Biliproteins

By Hugo Scheer[*]

Dedicated to Professor Hans-Herloff Inhoffen on the occasion of his 75th birthday

Biliproteins, covalently bonded complexes of proteins and bile pigments, serve as light-harvesting pigments in photosynthesis and light-sensory pigments of photosynthetic organisms. Recent developments in the biochemistry and biophysics of these pigments are reviewed and an attempt is made to describe their functions of light-harvesting and of information transduction on a molecular level.

1. Introduction

Cyanobacteria, red algae, and cryptophytes contain large quantities of blue and red pigments, which essentially determine the color of these organisms and may amount to 40% of the protein[1]. *Engelmann*[2] was the first to relate these pigments to photosynthesis; *Haxo*[3], *Emerson*[4], and *Gantt*[5], among others, recognized their function as light-harvesting pigments, mainly of photosystem II. In 1928, *Lemberg*[6] demonstrated that they contain bile-pigment chromophores; in accord with their origin and composition, these chromoproteins are thus called *phycobiliproteins.*

Pigments of this structure, but having completely different functions, exist in many other organisms. The most important compound of this group is *phytochrome.* This sensory pigment of green plants was discovered in 1945 by action-spectroscopy[7]. Owing to its instability and low concentration, it was first enriched and characterized by absorption spectroscopy as late as 1959[8]. Phytochrome is a photoreversibly-photochromic pigment. The position of the equilibrium between its two forms (R- and FR-form), the total concentration, and other factors are fundamental in regulating the development of plants[9]. Another group of photoreversibly-photochromic pigments, the phycochromes, were isolated from various cyanobacteria[10], and at least one phycochrome was also related to developmental processes like chromatic adaption[1,11,12].

These three groups of pigments are referred to as biliproteins (Table 1). This contribution deals with recent developments in their biochemistry and biophysics. Since the last comprehensive survey in this field[13a] several different aspects have been reviewed[1,5,9,13-25,31b]. It should be mentioned that besides these genuine biliproteins, there also exist bile pigment-protein aggregates lacking a covalent bond between both component parts. These include the physiologically important complex of bilirubin, which is only sparingly soluble in water, with serum albumin[26], and an increasing number of invertebrate pigments[13,27-30]. A selection of them is included in Table 1.

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Table 1. Natural occurrence and functions of biliproteins. The first three groups of pigments are the subject of this report.

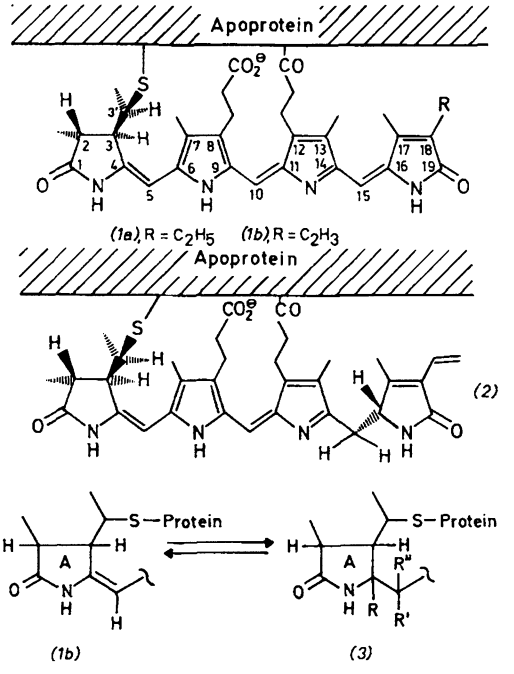
Pigment	Occurrence	Function	Chromophore structure	Ref.
Phycobiliproteins (*e.g.* phycocyanin (PC) allophycocyanin (APC) phycoerythrin (PE))	Cyanobacteria Red algae Cryptophytes	Antenna pigments of photosynthesis	Phycocyanobilin *(1a)*, phycoerythrobilin *(2)* etc.	see Text
Phytochrome	Higher green plants, certain algae, fungi (?), mosses, red algae (?)	Reaction-center pigments of photomorphogenesis	*(1b)*, *(3)* [a]	see Text
Phycochromes	Cyanobacteria, red algae	Possibly reaction center pigments of photomorphogenesis and chromatic adaption ("adaptachromes")	*(1a)* (?)	[1, 10–12]
Bilirubin-serum-albumin complexes	Vertebrates	Water-soluble transport form of bilirubin	—	[26]
Aplysioviolin	*Aplysia* (sea hare)	Defense excretion	Monoester of *(5)*	[29]
Turboverdin	*Turbo cornutus* (mussel)	Protective coloration (?)	[18-Ethyl]-[3-(2-hydroxyethyl)]-*(19)*, R = H	[30]
Pterobilin	Lepidoptera	Protective coloration (?)	IXγ-Isomer of *(19)*, R = H	[27]
Phorcabilins	Lepidoptera	Protective coloration (?)	Extended derivatives of pterobilin, similar to *(31)*	[27]

[a] The substituents R, R′ und R″ in *(3)* suggest that the double bond between C-4 and C-5 present in P_r is no longer noticeable in the absorption spectrum (see Section 2.3).

2. Structure of Chromophores

The two major chromophores of phycobiliproteins are *(1a)* (phycocyanobilin) and *(2)* (phycoerythrobilin)[*]. (In the formulas the bond to the protein is indicated.) *(1a)* is the blue chromophore of phycocyanins and allophycocyanins, *(2)* is the red chromophore of phycoerythrins. One or the other of them exists in each of the known phycobiliproteins, while R-phycocyanin (R-PC)[**] contains both. In addition, there occur several other chromophores with hitherto unknown structures, *e.g.* a phycourobilin in the γ-chain of B-phycoerythrin (B-PE)[**][31], the red chromophore in the α-chain of phycoerythrocyanin[32], the third chromophore (P_{590}) of PC from a *Hemiselmis species*[33] and the blue chromophore of PC from *Chroomonas*[34].

Strictly speaking, the structures *(1a)* and *(2)* have so far been unequivocally determined on only a few phycobiliproteins. Generally, they are identified by chromatographic comparison of the cleaved chromophores and spectroscopic investigations on denaturated biliproteins (see Section 2.3).



The chromophore *(1b)* of the R-form of phytochrome (P_r) is very similar to phycocyanobilin *(1a)*[35–38]. Instead of the ethyl-group it contains a vinyl-group at C-18[37,38], but the absolute configurations at C-2, C-3 and C-3′ are identical[39]. The structure of the P_{fr}-chromophore is still unclear; however, it is known that it contains a shorter conjugation-system than the P_r chromophore[35] but the same β-pyrrolic substituents[37]. Comparative investigations on free bile pigments have currently led to two models which would account for the properties of the P_{fr}-chromophore. It could be a geometrical (*Z,E*)-isomer[40] or a substitution product of the P_r-chromophore[41] (see Section 2.4).

