodium phosphate buffer (390 ml, 50 mM, pH 7.5), containing Na_4 EDTA (5 mM) and urea (8 M). Diazotized sulfanilic acid reagent (1 ml, 20 mM = 20 μmol) was added without delay under a vigorous stream of nitrogen, which was maintained for the reaction time (15 min). The reaction mixture was extracted 5 times with 200 ml portions of *i*-butanol. The contents of the extracts were determined spectrophotometrically from the known extinction coefficients of the azopigments **19** and **20**, *e.g.* 29800 at 521 nm [39], and of the 9-hydroxymethyl-pyrromethenones **21** and **22**, *e.g.* 27800 at 415 nm [8], with the proper corrections for the absorption of the azopigments at 415 nm. A total of 19.8 μmol (56% of theory) of the azopigments **19** and **20** was contained in the aqueous phase (13.9 μmol) and the organic phase (5.94 μmol). The latter phase contained quantitatively the hydroxymethyl-pyrromethenones **21** and **22** (15.3 μmol = 43% of theory)*. The *i*-butanol was evaporated and the residue extracted on a Buchner funnel with methanol until colorless. The orange filtrate was chromatographed (aluminia, neutral, activity "super I", Woelm, FRG; 6 × 2 cm). The column was first developed with methanol (500 ml) to remove the urea, then with water to remove the yellow mixture of **21**/**22**, whereas the red material (**19**/**20**) remained on the column. The mixture was evaporated to dryness (silylated glassware, to prevent strong adsorbtion on the walls) and rechromatographed under the same conditions to yield the hydroxymethyl-pyrromethenones **21** and **22** (5.76 μmol = 16.2% of theory). The material was finally precipitated from a concentrated and filtered methanolic solution (3 ml) with chloroform (3 ml) and a few drops of *n*-hexane, collected by centrifugation and dried over paraffin in high vacuum (yield: 0.7 mg). λ_{max} in water = 419, 266 nm. TLC: Two yellow spots of equal intensity (R_f = 0.35 and 0.41) on silica, chloroform/acetic acid = 85:15. The R_f of the starting material **13** under these conditions is 0.94.

Isomer identification of the 9-hydroxymethyl-pyrromethenones

The diazo reactions of the bilirubins IIIα (**14**) and XIIIα (**15**) were carried out as described above, but on a smaller scale. **14** (0.48 mg) yielded a single 9-hydroxymethyl-pyrromethenone (**21**), which co-chromatographs (TLC on silica, chloroform/acetic acid = 85:15, R_f = 0.41) with the faster migrating product derived from **13**. The R_f of **14** in this system is 0.94. λ_{max} in water: 421, 270 nm; in methanol: 424, 269 nm. **15** yielded also a single 9-hydroxymethyl-pyrromethenone (**22**), which co-chromatographs under the same conditions with the slower migrating product derived from **13** (R_f = 0.35, *vs.* 0.94 for **15**). λ_{max} in water: 410, 274 nm, in methanol: 416, 272 nm.

Reaction of mesobilirubin IXα (**3**) with diazotized sulfanilic acid (**17**)

3 (10.5 mg = 17.9 μmol) was reacted under identical conditions as **13**. The yields after partition between water and *i*-butanol were 20.2 μmol of the azopigments **23** and **5b** (based on the extinction coefficient of the vinyl-substituted azopigments (**19**/**20**) and 14.4 μmol of the 9-hydroxymethyl-pyrromethenones **24** and **7** (based on ε = 29500 for pyrromethenones with saturated side chains [7]. This corresponds to a total yield of 97% of the dipyrrolic degradation products. The 9-hydroxymethyl-pyrromethenones were purified twice on aluminia to yield 7.36 μmol of the mixture **24**/**7**. TLC: $R_f \approx 0.3$, two barely separated yellow spots (silica, chloroform/acetic acid = 85:15); R_f = 0.56 and 0.61 (same solvent, 75:25). λ_{max} in water: 404, 269, 230 nm; in sodium phosphate buffer (50 mM; pH 7.5) containing urea (8 M) and Na_4EDTA (5 mM): 409, 266 nm.

* ε of **19**/**20** was reported in chloroform/ethanol/hydrochloric acid (6 M) = 3:6:1 [39]. In this solvent mixture, the λ_{max} is shifted to 530 nm, indicating partial protonation, with ε however very similar to that at neutral pH ([40] cited in [5]). For **21**/**22**, the extinction coefficients of the respective 9-alkoxy-pyrromethenones [8], bearing the same conjugation system were used. The ε values determined for the yellow products **21** and **22** obtained in aqueous urea solution were too low by a factor of three for compounds with a pyrromethenone structure [7], very likely due to insufficient removal of urea.

Reaction of mesobilirubin IXα (**3**) with diazotized ethylanthranilate (**18**)

The reaction was carried out as described above with the reagent **17**. For the quantitative evaluation, the same extinction coefficients have been applied as for the sulfanilic acid azopigments [39].

Reaction of octaethylbilirubin (**10**) with diazotized ethylanthranilate (**18**)

The diazo reagent **18** (9 μmol) was added to **10** (1 mg = 1.8 μmol), dissolved in ethanol (10 ml). After 10 min, the reaction mixture was partitioned between water and chloroform. The organic extract was washed with water, dried over NaCl and evaporated to dryness. Preparative TLC (silica, carbon tetrachloride/acetone = 95:5) yielded a faster moving red and a yellow zone. The faster migrating zone yielded the tetraethylated azopigment **11** (1.62 μmol = 45% of theory). λ_{max} (ε) in chloroform: 518 (30000), 322, 280 nm. TLC: R_f = 0.7 (silica, carbon tetrachloride/acetone = 95:5); R_f = 0.75 (silica, benzene/ethyl acetate = 90:10). ^{1}H NMR: 13.35, 10.41 (N-H), 6.83–8.11 (phenyl-H), 5.86 (5-H), 4.58 ($OC\underline{H}_2$–CH_3), 2.27–2.78 ($C\underline{H}_2$–CH_3), 0.78–1.67 ppm ($CH_2C\underline{H}_3$, OCH_2–$C\underline{H}_3$).

Reaction of dihydrooctaethylbilirubin (**16**) with diazotized ethylanthranilate (**18**)

16 yielded under identical conditions as described for **10** and separation in the same solvent system three colored products (red, R_f = 0.7; orange, R_f = 0.6; yellow, R_f = 0.2; additional yellowish-brown and blue by-products were occasionally observed). The red azopigment obtained in 4.5% yield and the yellow pigment were identical to the red (**11**) and yellow pigments (**12**), respectively, derived from **10** according to co-chromatography in different TLC systems. The orange azopigment (**25**) was obtained in 3% yield. TLC: R_f = 0.6 (silica, carbon tetrachloride/acetone = 95:5); R_f = 0.2 (C-8 reverse phase bonded silica, methanol). λ_{max} (ε) in chloroform: 480, 325, 280 nm. ^{1}H NMR: 6.9–8.1 (phenyl-H), 6.42 (5-H), 4.2–4.6 ($OC\underline{H}_2$–CH_3), 2–2.6 (C–$C\underline{H}_2$–CH_3), 1.72–1.76 (CH–C–$\underline{H}_2$–CH_3), 0.69–1.1 (CH_3).