2.1. Cleavage of Chromophores

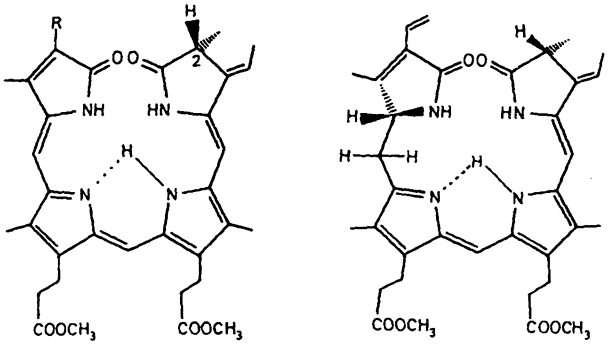
For a long time elucidation of the structures of biliprotein-chromophores was complicated by their covalent bonds to

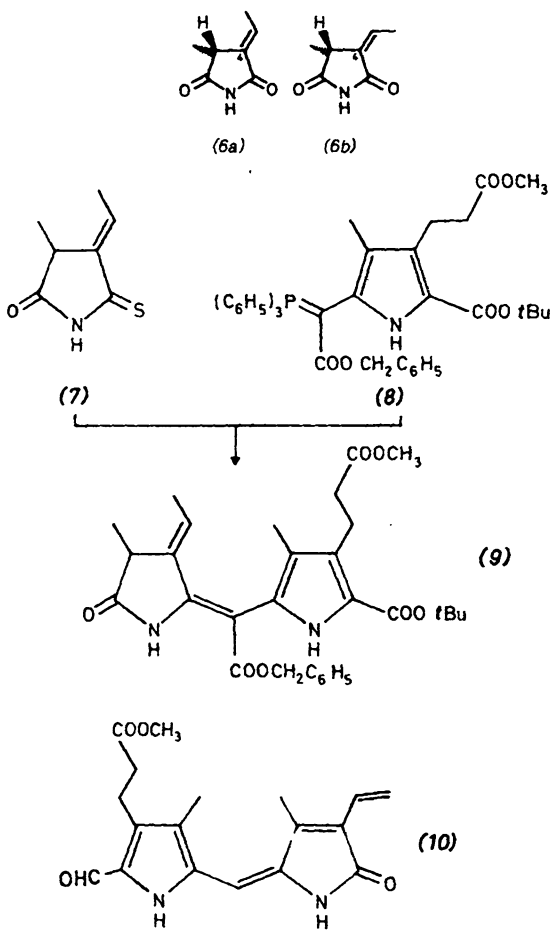
[*] The nomenclature of the bile-pigments has been modified several times in recent years. Four systems were and are used concomitantly. Trivial names and the numbering system of *H. Fischer*, which correlates the bile pigments with the porphyrins, are mainly used in the older literature [for example *(19)* = biliverdin IXα]. The numbering system, still used today and shown in formulas *(1)* and *(23)*, results from the first attempt of a rational nomenclature. It allows no direct correlation between the C-atoms of macrocyclic tetrapyrroles, and the linear tetrapyrroles derived therefrom. Apart from using a certain number of trivial-names, it is based on the completely saturated "bilan". An exchange-nomenclature is possible for both systems: A new substituent replacing the original one of a parent compound is placed in square brackets [*viz.* [*(18)*-vinyl]-*(1a)* = *(1b)*]. The nomenclature used in this article corresponds to the VIth Memorandum of the IUPAC Nomenclature Commission. Aside from a limited number of trivial-names [*e.g. (19)* = biliverdin] it is based on bilin which, *in natural bile pigments*, contains the maximum number of non-cumulated double bonds [cf. *R. Bonnett*, in [24a], Vol. 1, 1978, p. 1; example: *(23)* = 2,3,7,8,12,13,17,18-octaethyl-2,3-dihydro-1,19[21*H*,24*H*]-bilindione]. The nomenclature recently proposed by the IUPAC (Pure Appl. Chem. *51*, 2251 (1979)) is similar.

[**] Abbreviations: PC = phycocyanin, APC = allophycocyanin, PE = phycoerythrin, P_r, P_{fr} = phytochrome in the R- or FR-form (see Sections 4.3 and 5.2). Prefixes indicate the parent organisms: C = cyanobacteria, R = red alga, B = *Bangiales* (an order of red alga), K = cryptophytes.

the proteins, which preclude a cleavage without chemical modification. Depending on the conditions of cleavage, different phycobilins result from one and the same pigment, so that the nomenclature became very complex (cf. [13,22,24]).

The best characterized products are the ethylidenebilins *(4a)* and *(5)*, which are formed as main products on treatment of the corresponding biliproteins with the chromophores *(1a)* and *(2)*, respectively, in refluxing methanol[42] or long chain alcohols[43], as well as by treatment with HBr/trifluoroacetic acid (TFA)[44]. The cleavage proceeds by stereoselective elimination of the C-3-thio ether[45]; in *(2)* and/or *(5)* the asymmetric C-16 can epimerize at the same time[46,47]. The structures, suggested originally from ^{1}H-NMR and MS data[48-51] and from chromic acid degradation[13,52,53], have been confirmed by total syntheses[46,54].



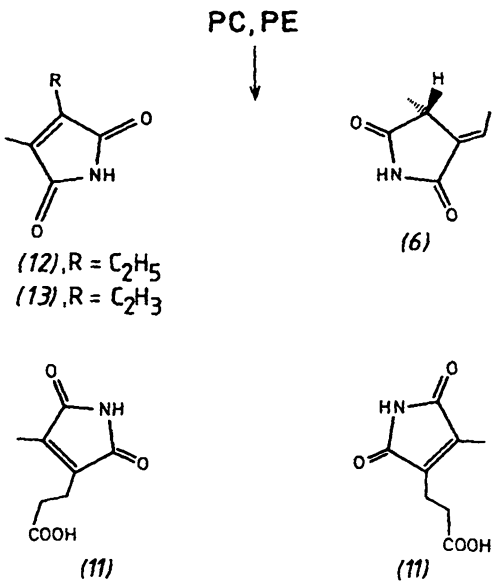


Scheme 1.

The key reaction in the synthesis is the regioselective coupling of rings A and B, which is possible in good yields by condensation of the monothioimide *(7)* with the pyrrole ylide *(8)*; condensation of the product *(9)* with the CD-fragment *(10)* yields *(4b)* (Scheme 1). For thermolabile substituents[54], sulfur-contraction[54a] can be used instead. By the first procedure the 18-vinylbilindione *(4b)* (="P_r-bilin") was obtained[38]. It could be correlated with *(1b)* by treatment of the latter with HBr/TFA[55]. The stereochemistry of *(4a)* and *(5)* at C-2 was established by chromic acid degradation to the imide *(6)* with known absolute (4*R*)-configuration[56], while that of *(5)* at C-16 was established by asymmetric synthesis and correlation with (4*R*,16*R*)-urobilin[46,57].