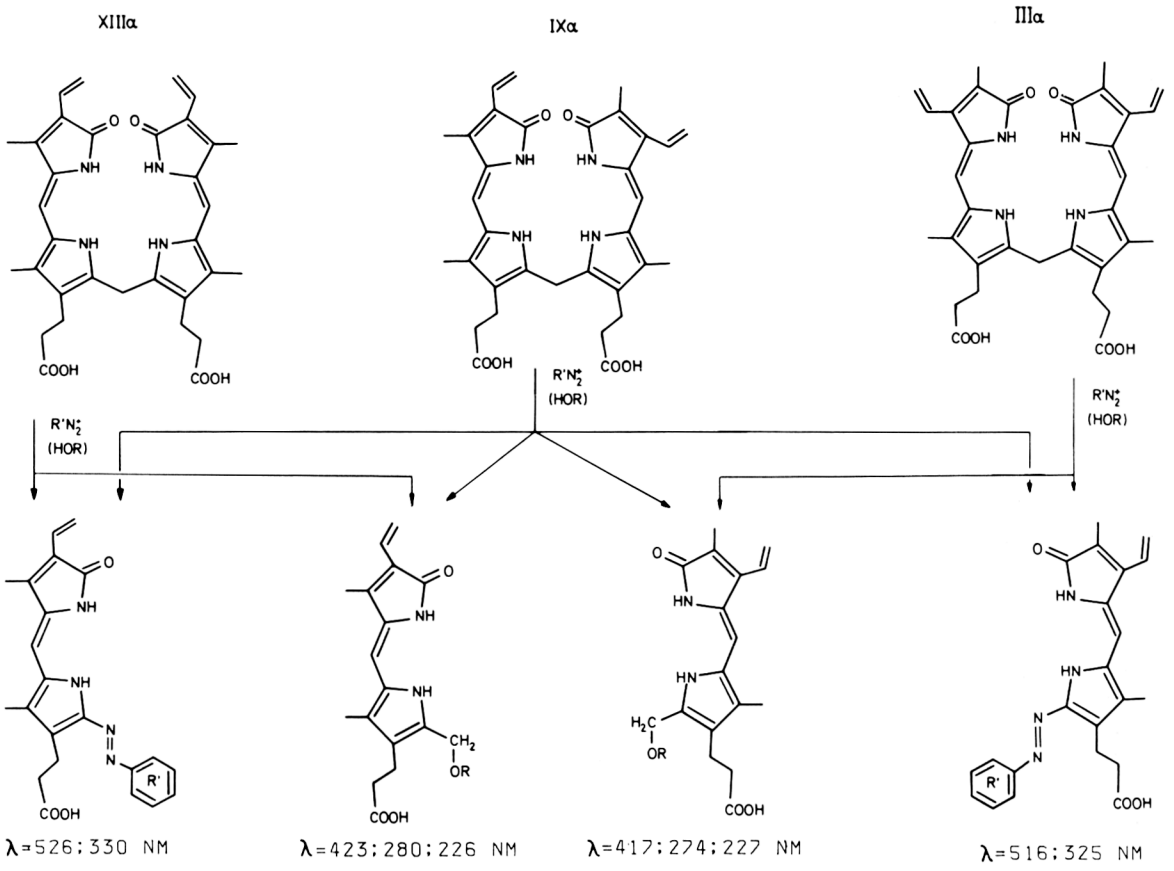
Reaction of phycorubin (**2**) with the diazoreagents (**17**) and (**18**)

2 ml of a solution of **2** in 50 mM potassium phosphate buffer, ph = 7.5, containing 5 mM Na_4EDTA and 8 M urea, were treated with various concentrations of **17** or **18**. The reaction was followed spectrophotometrically in the absorption range 700–300 nm. After the end of the reactions, usually 5–10 min, the reaction mixtures were extracted with organic solvents. Standard procedure: Half a volume *i*-butanol was added to the buffered solution. The phases were mixed by vortexing for 1 min and then separated by centrifugation. The extraction of the aqueous phase was repeated twice. The contents of the aqueous and of the combined organic phases were determined spectrophotometrically. Alternatively, the solutions were lyophilized after the diazoreaction. The lyophilisate was resuspended in 3 ml methanol and centrifuged for 3 min in a laboratory centrifuge. The pellet was extracted twice with 2 ml methanol. The first pellet was dissolved in 2 ml phosphate buffer, containing 6 M urea. The aqueous phase and the combined methanolic extracts were again analyzed spectrophotometrically.

Results

*Diazoreaction of bilirubins (**3**, **13–15**) (see Scheme 1)*

Addition of a 1.2 molar excess of diazotized sulfanilic acid (**17**) to bilirubin IXα (**13**), dissolved in phosphate buffer containing 8 M urea, resulted in an immediate color change from yellow to red. The bilirubin absorption band (445 nm) was replaced by new absorptions at 520, 430, 327 and 280 nm (Fig. 1). Partition between water and *i*-butanol yielded two pigment fractions: The red azopigments **19** and **20** with their characteristic absorptions at 521, 321 and 280 nm [41], which remained to the most part in the aqueous phase, and the yellow 9-hydroxy-methyl-pyrromethenones (λ_{max} = 419, 266 nm, Fig. 2), which were quantitatively extracted into the organic phase. Analytical TLC of this phase revealed two yellow spots of equal intensity, which were much more polar than the starting material. The isomer identification was achieved by comparison with the similarily prepared yellow fractions from the symmetric bilirubins **14** and **15** (see Scheme). Both gave only one spot each on TLC, which were identical in



Scheme 1. Diazoreaction of the bilirubins (**13**–**15**).

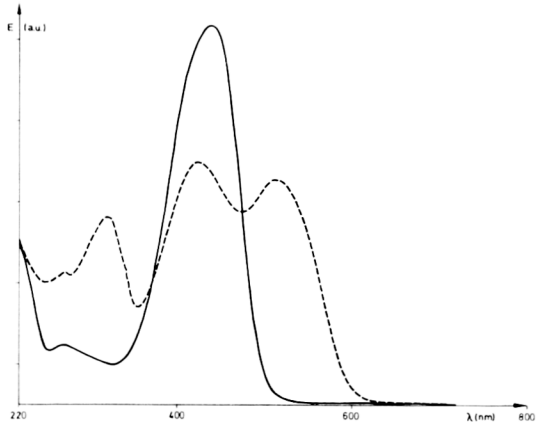


Fig. 1. Reaction of bilirubin IXα (**13**) with diazotized sulfanilic acid (**17**) in phosphate buffer (50 mM, pH 7.5), containing 8 M urea. Uv-vis spectra of the reaction mixture before (———) and 15 min after (– – –) the addition of the diazonium salt solution in a 1.2:1 molar ratio.

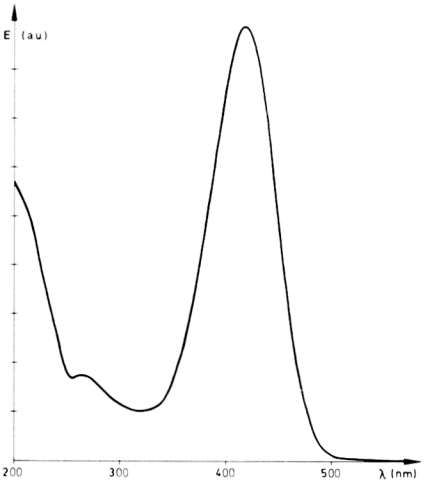


Fig. 2. Uv-vis spectrum of the 9-hydroxymethyl-dipyrromethenones **21** and **22** in water.

cochromatography with the faster and slower migrating spots from **13**, respectively, which were thus assigned to the pyrromethenones **21** and **22**. The reaction of mesobilirubin IXα (**3**) followed the same principles, but the two yellow fractions could only be partially separated by TLC.

The experiments demonstrated for compounds **3** and **13** a strong dependence of the yield and product distribution of the diazoreactions on the amount of diazonium salt **17** or **18** added. **13** reacted with a 1.2 molar excess of **17** quantitatively to the azopigments (1.11 mol per mol **13**) and pyrromethenones (0.86 mol per mol **13**; combined yield of 99% of theory). Similar yields are obtained with the mesopigment (**3**). If less diazonium reagent is used, the bilirubin reacts only incompletely, which renders the spectrophotometric analysis impossible due to the similar spectra of bilirubins and the oxypyrromethenones. The combined yield of the products decreased steadily with increasing amounts of reagent added, *e.g.* to 0.88 mol per mole bilirubin (44% of theory) with **17**:**13** = 7. Thus, the formation of 2 mol of azopigment per mol bilirubin was never observed. The decrease in yield is likely to be due to the reaction of the azopigments **19** and **20** with excess diazoreagent, because the spectrum of the reaction mixture obtained after addition of only 1.2 mol **17** remained stable upon standing. The yield of the azopigments **19** and **20** was also temperature dependent. With a seven-fold excess of diazonium reagent, 0.88 mol/mol of **13**, were obtained at ambient temperature after 3 min reaction, 1.62 mol/mol at 0 °C: the yield decreased with increasing reaction time (30 min) to 0.65 and 1.43 mol/mol, respectively. The yields were finally also dependent on the diazonium salt used. They were generally determined to be lower by about 30% when **18** was used instead of **17** in the reaction of **3**, which could in part be due to the less clear-cut separation of the products by solvent partition. (All yields have been determined by assuming the same extinction coefficients, irrespective of the solvent and the type of diazonium salt used.)

*Diazo degradation of the biliverdins **8** and **9** (see Scheme 2)*

Reduction to the bilirubins **10** and **16**

The rubins **10** and **16** have been prepared by reduction of the corresponding biliverdins **8** and **9**, respectively, with sodium borohydride under nitrogen atmosphere. Isosbestic points, linear absorption difference diagrams [30] and TLC analysis of the product mixture proved a 1:1 conversion of the fully unsaturated biliverdin **8** to the rubin **10** up to about 75% completion of the reaction. Later on, the produced rubin reacts further with excess reagent to products without absorption maxima above 280 nm. Optimum reaction conditions are a 100-fold excess of the reagent at 30 °C.

The A-dihydrobilindion **9** reacts in the beginning also quantitatively to the dihydrorubin **16**, but the reaction is more sluggish and the product is more prone to further reactions. Optimum conditions are a 200-fold excess of the reagent at 70 °C. The low yield (30%) is compensated by a quantitative recovery of unreacted **9**.

The structures **10** and **16** are established by the dissapearence of the 10-methine signal and the appearance of a singlet (2H = 10-H_2) around 4 ppm in the ^{1}H NMR spectrum. The uv-vis extinction coefficients of 60.0 and 30.3 × 10^3 for the long-wavelength bands of **10** and **16** are in agreement with the presence of two and one dipyrromethenone chromophores, respectively [10]. **16** has a second absorption band at 275 nm, which is assigned to the A-dihydrodipyrromethenone moiety. It is noteworthy, that the absorption bands of both rubins, **10** and **16**, are structured (see *e.g.* Fig. 3) and that the structure is solvent dependent. This effect has been investigated in some detail for bilirubins [42], and was correlated

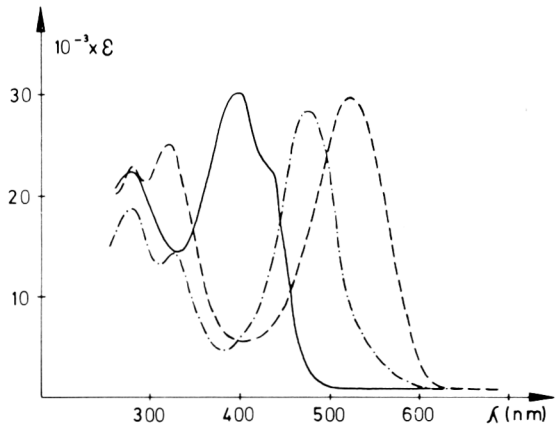
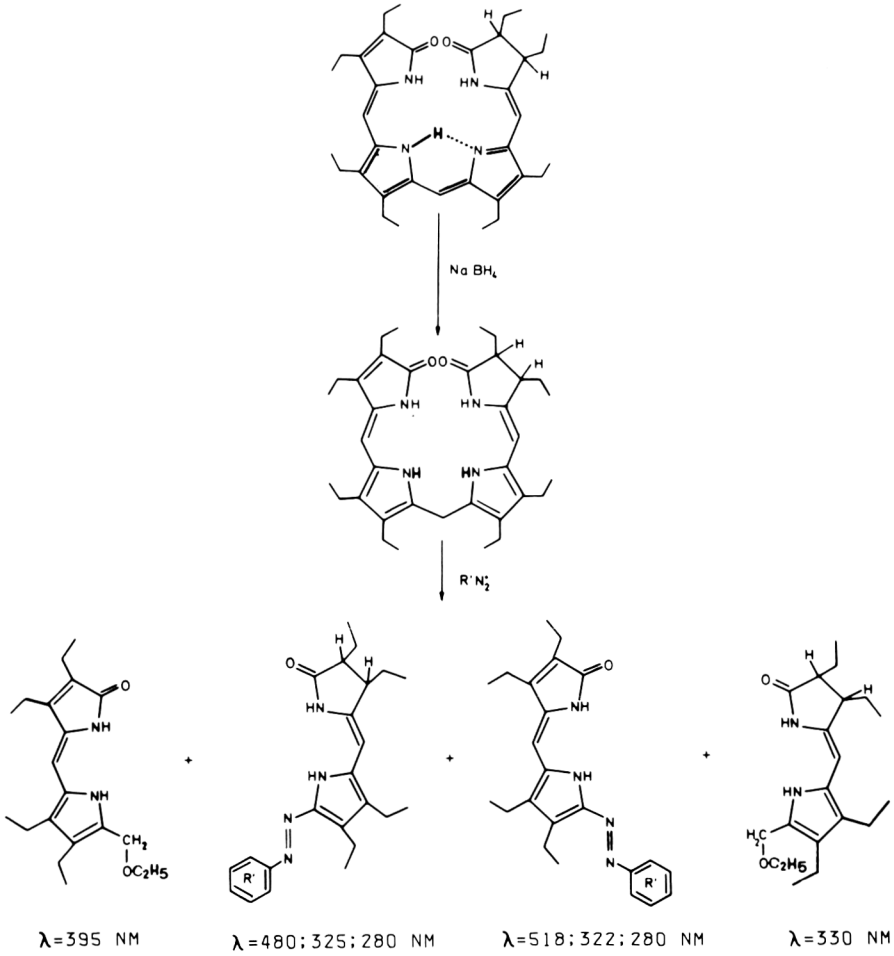