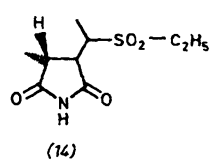
2.2. Degradation Reactions of Biliproteins

On oxidation with chromic acid, tetrapyrroles are degraded to cyclic imides possessing (at least in principle) the same β-pyrrolic substituents. This method, originally introduced by *H. Fischer*, has in the meantime been improved several times and standardized, and has especially been applied to biliproteins by *Rüdiger et al.*[13,53]. The "hydrolytic" chromic acid degradation of phycocyanin (PC) at 100 °C



yields the three imides *(6)*, *(11)* and *(12)*, while that of phycoerythrin (PE) and P_r[37] affords the imides *(6)*, *(11)* and *(13)*, the latter of which readily decomposes under the reaction conditions. On oxidation at ambient temperatures, no *(6)* is formed, and *(11)* can only be extracted in about 50% yield, thus indicating protein bonding to the respective rings[13].

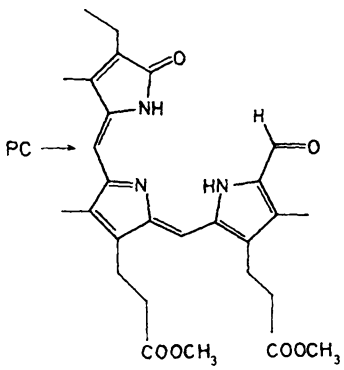
Under these conditions, the degradation of the tetrapyrrole is accompanied by oxidation of the thio ether to sulfone



(14), which eliminates $-SO_2-C_2H_5$ to give *(6)*[58] in the presence of aqueous ammonia. This reaction sequence not only demonstrates the protein-bond of ring A, it is also ste-

reoselective[45,58,59] and leads, with the well-known 4*R*,*E*-configuration of the imide *(6a)*[56], to the 2*R*,3*R*;3′*R*-configuration of *(1a)*, *(1b)*, *(2)* and *(3)*. A possible second binding site[13] is still controversial[22,60-65].

During chromic acid degradation the information concerning α-pyrrolic and methine-substituents is lost and, *e.g.*, hydrolysis of the β-pyrrolic substituents cannot be excluded. The milder degradation procedure with chromate yields imides of the terminal rings, whereas the inner ones yield pyrroledicarbaldehydes[13,53]. Thus, it is possible to differentiate, *e.g.*, between isomeric biliverdins, and to assign the IXα-type substitution to *(1)-(5)*. A recently developed milder



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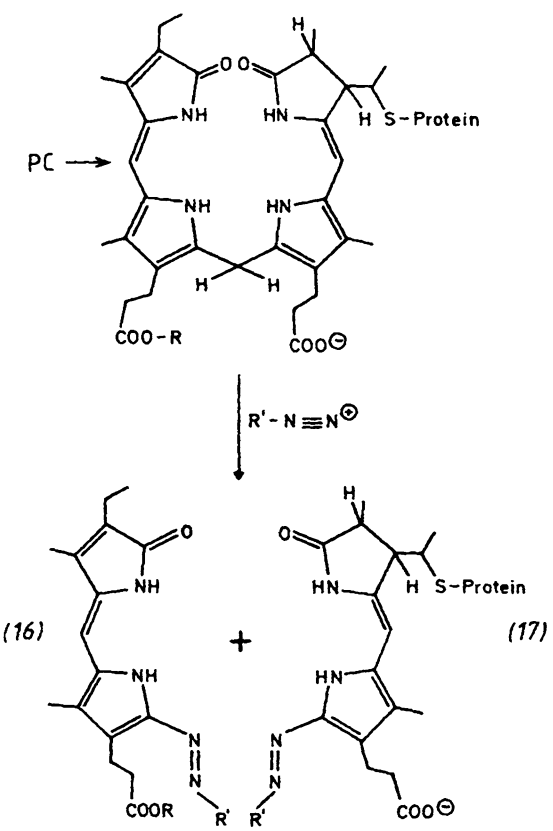
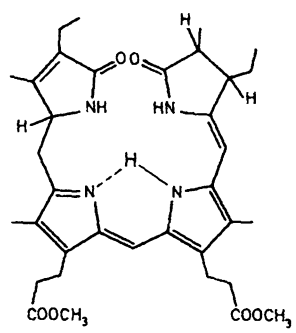
method of oxidation enables regioselective cleavage of biliprotein chromophores like *(1a)* at the methine bridge next to ring A, to give formyltripyrrinones. The method is specific for A-dihydrobilindiones[66,67]. Thus, tripyrrinone *(15)* was obtained in this way from PC. Another selective cleavage



reaction, in this case however between rings B and C, is the diazo reaction[68]. This reaction, important in the medical analysis of bilirubin[69], can be applied to the higher oxidized bilindiones present in the biliproteins, only after a pretreatment[70]. The protein first has to be unfolded by addition of urea, and then the central methine bridge of the chromophores has to be reduced by $NaBH_4$. The complete reaction sequence can be carried out under very mild conditions (4 °C, pH = 7). The fragments *(16)* and *(17)* were obtained from PC. *(17)* confirms again the binding of ring A to the protein; both of the last mentioned reactions also indicate the existence of a second bond at ring C, at least in PC of *Spirulina Platensis*, used in these studies. The proportion of free, extractable *(16)* is increased after hydrolytical pre-treatment; *(15)* is only obtainable in this way.

2.3. Spectroscopy of Denatured Pigments

A sensitive, non-destructive method for the analysis of biliprotein chromophores is UV-VIS absorption spectroscopy. In the native pigments the spectral properties are strongly dependent on the protein environment (see Section 3), but the non-covalent interactions responsible for these effects are decoupled by complete unfolding using urea, guanidinium chloride, or heat. Although still covalently bound to the protein, the chromophores can be correlated with free bile pigments of known structure. The identification is improved by measuring the free bases as well as the cations, anions, and metal-complexes; for an exact characterization the most suitable are cations and zinc-complexes[35,36,61,71,72]. The method has also been successfully applied for the quantitative determination of the number of chromophores (Table 3)[61,72]; and by acid-base titration the pK-values of protonation and deprotonation are available as additional important parameters[35,36].