Fig. 3. Reaction of the 2,3-dihydrobilirubin **16** with diazotized ethyl anthranilate (**18**) in ethanol. Spectra of the educt (———), of the 2,3-dihydro azopigment **25** (–.–.–) and of the fully unsaturated azopigment **11** (– – – – –).



Scheme 2. Reduction of 2,3-dihydrooctaethylbiliverdin (**8**) and diazoreaction of its rubin (**16**) to azopigments (**11** and **25**) and pyrromethenones (**12** and **26**).

to the presence of more than one species in solution. The similar split in **16** bearing only one chromophore, excludes an intramolecular interaction of the two halves as the origin for this effect.

Diazoreaction of rubins **10** and **16**

All reactions were carried out in ethanol or methanol with diazotized ethylanthranilate (**18**), yielding products, which can be purified readily by chromatography on silica. The rubin **10** reacts smoothly to yield the red azopigment **11**. With only a 2-fold molar excess of the reagent over the rubin, 0.8 mol azopigment per mol **10** (40% of the theoretical 2 mol of dipyrrolic products) were isolated after a reaction time of only 30 sec. The yield increased only slightly with larger excess of reagent and prolonged reaction time (*e.g.* 0.9 mol of **11** per mol **10** with a 5-fold excess of reagent and 10 min reaction time) and was not significantly affected by reaction temperatures in the range of 4–30 °C. With a 15–20-fold excess, however, after 30 min a bleaching of the solution occurs. The yield of **11** never exceeded 1 mol per mol of the educt (**10**). Obviously, the electrophilic attack at either C-9 or C-11 leads to the cleavage at the respective position, but the remaining "second half" still bearing the C-10 is no subject to further attack by the diazonium salt. The 9-ethoxymethyl-dipyrromethenone **12** has accordingly been isolated from the reaction mixture of **10** as the second product.

The diazoreaction of the A-dihydrorubin (**16**) proceeds under the same conditions to produce two azopigments. The first, red one was identified as **11** and is then derived from the dipyrromethenone half of **16** (attack at C-11). The second, orange pigment is identified as the 9-azo-2,3-dihydrodipyrromethenone

25 by ^{1}H NMR and its blue shifted absorption maximum. It is then derived by electrophilic attack of **18** at C-9. The attack of the diazonium salt is preferential at C-11, and a regioselectivity of 3:2 is estimated by assuming identical extinction coefficients for the two azopigments **11** and **25**.

*Diazo degradation of phycocyanins (**1**) (see Scheme 3)*

Reduction to phycorubins (**2**)

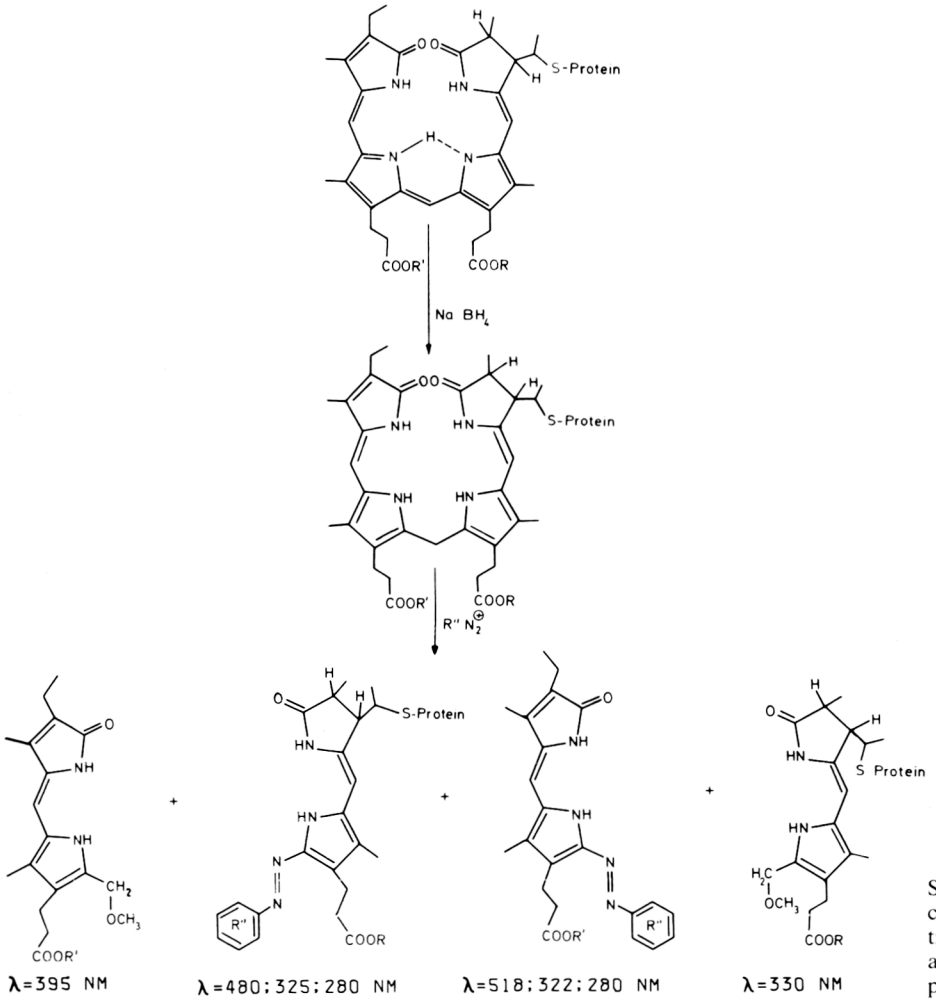
The phycorubins have been prepared by sodium borohydride reduction of the urea denatured phycocyanins as reported previously [10, 28]. Phycorubin should be used without delay for the subsequent diazoreaction, because it does not only react further with excess sodium borohydride, but is also unstable after its removal. Excess $NaBH_4$ interferes with the subsequent diazo reaction. It is removed on a desalting column in the presence of urea, to keep the polypeptide chains in the denatured state.