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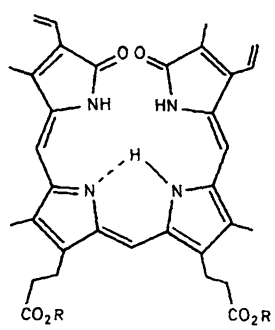


(19)

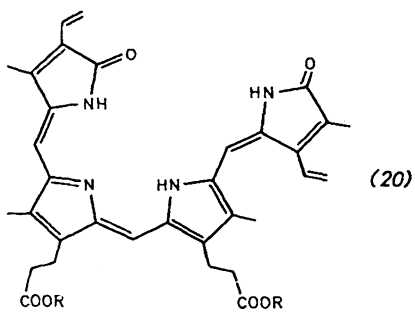
Denaturated PE is spectroscopically very similar to *(18)*; it contains the rhodin-chromophore *(2)*[71]. Denaturated PC and P_r absorb at longer wavelengths than *(18)*, but at shorter wavelengths than bilindiones such as *(19)*. The spectroscopic similarities to *(23)* indicate the 2,3-dihydrobilindione conjugation system for the chromophores of both pigments. The 18-vinyl group of *(1b)* leads to a small red-shift of the absorption as compared to pigments containing *(1a)*[35]. At pH ≤ 5.2 denaturated P_{fr} has an absorption maximum at 610 nm; thus, in contrast to native phytochrome the denaturated P_{fr} absorbs at *shorter wavelengths* than denaturated P_r under the same conditions ($\lambda_{max}^{cation} = 660$ nm). Therefore, P_{fr} has a

shorter conjugation system than P_r[35]. Analogous results have been obtained when proteolytic digestion is used instead for the uncoupling of the chromophore[55]. The A-dihydrobilindione structure of the chromophores *(1a)* and *(1b)* and the thioether-bond have recently been proved, too, by NMR spectroscopy of the respective bilipeptides[38,65]. This method also afforded independent proof of the presence of the 18-vinyl group in *(1b)*[38].

2.4. Reactivity of Chromophores

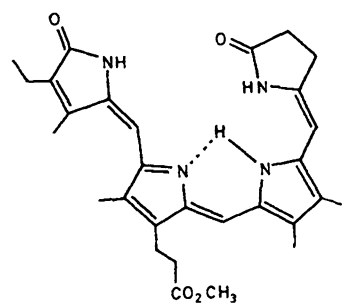
The different spectra of both forms of denaturated phytochrome (P_{fr} and P_r) demonstrate that the chromophores have different molecular structures. Conformational[73] changes, protonation-deprotonation[74] and the like, can thus be excluded as the *sole* reactions during phytochrome-transformation, and photochemical reactions gain a special interest. The primary reaction of the phytochrome system is the photochemical transformation of P_r into P_{fr}, or *vice versa*. The reaction has several spectroscopically well defined intermediates, which are different for the forward and back reactions, respectively. The structures of the intermediates are still unknown, but the results of flash[75-78] and of low-temperature spectroscopy[77,79-82] as well as of dehydration experiments[82-84] have shown, that only the first step(s) are photochemical one(s), followed by dark-reactions (cf. [18]). Owing to the formal analogy with the reactions of rhodopsin[85], an analogous nomenclature[84] has been used.

In the context of these results, photochemical reactions of free bile pigments[86] and especially of bilindiones related to the P_r-chromophore *(1b)* were investigated. From the criteria, determined by the natural system, it should be possible to obtain some evidence regarding the structure of P_{fr}, the reaction pathway, and the primary signal of the phytochrome system. There exist two well known photochemical reactions for the conversion $P_r \rightarrow P_{fr}$ which meet most criteria: 1. geometrical isomerization at the double-bond between C-4 and C-5 or C-15 and C-16; 2. oxidative substitution at the C-5 methine bridge. It has been known for some time that dipyrromethenones form stable geometric (*Z,E*)-isomers which are in photochemical equilibrium[87,88]. Photochemical studies on pterobilin ("biliverdin IXγ") gave first indications, that corresponding isomers also exist in the case of bilindiones[27]. The isomerization reactions have been investigated systematically by *Falk et al.* with partial structures and integral bile pigments. They could show that bilindiones isomerize at one or at both terminal methine bridges and they have isolated and characterized the resulting products, like *(20)*[40,88,89]. Starting with an *E*-configurated formylpyrromethenone *Gossauer et al.*[90] recently also achieved the total synthesis of (*E,Z,Z*)-bilindiones. In comparison to the (*Z,Z,Z*)-educts, the absorption maxima are displaced to shorter wavelengths, *e.g.* to regions expected for a P_{fr}-chromophore model. This shift has been rationalized in terms of a simultaneous change of the configuration at the double bond between C-4 and C-5 in *(20)* and of the conformation of the neighboring single bond between C-5 and C-6. By this mechanism the *E*-configurated double bond is partially uncoupled from the remaining π-system[91]. For the thermal reisomerization $\Delta G^0 = -20$ kJ/mol and $\Delta H^+ = 105$ kJ/mol were determined[92]. Non-symmetrically substituted bilindiones yield two (*E,Z,Z*)-isomers[40]. A regioselective isomerization is observed with less symmetrical educts[93].



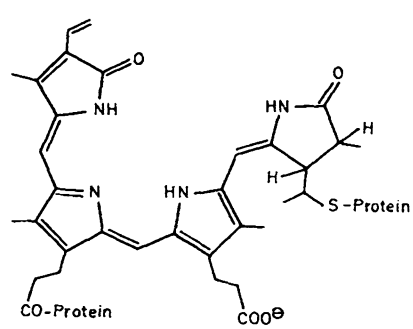
(20)

As a consequence of the hydrated ring A—and additionally of the 18-vinyl group in the case of P_r [*(1b)*]—the chromophores of biliproteins are perturbed from symmetry. Ramachandran calculations indicate a preferred isomerization of the Δ^4-bond next to the hydrogenated ring for steric reasons,