*Diazo reaction of phycorubins (**2**)*

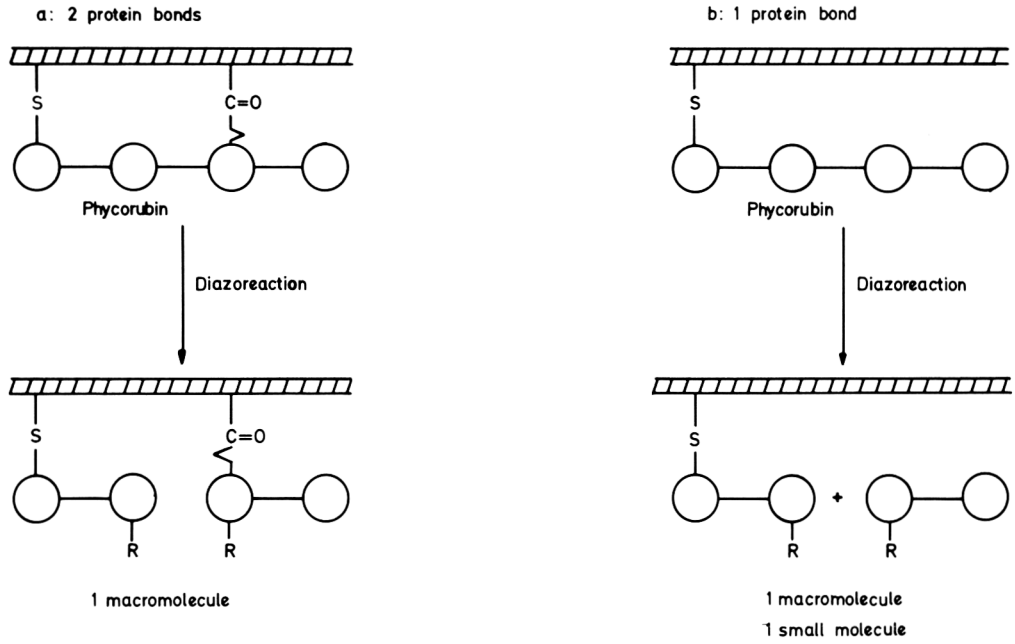
Qualitative results

The denatured phycorubins were treated without delay with the diazo reagents* in the presence of urea (8 M). The reaction can be followed spec-

* Denatured PC is unstable, too, against diazonium salts. The spectrum of the product mixture shows broad bands at around 320, 400, 480 and 500 nm, which are on top of a featureless absorption in the blue and green spectral region. The general appearance is similar to the product mixture, obtained by oxidation of denatured PC. Solvent extraction proved ineffective to separate any products, which are thus believed to be still tetrapyrroles.



Scheme 3. Reduction of phycocyanin (**1**) and diazoreaction of phycorubin (**2**) to azopigments (**5a** and **4**) and pyrromethenones (**6** and **7**).



Scheme 4. Model of the diazo cleavage of phycorubin, taking into account one (b) or two (a) protein bonds.

trophotometrically (decrease at 416 nm, increase at 400 nm due to the pyrromethenone derived from rings C/D, and at 480–520 nm due to the azopigments**). It is generally complete 20 min after addition of the diazo reagent, and the yield is rather insensitive to temperature in the range from 0 to 25 °C. The overall product yield (determined spectrophotometrically) is again better with diazotized sulfanilic acid (**17**, $\leq$ 70%), than with diazotized ethylanthranilate (**18**, $\leq$ 50%), if the same molar ratios of the reagents were used. The latter was nonetheless used in all of the following experiments, to allow a clear-cut separation of low molecular weight products from the peptide-bond high molecular weight ones by solvent extraction (according to Scheme 4).

The degradation has been optimized with PC from *Spirulina platensis*. Two different work-up methods have been tested. The first consisted of a thorough extraction of the reaction mixture with 2-butanol, which removes all yellow pyrromethenone **7** (λ_{max} = 410 nm) and red azopigment **5a** (λ_{max} = 520 nm) from the aqueous phase and leaves back the orange azopigment **4a** (λ_{max} = 480 nm) and the colourless pyrromethenone **6**. The complete separation of the spectrally overlapping azopigments during extraction has been ensured by second derivative spectroscopy* (Fig. 4). The foregoing results prove qualitatively the absence of a second covalent bond to the protein in the C/D ring moiety. The stability of **7** and **5a** was unsatisfactory, however, with this extraction method. All products of the diazo reaction are unstable (see below), and 2-butanol seemed to enhanced in particular the degradation of the extractable products derived from rings C and D. The alternative work-up was to extract the lyophilized reaction mixture with methanol. It is less sensitive to an excess of the diazo reagent, and gave excellent recoveries of **7** ($\approx$ 85%) and of the azopigments **4a** and **5a** ($\approx$ 95%). The drawback is the solubilization of some protein bound **4a** into the organic phase during repeated extraction, due to the large amount of urea present. The absence of the latter has been used as a criterion for a good separation.

The product yield was much better than with the dihydrorubin **16** and reached almost that observed with free bilirubins (**13**–**15**), with maximum yields

** The pyrromethenone originating from rings A/B, becomes only discernible after removal of the diazo reagent as a long wave-length shoulder of the protein band at 280 nm.

* Second derivative spectroscopy showed, interestingly, a double maximum for both azopigments, indicating the presence of two species. *Z,E*-isomerism has been described for bilirubin azopigments [43].

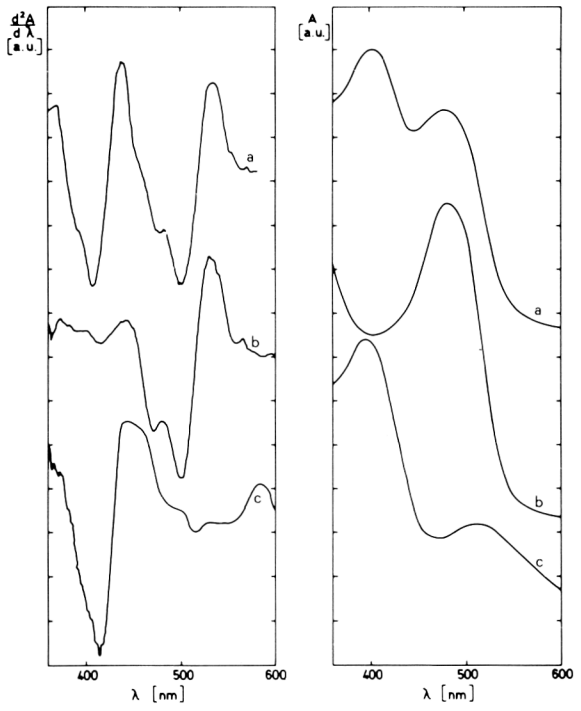


Fig. 4. Reaction of phycorubin (**2**) with diazotized ethylanthranilate (**18**) in phosphate buffer (50 mM, pH 7.5) containing urea (8 M). Uv-vis spectra (right column) and their second derivatives (left column) of a) the crude reaction mixture before extraction, b) the aqueous phase containing **4a** after extraction with 2-butanol and c) the 2-butanol phase, containing **7** and **5a**.

(70%) at a ratio of 1.2 for the diazonium salt to the phycorubin chromophore. The peptide-bound chromophores thus behave essentially identical to the free bilirubins. The product analysis is difficult, if less than 1 mol of the diazo reagent is used, due to the spectral overlap of the cleavage product **7** with the phycorubin educt. The latter is not extracted into the organic phase, but yields additional extractable **7** and **5a** upon further addition of the diazo reagent.

As observed for the free bilirubins, all products of the diazo reaction are unstable. The products **7** and **5a**, originating from the fully unsaturated ring C/D fragment, are surprisingly less stable than the product **4a**, bearing the generally more reactive [24] hydrogenated ring A (Scheme 3), which may be due to the attached peptide chain. As one consequence of this differential stability, the ratio **5a**:**4a** decreases with increasing excess of added reagent. The regioselectivity of the attack at C-11 *vs.* C-9 is thus difficult to determine, but can be estimated to $\leq$ 1:4 from the product analysis, obtained with $\leq$ 1 mol added. This is clearly at variance with the data obtained with the model pigment **16**, bearing the same conjugation system as does phycorubin. It may indicate again a rather pronounced influence of the protein, of the solvent, and possibly also of the carboxylic acid side chains, which are known to play a crucial role in the conformation of bilirubins [6]. The 2,3-dihydro-azopigment was less stable, if the reactions were carried out with diazotized sulfanilic acid.

If the reaction is carried out with diazotized ethyl anthranilate, and the resulting reaction mixture extracted with 2-butanol, then transfered into chloroform and esterfied with diazomethane, a single azopigment (methyl ester of **5a**) is obtained. If the reaction mixture of mesobilirubin (**3**) with diazotized ethyl anthranilate was worked up similarily, two isomers (methyl ester of **5a** and **23a**) could be separated on TLC [41].

Quantitative results

The quantitative results of the diazo reaction with the different phycocyanins and with the model pigment **3** are summarized in Table I. All reactions have been carried out with only little excess of the reagent added, to avoid the aforementioned destruction of the reaction products. The data for the free pigment **3** have been included as a standard to check the partition of the products between water and 2-butanol during work-up. The yields for the extracted pigments contain losses during the extraction procedure. These losses amount to 10–20% for the azopigments **5a** and **23a**. In the case of the yellow 9-hydroxymethyl-dipyrromethenones, they depend on the diazonium salt/chromophore ratio and amount to about 50% at the commonly used conditions, when the reaction was carried out with diazotized ethylanthranilate and the products extracted with *i*-butanol. If these corrections are taken into account, the reaction gives essentially a quantitative yield of the free pigments **7** and **5a**, derived from the ring C/D-half of the phycocyanin chromophore.

Discussion

The diazo reaction in aqueous solution

Bilirubin IXα is the major mammalian bile pigment. It has two essentially identical chromophors which differ only in their substitution-patterns at the

Table I. Quantitative product analysis of the diazoreaction of mesobilirubin (**3**) and the phycorubins (**2**) derived from PC from two different cyanobacteria.

Tetrapyrrolic Pigment	Reagent Ratio [mol/mol]	Total yield[a] of Dipyrrolic Pigments		Fraction of individual Dipyrrolic Pigments[b]					
				5a		**7**		**4a**	
		before extraction	after extraction[f]	aq.	org.	aq.	org.	aq.	org.
Mesobilirubin (**3**)	1.2	57	54	–	52[c]	11[d]	37[d]	–	–
PC ($\alpha\ \beta$) *Spirulina*	1.2	42	37	–	22	–	46	32	–
PC α *Spirulina*	1.2	46	34	–	≤1	–	50	50	–
PC β *Spirulina*	1.1	55	34	–	6	–	44	50	–
PC $\alpha\ \beta$ *Mastigocladus*	1.2	43[e]	37	–	–	–	43	57	–

[a] Calculated for a theoretical yield of 2 mol dipyrrolic pigments/mol tetrapyrrole.
[b] In percent of the total yield of dipyrrolic pigments after extraction.
[c] Mixture of **5a** with its isomer.
[d] Mixture of **7**/**24**.
[e] Only **4** taken into account, due to interference in the 410 nm region.
[f] All extractions with *i*-butanol, except for PC ($\alpha\ \beta$) from *Spirulina*, which was extracted with methanol after lyophilization.

lactam rings. During the diazo reaction, it is cleaved to yield at least principally a mixture of azopigments and 9-oxypyrromethenones. If the reaction is carried out in methanolic solution, both types of pigments are indeed found irrespective of the excess of reagent used [8]. In water, this is only true with little excess of the diazonium salt. The reaction mixture of bilirubin IXα (**13**) with a large (*e.g.* 7-fold) excess of diazotized sulfanilic acid (**17**) in aqueous buffer containing 8 M urea, yields exclusively azopigments. Pyrromethenone-like products could not be identified spectroscopically. However, the yields of azopigments still never reached the theoretical value of 2 mol per mol bilirubin and decreased with prolonged reaction time. This indicates that under these conditions both the azopigments and the oxypyrromethenones are unstable in the presence of excess diazoreagent. The intermediate formation of the oxypyrromethenones is nonetheless evidenced from experiments using only a 1.2-fold excess of diazoreagent over bilirubin. The UV-vis spectrum obtained (Fig. 1), was virtually identical with that from the reaction in methanol [8].

It was again possible to separate a fraction absorbing around 430 nm from the azopigments (**19, 20**) by solvent extraction. The UV-vis spectrum of the purified yellow fraction (Fig. 2) resembled that of the corresponding fraction from the reaction in methanol [8]. Again, it could be separated by TLC in two isomers, which were correlated to the different molecule halves of bilirubin IXα (**13**) by comparison with products derived from the symmetric bilirubins IIIα (**14**) and XIIIα (**15**) (Scheme 1). The products isolated from the aqueous solvent were more polar than those from methanol. Although further data for the products described here were not yet obtained, the 9-hydroxymethyl structures **21** and **22** were tentatively assigned to the products, taking into account the strong analogy to the well investigated reaction in methanol [8].

The influence of the hydrogenated ring A

Plant bile pigment chromophores differ from the mammalian ones by containing a hydrogenated ring A (see *e.g.* phycocyanobilin (**1**)). 2,3-dihydro octaethylbiliverdin (**9**) has been demonstrated by spectroscopic and chemical evidences [10, 35, 44] to be a useful synthetic analogon for the chromophores of the two biliproteins, PC and PR [44]. The corresponding rubin (**16**) was, therefore, chosen as a synthetic model for phycorubin (**2**). By comparing the reaction of **16** with that of the fully unsaturated

analogue **10**, the influence of the hydrogenated ring A was studied.