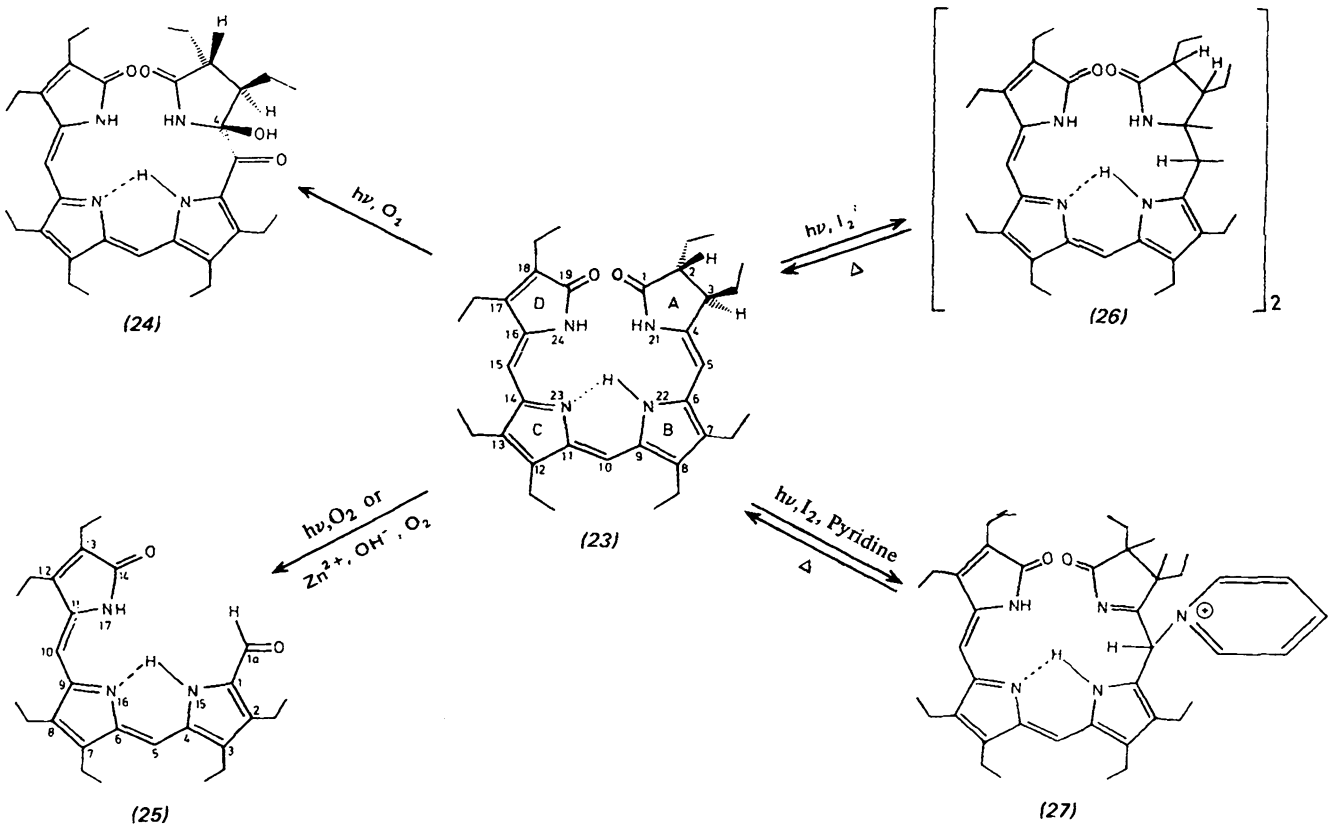
(21)

thus products of the violin spectral type are expected[94]. This assumption has recently been confirmed experimentally[90b]. Δ^{15}-*E*, but not Δ^4-*E*-configurated 2,3 dihydrobilindiones are accessible by total synthesis, the former having a rhodin-type spectrum due to uncoupling of ring D[90b]. A violin-type spectrum is then expected for the hypothetical, less stable Δ^4-*E*-isomer.



(22)

The hypothetical (*E,Z,Z*)-isomer of *(1b)* [cf. *(22)*] would be spectroscopically comparable with the P_{fr}-chromophore. The reaction mechanism is as yet only difficultly reconcilable with the properties of phytochrome. The photoisomerization of bilindiones does not proceed directly, but rather *via* intermediate rubinoid products formed in a dark reaction. Due to their low electron density at C-10[95], the bilindiones reversibly add nucleophiles[89b,96-99] and the yellow pigments thus formed are the substrates proper for isomerization[89b]. In accord with their absorption maximum at 450 nm, blue light is especially effective for isomerization[89b], whereas the action-spectrum of the $P_r \rightarrow P_{fr}$ transformation has its maximum at 660 nm in the red spectral region, the absorption-



Scheme 2.

maximum of the P_r-chromophore. The mechanism of reaction is, however, very dependent on the substrate structure, and direct photochemical reactions basically seem to be possible in all cases in which the geometry of the tetrapyrrole skeleton differs from the flat uniform helix. Examples are *N*-alkylated bilindiones[93] and non-(*Z,Z,Z*)-isomers[92]. It still remains open, how the asymmetry of the natural chromophores and their protein environment can affect the course of reaction.

The second model reaction for the $P_r \rightarrow P_{fr}$ transformation is a photochemical oxidation of the P_r-chromophore *(1b)*. Denaturated P_r is oxidized by strong oxidizing agents (Fe^{III}, Ce^{IV}) to products, spectroscopically similar to denaturated P_{fr}[35]. The phytochrome transformation had already been correlated with redox-reactions[100-102]; and the spectra indicate a photochemical oxidation of the chromophore during P_{fr}-formation. This possibility was studied using *(23)* as a model for P_r. Comparison of *(23)* and *(19)* allows one to further analyze, whether a hydrogenated ring has similarly pronounced effects on the reactivity of bile pigments, as it has in the cyclic tetrapyrroles (cf. *e.g.* [103]). The products of photooxidation of *(23)* are summarized in Scheme 2. In presence of oxygen, the purpurins *(24)* and *(25)* are formed in a regioselective, self-sensitized singlet-O_2 reaction[66].

(25) is likewise accessible by a smooth dark-reaction[19], which is also suitable as a degradation reaction for biliproteins[67]. Spectroscopically similar products were observed during the dark reaction of *(19)* with singlet-O_2[104]; the typical spectrum with two bands[66], however, makes the proposed endoperoxide structure[104] unlikely and rather suggests the formation of purpurins as well. The influence of the hydrogenated ring on these reactions is very pronounced. *(23)* reacts much more rapidly than the fully unsaturated analogue and other bilindiones, and the reaction is regioselective at C-5. Although the spectroscopic characteristics of *(24)* and *(25)* correspond to denaturated P_{fr}, like other purpurins[109] they regenerate *(23)* in only small yields and under drastic conditions.

With regard to the reversion, especially the products *(26)* and *(27)*, derived from anaerobic photooxidation, seem attractive, because they correspond in their spectroscopic characteristics to denatured P_{fr} and because they can regenerate *(23)*, at least thermally. These reactions, too, are specific for the A-dihydrobilindione *(23)* and are regioselective at the C-5 methine bridge; corresponding reactions at C-15 or with fully unsaturated bilindiones were only observed as rare exceptions[106]. The dimerization of *(23)* to *(26)* is reminescent of the reversible photodimerization of pyrimidines in nucleic acids (cf. *e.g.* [107]). In phytochrome, the presence of two chromophores would be a precondition, which seems unlikely from the known data[14,18,21,55]. The formation of the pyridiniobilindione *(27)* demonstrates, however, that in principle other partners may take place in the reaction besides a second bilindione[41]. Probably, the reaction proceeds in two steps. The educt is first oxidized in one or two one-electron steps. Such a series of oxidations has been established electrochemically with bilindiones[108] and is supported by observation of long-lived cation radicals during oxidation of Zn-*(23)*[109]. In the second step, the nucleophilic addition of pyridine at C-5 takes place. Obviously, the reactivity of the intermediates is reversed. The *(27)* that is formed, formally possesses the conjugation system of the educt *(23)*; but its ab-

sorption spectrum is shifted to shorter wavelengths, because the Δ^5-bonding is twisted by the steric hindrance between the rings A, B and the bulky C-5 substituent[41,110].