Both rubins **10** and **16**, were synthesized by reaction with sodium borohydride from the corresponding verdins, **8** and **9**. It is noteworthy, that the reaction of the hydrogenated verdin (**9**) is less smooth and requires higher temperatures than the reaction of **8**. Theoretical calculations indicated a decreased reactivity towards nucleophiles at C-10 of 2,3-dihydrobiliverdins like **8** [45]. The reactions of both, **8** and **9**, did not stop at the stage of the rubin, but with too large excess of reagent proceeded further to colorless, spectroscopically not identified products. The reaction conditions were therefore adjusted such, that the reaction did not go to completion. Thus, an apropriate yield of the rubin was obtained, and the remaining starting material could be recycled. The resulting alkyl-substitued rubins **10** and **16** are prone to easy autoxidation, since they lack the stabilizing ridge-tile conformation as induced in natural bilirubin IXα (**13**) by intramolecular H-bonding [6, 46]. The diazoreaction of octaethylbilirubin (**10**) was carried out in alcoholic solvents with diazotized ethylanthranilate (**18**), thus allowing for TLC investigation of the products. It resembled strongly the reaction of bilirubin IXα (**13**) in methanol with diazotized sulfanilic acid (**17**) described previously [8], with regard to product distribution and reactivity. Irrespective of the molar excess of diazoreagent used (2–15-fold), only one mol of azopigment (**11**) was formed per mol of **10**. Even prolonged treatment with a 15-fold molar excess of reagent did not result in a higher yield of azopigment, but rather in a bleaching of the red color. Instead, as in the reaction of **13** mentioned above [8], a second (yellow) product was identified spectroscopically and by TLC. It arises from the addition of a solvent molecule to the suggested intermediary azafulvenium ion, formed after the attack of the diazonium ion at either C-9 or C-11 of the rubin [8, 9]. In analogy to the 9-oxymethyl-pyrromethenone structures found for the products of **13** [8], it is likely that the product of **10** has structure **12** (ethanolic solvent).

As expected from its structure (**16**), two azopigments (**11** and **25**) were obtained from 2,3-dihydrooctaethylbilirubin (**16**). Attack of the diazonium salt at C-11 leads to the formation of the red azopigment **11**, which is identical to that obtained from **10**, while an orange azopigment (**25**), λ_{max} = 480 nm arises from attack at C-9. A quantitative analysis, based on TLC separation of the reaction mixture under the assumption of equal extinction coefficients for both azopigments gives values of 60% (**11**) and 40% (**25**), thus indicating a 3:2 preferential attack at C-11. Due to the interference of the broad absorption bands of the azopigments, the reaction mixture showed an absorption maximum around 500 nm, which can be resolved by second derivative spectroscopy. The reaction should likewise yield two 9-oxymethyl pyrromethenones. The fully unsaturated **12** is readily identified by spectral and TLC comparison with the product obtained from **10**. The expected second pyrromethenone (**24**) (see Scheme 3) has a vinylpyrrole structure. Such compounds absorb around 315 nm [7] and thus give no colored spot on TLC. **24** could also not be identified spectroscopically in the reaction mixture due to interference with the short wavelength absorption bands of the azopigments and was not investigated further.

Presence of a peptide bond to rings C or D in PC

The reaction mixture of phycorubin (**2**) with either one of the diazonium reagents had a maximum at 486 nm (Fig. 4), which is close to that of the 2,3-dihydro azopigment **4** (Fig. 3). This indicates a reversed regioselectivity as compared with the reaction of the dihydrorubin **16** with a preferential attack at C-9 (the data presented below suggest a ratio of about 6:1). This regioselectivity would explain earlier results with PC from *Spirulina platensis,* which yielded the free azopigment **5a** in low yield only. It should be noted in this context, that a chromophore heterogeneity has recently been suggested for PC from *Synechocossus* 6301 [48]. According to NMR studies with chromopeptides, the classical molecular structure **1** with a 3^1-thioether bond is assigned to two of the chromophores (α and β^1), whereas an isomeric structure with a 18^1-thioether bond was assigned to the third one (β^2). The diazo reaction should then yield the isomeric azopigments **5a** and **23a** in a 2:1 ratio. While the spectroscopic results for the diazoreaction of these chromophores should be very similar, the two isomers can be separated by TLC. In our studies with PC from *Spirulina platensis,* only the azopigment **5a** was found, thus arguing against such heterogeneity in this organism.

The former qualitative studies have proved that at least part of the chromophores do not contain a second protein bond in the C,D-ring moiety. Like

the diazo reaction of the dihydrorubin **16**, that of phycorubin should yield four different pigments (Scheme 3), *e.g.* the two azopigments (**4a** and **5a**) identified previously [8], and two 9-hydroxy-dipyrromethenones (**7** and **6**). A complete quantitative study would then require the analysis of all four products. As discussed above for the reaction of **16**, this is not possible by spectrophotometry.

The problem can be circumvented satisfactorily by solvent extraction. Upon extraction of the diazo reaction mixtures of the free bilirubins **3**, **13**–**15** with organic solvent, all dipyrromethenones were found in the organic phase. Since the absorption maxima of the dipyrromethenones **7** and **6** are well separated, any dipyrromethenone (**7**) remaining in the aqueous phase during work-up of the diazo reaction with phycocyanins would indicate a linkage to the peptide chain at the rings C or D. This has neither been observed with PC from *S. platensis,* nor with its isolated subunits and shows, that no such bond is present in any one of its three chromophores. This result is further supported by second derivative spectroscopy. Although this did not allow the quantitation of the two azopigments, it was sufficient to establish the purity of each of the two with the other to within $\approx 10\%$. Any azopigment not bound to a peptide, would be rendered extractable into organic solvents by the use of the esterified diazonium reagent **18**. Derivative spectroscopy of the aqueous phase after extraction did not show any contamination of the protein bound orange pigment **4** with red pigment, derived from rings C and D.

The quantitative evaluation of the reaction (Table I) has furthermore shown, that the yields of the dipyrrolic pigments derived from PC of *Spirulina platensis* and the isolated subunits are very similar to that observed for the free bile pigment, mesobilirubin (**3**). Taken together, the results provide very strong evidence, that there is no second chromophore-protein bond in PC from this species.

The quantitative results for PC from *Mastigocladus laminosus* were comparable to those, obtained with the pigments from *S. platensis*. Both, the yields and the product ratios, are essentially the same as with the other phycocyanins. These data suggest, that there is no second chromophore peptide bond in the ring C/D fragment in the pigment from this species, too. The results are nonetheless more ambigious, because some yellow pigment ($\lambda_{max} \approx$ 390 nm) remained in the aqueous phase upon extraction of the reaction mixture. This non-extractable pigment has, however, a different chemical stability than the pyrromethenone **7** and we thus ascribe it to a product derived from some other material, *e.g.* the peptide chain or some impurity. This assignement is also supported by the quantitative analysis of the other products discussed above.