Regarding the latter as a model reaction would require P_r to contain both an oxidant and a nucleophile. According to present day knowledge, amino acid residues are the only candidates, because phytochrome is transformed in solution and contains only carbohydrates[111] besides the chromophore. For oxidation, cystine residues are possible candidates, for addition tryptophan, tyrosine, serine, cysteine and the like. There do in fact exist certain indications to this effect; thus, P_{fr} contains one to two accessible SH-residues more than P_r[112,113], and *(23)* reacts with *e.g.* derivatives of tryptophan to give violins[106]; but here again a decision is not yet possible.

3. Chromophore-Protein Interactions

3.1. Molecular Ecology

The chromophores of native and denaturated biliproteins differ so conspicuously in their properties (Table 2) that a relationship between the two may seem unlikely. Phycobiliproteins can be denaturated reversibly and in excellent yields with urea or guanidinium chloride[22]. Since the covalent bonds between chromophore and protein are retained, the different properties of the chromophores in native and denatured pigments, respectively, are exclusively due to non-covalent interactions with the native protein. These interactions are essential in optimizing the chromophores for their function of photoreception. This includes (a) an increase in the extinction-coefficient by nearly one order of magnitude, leading to an increased light-absorption, (b) the suppression of radiationless deactivation to diminish energy losses in favor of fluorescence (phycobiliproteins) or of photochemical reactions (phytochrome), and (c) the chemical stabilization of chromophores (Table 2). In addition, also the positions of the absorption maxima of certain chromophores are variable between remarkable limits [*e.g.* 80 nm for *(1a)*] and thus permit a "fine tuning" of the energy transfer. The question as to the origin of these optimizations, which may be paraphrased under the heading "molecular ecology", is essential for an understanding of the function of biliproteins. Geometrical transformations, protonation-deprotonation, and a restricted flexibility of chromophores seem to be some of the essential factors.

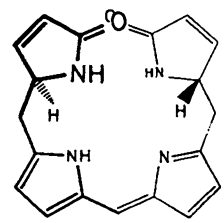
Table 2. Molecular ecology of the biliproteins. Comparison of the properties of native bilindiones, with those of denatured pigments and free bilindiones of similar structures.

	Free bilindiones or denatured biliproteins	Native biliproteins
UV/VIS absorption spectra (see Fig. 1)	Broad bands, intense ($\varepsilon \approx 35000$) [a] in the near UV, weak ($\varepsilon \approx 15000$) [a] in the visible range	Narrow bands, weak ($\varepsilon \approx 15000$) [a] in the near UV, intense ($\varepsilon \approx 100000$) [a] in the visible range
Photochemistry	Mainly radiationless deactivation, low quantum yields ($\leq 10^{-3}$) for fluorescence or photochemical reactions	High quantum yields for fluourescence (≥ 0.6 in phycobiliproteins) or photochemical reactions (≈ 0.15 in phytochrome)
Chemical stability	Poor; ready formation of metal complexes, facile nucleophilic addition or reduction at C-10, sensitive towards photooxidation	Good; little to no reaction with these reagents

[a] All extinction coefficients are given with respect to one chromophore. They have to be multiplied by the number of chromophores (Table 3) to obtain the extinction coefficients of the respective biliprotein. Somewhat varying ε-values are cited in the literature, due to different methods of determination.

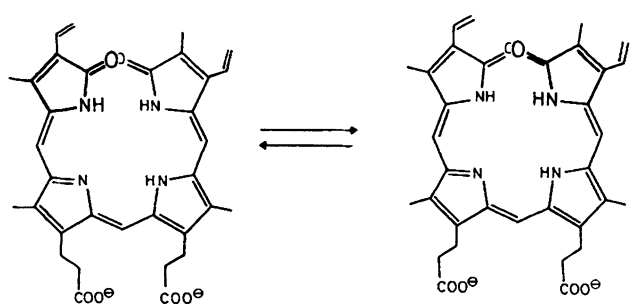
3.2. Geometry of Chromophores

The first detailed analysis of the geometry of free bile pigments stems from *Moscowitz et al.*[114], postulating a cyclohelical porphyrin-like structure in solution for urobilins [*(28)*; for sake of clarity, the substituents have been omitted], with the sign of the twist determined by the absolute configuration of the α-pyrrolic centers C-4 and C-16. Cyclohelical



(28)

structures have also been established for bilindiones, like *(19)*, both by X-ray analysis[115,116] as well as by ^{1}H-NMR[117,118] measurements and measurements of solvent-induced circular dichroism (SICD)[117,119] in solution. In the formally achiral bilindiones of the biliverdin type, this results in a uniform population of two enantiomeric conformations *(29a)* and *(29b)* which differ in their sign of rotation and are in equilibrium with each other ($\Delta H_0^{\neq} = 42$ kJ/mol for a derivative of *(19)*[120]). The equilibrium may be shifted sufficiently by dissolution in chiral solvents[117] or by adsorption to the chiral biopolymer serum-albumin[121] to allow the observation of typical Cotton effects of inherently dissymmetric chromophores. In support of this, purpurins like *(25)* containing planar chromophores[122] give no SICD-effect[123].



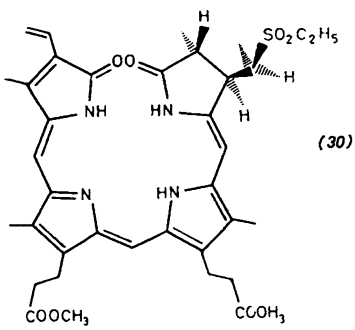
(29a) *(29b)*

The *E,Z,Z*-isomeric bilindiones are SICD-negative[90], too, thus supporting the suggested *anti-,syn-,syn*-conformation [Formula *(20)*][91].

The state of equilibrium between the two helical forms may also be shifted by asymmetric centers within the molecule. The high rotational values of optically active urobilins are due to the asymmetric centers C-4 and C-16 having the

same absolute configuration by which one form is preferentially populated for steric reasons[114]. Since the absolute configuration of the helix can be derived from the sign of rotation of helical chromophores, the absolute configuration of the assymmetric centers is thus also indirectly accessible[46,57,94b,114].