It is concluded, that in the two phycocyanins studied, none of the three chromophores has a linkage at the C,D-ring half and is bound to the protein *via* a thioether bond to a ring A. It should be emphazised, however, that a second bond in the A,B-ring half, cannot be excluded by the technique used and requires a complementary cleavage method [28]. The recent results of Rapoport and Glazer [48] also suggest a strong species dependence on the chromophore binding situation. Further biochemical and higher resolution x-ray work [49] is required to solve these questions.

Acknowledgements

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[1] P. Hefferle, W. John, H. Scheer, and S. Schneider, Photochem. Photobiol. **39,** 221 (1984).
[2] P. Ehrlich, Charite Ann. **8,** 140 (1983).
[3] A. A. H. v. d. Bergh and P. Muller, Biochem. Z. **77,** 90 (1916).
[4] K. P. M. Heirwegh, J. Fevery, J. A. T. P. Meuwissen, F. Compernolle, V. Dermet, and F. P. v. Roy, Meth. Biochem. Anal. **22,** 205 (1974).
[5] T. K. With, Bile Pigments, Academic Press, New York 1968.
[6] A. F. Mc Donagh, in: The Porphyrins, (D. Dolphin, ed.), **Vol. 6,** 293, Academic Press, New York 1979.
[7] A. Gossauer and H. Plieninger, in: The Porphyrins (D. Dolphin, ed.), **Vol. 6,** 598, Academic Press, New York 1979.
[8] W. Kufer and H. Scheer, Tetrahedron **39,** 1887 (1983).
[9] D. W. Hutchinson, B. Johnson, and A. J. Knell, Biochem. J. **127,** 907 (1972).
[10] W. Kufer and H. Scheer, Z. Naturforsch. **37c,** 179 (1982).
[11] W. Rüdiger, Ber. Deutsch. Bot. Ges. **92,** 413 (1979).
[12] W. Rüdiger and H. Scheer, in: Photomorphogenesis-Encyclopedia of Plant Physiol (W. Shropshire, jr., and H. Mohr, eds.), **Vol. 16,** 119, Springer Verlag, Heidelberg 1983.
[13] E. Koest-Reyes, H.-P. Koest, and W. Rüdiger, Liebigs Ann. Chem. **1975,** 1594 (1975).
[14] E. Koest-Reyes and H.-P. Koest, Eur. J. Biochem. **102,** 83 (1979).
[15] G. Muckle, J. Otto, and W. Rüdiger, Hoppe-Seyler's Z. Physiol. Chem. **359,** 345 (1978).
[16] G. Klein, S. Grombein, and W. Rüdiger, Hoppe-Seyler's Z. Physiol. Chem. **358,** 1077 (1977).
[17] G. Klein and W. Rüdiger, Liebigs Ann. Chem. **1978,** 267 (1978).
[18] G. Frank, W. Sidler, H. Widmer, and H. Zuber, Hoppe-Seyler's Z. Physiol. Chem. **359,** 1491 (1978).
[19] A. N. Glazer, C. S. Hixson, and R. J. De Lange, Anal. Biochem. **92,** 489 (1979).
[20] V. P. Williams and A. N. Glazer, J. Biol. Chem. **253,** 202 (1978).
[21] D. A. Bryant, D. S. Hixson, and A. N. Glazer, J. Biol. Chem. **253,** 220 (1978).
[22] J. C. Lagarias, A. N. Glazer, and H. Rapoport, J. Am. Chem. Soc. **101,** 5030 (1979).
[23] J. C. Lagarias and H. Rapoport, J. Am. Chem. Soc. **102,** 4821 (1980).
[24] H. Scheer in: Light Reaction Path of Photosynthesis (F. K. Fong, ed.), p. 7, Springer Verlag, Berlin 1982.
[25] P. Freidenreich, G. S. Appel, and A. N. Glazer, J. Biol. Chem. **253,** 212 (1978).
[26] W. Rüdiger, Hoppe-Seyler's Z. Physiol. Chem. **350,** 1291 (1969).
[27] R. F. Troxler, A. B. Brown, and H.-P. Koest, Eur. J. Biochem. **87,** 181 (1978).
[28] W. Kufer, C. Krauss, and H. Scheer, Angew. Chem. **94,** 455 (1982); Angew. Chem. Int. Ed. Engl. **21,** 446 (1982), Angew. Chem. Suppl. **1982,** 1050 (1982).
[29] J. A. S. Cavaleiro and K. M. Smith, J. Chem. Soc. Perkin I **1973,** 2149 (1973).
[30] H. Mauser, Z. Naturforsch. **23b,** 1025–1030 (1968).
[31] A. F. Mc Donagh and F. Assisi, J. Chem. Soc. Chem. Commun., 117 (1972).
[32] A. F. Mc Donagh and F. Assisi, FEBS Lett. **18,** 315 (1971).
[33] M. S. Stoll and C. H. Gray, Biochem. J. **163,** 59 (1977).
[34] H. Fischer and H. W. Haberland, Hoppe-Seyler's Z. Physiol. Chem. **232,** 236 (1935).
[35] W. Kufer and H. Scheer, Hoppe-Seyler's Z. Physiol. Chem. **360,** 935 (1979).
[36] A. N. Glazer and S. Fang, J. Biol. Chem. **248,** 663 (1973).
[37] R. W. Castenholz, Schweizer Z. Hydrol. **35,** 538 (1970).
[38] Ein Autorenkollektiv (ed.), Organikum, p. 492, VEB Verlag der Wissenschaften, Berlin 1964.
[39] J. Th. G. Overbeek, C. L. J. Vink, and H. Deenstra, Rec. Trav. Chim. Pays-Bas **74,** 85 (1955).
[40] J. Fog (1957), cited in T. K. With "Bile Pigments", p. 65 (1968).
[41] N. Blanckaert, K. P. M. Heirwegh, and F. Compernolle, Biochem. J. **155,** 405 (1976).
[42] S. Braslavsky, A. R. Holzwarth, and K. Schaffner, Angew. Chem. **95,** 670 (1983); Angew. Chem. Int. Ed. **23,** 666 (1983).
[43] M. Salmon, J. Heterocycl. Chem. **14,** 1101 (1977).
[44] H. Scheer, Z. Naturforsch. **31c,** 413 (1976).
[45] H. Falk, K. Grubmayr, G. Kapl, N. Müller, and U. Zrunek, Mh. Chem. **114,** 753 (1983).
[46] H. Fischer and J. Aschenbrenner, Hoppe-Seyler's Z. Physiol. Chem. **232,** 236 (1936).
[47] W. Kufer, Dissertation, Universität München 1980.
[48] H. Rapoport and A. N. Glazer, in: Optical Properties and Structure of Tetrapyrroles (G. Blauer and H. Sund, eds.), 411, De Gruyter, Berlin 1985.
[49] T. Schirmer, W. Bode, R. Huber, W. Sidler, and H. Zuber, Reprinted from J. Mol. Biol. **184,** 257 (1985).