In the biliprotein-chromophores *(1)—(3)*, ring A has at least three asymmetric centers. The different steric hindrance

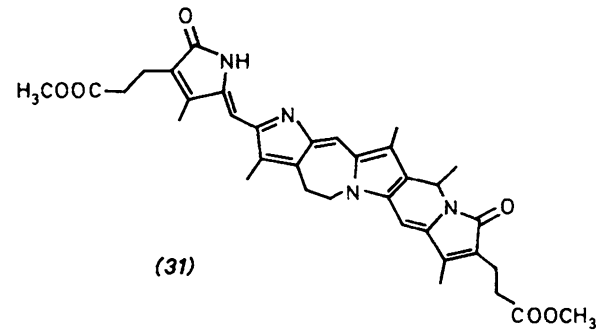


(30)

of the two forms has been estimated by Ramachandran calculations on *(30)* as a model of denaturated PC and P_r to result in an energy difference of $\Delta G^0 = 4—8$ kJ/mol[94]. Accordingly, denaturated PC exhibits a rather strong optical activity ($\Theta_{605} = 133000$[124]). In the chromophore *(2)* of PE, the influence of the asymmetric centers of ring A is opposite to the influence of the asymmetric C-16; accordingly the optical activity of denaturated PE is much lower[144].

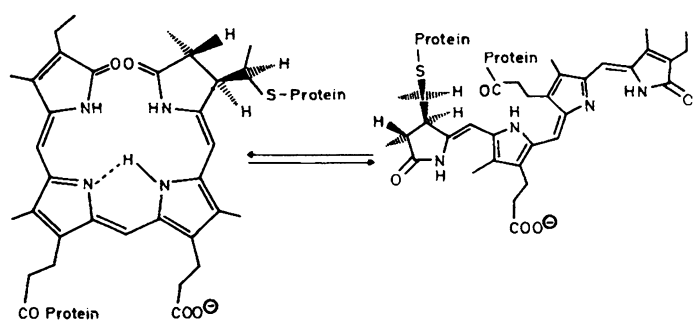
The hitherto described effects can only be rationalized in terms of a preferential cyclohelical conformation of free bile pigments of the biliverdin type. This conformation is, however, not rigid but rather flexible, and it also exists in equilibrium with more extended conformations. The broad absorption bands, which are unstructured even at low temperatures[19,125], refer to this situation, as well as the exceptions in planar pigments like *(25)*[66]. Recently, the problem was investigated in detail by the Mülheim group. It was possible, by fluorescence spectroscopy, to differentiate at least two conformer populations in biliverdin[119]. The form fluorescing at longer wavelengths was identified as a cyclic conformation, since only this one showed an SICD effect; the form emitting at shorter wavelengths was associated with a more extended conformation because of its higher ratio of absorption of the visible and near UV-band, Q_{uv}^{vis} *(vide infra)*. The equilibrium between both forms is dependent on temperature and solvent; the open form is preferred in viscous H-donor solvents[126], especially in lipid-vesicles[127]. Strong intramolecular H-bridges similar to those of bilirubin[128] were also implicated as the origin of the formation of isomers in biliprotein cleavage products. These H-bridges were only observed in the case of the free acids[43].

The denaturated biliproteins are so similar in their properties to free bile pigments of corresponding structure [*(18)* for PE, *(23)* for P_r and PC], that they also are expected to assume preferentially cyclohelical conformations. The modified spectroscopic properties of native biliproteins (*e.g.* PC, Fig. 1) originate mainly from their chromophores being rigidly fixed by the protein in an extended conformation[19,125]. The intensity ratio Q_{uv}^{vis} of the visible with respect to the near UV-band is mainly determined by the geometry of the chromophore. In the case of cyclohelical conformations Q_{uv}^{vis} is small and thus similar to that for a porphyrin, whereas in extended conformations it is large and thus similar to that for a polyene. This is one essentially consistent result obtained from MO-calculations by several groups[80,91,121,131,134] and is confirmed by the spectral data of free bilins of known conformation. $Q_{uv}^{vis} = 0.25$ in cyclohelical *(19) (vide supra)*, but is increased to 6.4 in isophorcabilin *(31)*, which by reason of its additional intramolecular bridges can only assume extended conformations[27b].



(31)

A value of $Q_{uv}^{vis} = 0.15$ was measured recently for a 21,24-methanobilindione, which necessarily possesses the *all-syn,Z*-conformation[32]. The variation in the Q_{uv}^{vis} of bilindiones due to formation of cations, anions and Zn-complexes is comparatively small, and the absorption in the near UV, in particular, remains essentially unchanged[36].



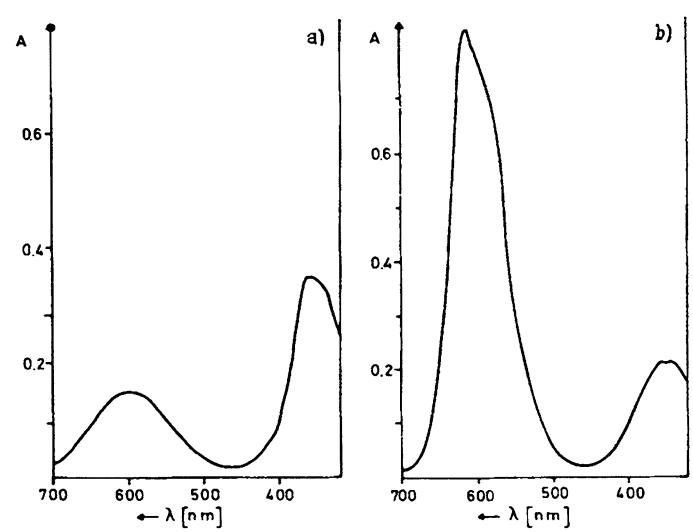


Fig. 1. Spectrum of denatured (a) and native phycocyanin (b) from *Spirulina platensis* (same concentrations in (a) and (b)). The denaturation by urea (8 M) is reversible; similar changes are observed upon thermal denaturation. The formulas depict the type of conformation for the two forms: cyclohelical for (a) and extended (in a sterically unhindered conformation) for (b).

In denaturated PC at pH 7.5, $Q_{uv}^{vis}=0.43$; it is increased to 4.1 in native PC, with both bands changing their intensity in opposite directions (see Fig. 1)[125]. Similar effects are observed with P_r[55]. Thus, the chromophores of both native pigments are present in an extended conformation. There is an interesting aspect of these findings: the extended conformations of bilindiones are higher in energy than the cyclic ones[92,94] and the necessary energy has to be provided by the protein. The energies of folding of several phycocyanins are indeed much lower than in other proteins of comparable size[135], *i.e.* the difference is possibly due to the strained chromophores[129]. In native PE, Q_{uv}^{vis} increases to a comparable extent as found in PC, as compared with the denaturated pigment[139,144]. To our knowledge, the relation between the absorption and geometry of chromophores of PE has only once been studied theoretically[144a], and extended pigments corresponding to bilindione *(31)* possessing the tetrahydro-conjugation system of rhodin *(18)* have so far not been reported. On the basis of calculations, however, it is likely that Q_{uv}^{vis} is also a certain indicator of the geometry of molecules, which would suggest extended chromophores for native PE, too.

The high ellipticities of the long-wavelength CD-bands of native PC as well as of PE would indicate an inherently dissymmetric chromophore, and therefore a more or less uniform twist of the extended chromophore. Generally, the bands have a positive sign. P_r is a remarkable exception in having a negative CD-band despite it presumably[37] having the same absolute configuration of the asymmetric centers as P_{fr} at ring A[135a]. At least in this case, an exciton-coupling would seem to be unlikely since phytochrome carries only one chromophore.

The picture is more complex with P_{fr}, the active form of phytochrome. On the one hand, Q_{uv}^{vis} of native P_{fr} is only half that in native P_r (see *e.g.* [18]) and, on the other, the absorption maximum of the native pigment is extremely red-shifted. Denaturated P_{fr} absorbs in its protonated form (pH = 2) at 610 nm[35], corresponding to a maximum for the free base at about 570 nm[35,71]. The absorption maximum of native P_{fr} ($\lambda_{max}=730$ nm) is shifted to the red by more than 160 nm ($\triangleq 3850\ cm^{-1}$). While the lower value of Q_{uv}^{vis} is still compatible with a rather extended conformation, the pronounced red-shift cannot be explained by a conformational change alone. Since the deprotonation of bilindiones leads to red-shifts of the same magnitude [*e.g.* 3800 cm^{-1} in *(23)*][36] this effect was rationalized as a deprotonation of the P_{fr}-chromophore[35] in the native pigment. Recent MO-calculations on chromophores with the conjugation system of *(3)* support this interpretation[133].

3.3. Charge and Chromophore-Chromophore Interactions

In addition to geometrical transformations, electric charges in the vicinity of the chromophore are possibly important factors for the functionally important fine-tuning (see Section 5.1) of the absorption maxima of phycobiliproteins. Phycobiliproteins contain up to four chromophores on one subunit and up to six within the ($\alpha\beta$)-monomer (Table 3). Although they frequently have the same molecular structure [*e.g. (2)* in C-PE] and by and large the same geometries,

Table 3. Classification and properties of biliproteins.

Type [a]	λ_{max} [nm] (VIS)	Chromophore α-	Chromophore β-chain	Chromophore γ-	Protein structure Monomer	Protein structure Aggregation	Mol.wt. [k] α-	Mol.wt. [k] β-chain	Mol.wt. [k] γ-	Occurrence	Ref.
APC I	656	1 × *(1a)*	1 × *(1a)*	?	$\alpha_1\beta_3\gamma$	1	18	18	30	Cyanobacteria, Red algae	[152, 181, 182]
II, III	650	1 × *(1a)*	1 × *(1a)*	—	αβ	(1), 3, 6	16	18	—	Cyanobacteria, Red algae	[1, 147, 149, 153, 154, 181–183]
B	670	1 × *(1a)*	1 × *(1a)*	—	αβ	(1), 3, 6	16	20	—	Cyanobacteria, Red algae	[148b, 184]
C-PC	635 [e], 620, (590) [f]	1 × *(1a)*	2 × *(1a)*	—	αβ	1, 3, 6	16	20	—	Cyanobacteria	[1, 13, 15, 20, 22–25, 147, 185–187]
PEC [b]	590, 568	1 × PXB [h]	2 × *(1a)*	—	αβ	3	17	20	—	Cyanobacteria	[32]
R-PC	620, 555	1 × *(1a)*	1 × *(1a)*, 1 × *(2)*	—	αβ	3, 6	18	20	—	Red algae	[72, 153, 188]
K-PC [b]	645, 610, 580 *etc.*	1 × *(1a)* *etc.* [33] [i]	1 × *(1a)*, 1 × *(2)* (?)	—	$\alpha\alpha'\beta_2$	1	9, 10	16	—	Cryptophytes	[33, 34, 189]
C-PE	575 [e], 560, 540	2 × *(2)*	3–4 × *(2)*	—	αβ	1, 3, 6	17	21	—	Cyanobacteria	[1, 13, 15, 20, 22–25, 154]
R-PE [c]	568, 540, 498 [g]	2 × *(2)*	4 × *(2)*	2 × PUB [j], 2 × *(2)*	$\alpha_6\beta_6\gamma$	1	19	19	35	Red algae	[22, 190]
b-PE	575 [e], 565, 540	2 × *(2)*	4 × *(2)*	—	αβ	3	19	19	—	Red algae	[153, 190]
B-PE	565, 545, 498 [g]	2 × *(2)*	4 × *(2)*	2 × PUB [j], 2 × *(2)*	$\alpha_6\beta_6\gamma$	1	19	19	35	Red algae [h]	[31, 153, 192–194]
K-PE	545–565	? × *(2)*	? × *(2)*	—	αβ	2	10	17	—	Cryptophytes	[1, 195–198]
Phytochrome P_r [d]	660	1 × *(1b)*	—	—	α	(1) 2	120	—	—	Green plants and others	[9, 13, 14, 18, 21, 191, 199–203]
P_{fr}	730	1 × *(3)*	—	—	α	(1) 2	120	—	—	Green plants and others	

[a] The prefixes are derived from the parent organisms: C = Cyanobacteria. R = red algae. B = *Bangiales* (on order of red algae). K = cryptophytes, b and B refer to different quaternary structure. [b] Phycoerythrocyanin. [c] R-PE is possibly a glycochromoprotein [25, 226]. [d] Phytochrome is probably a glycochromophosphoprotein [111, 201]. [e] Special form, possibly aggregate [147, 182]. [f] Shoulder, resolved below 77K [125, 137, 138, 151a]. [g] Shoulder due to phycourobilin chromophores [i]. [h] Chromophore of unknown structure $\lambda_{max,pH\,1-3,0}^{denaturated}=600$ nm. [i] Additional chromophors of unknown structure: $\lambda_{max,pH\,\sim 2,0}^{denaturated}=690$ nm [33, 34, 189]. [j] Phycourobilin chromophore, detailed structure unknown: $\lambda_{max,(1\,M\,HCl)}^{denaturated}=498$ nm [22]. [k] Approximate value 10^{-3}. [l] A pigment similar to B-PE has also been reported for a marine cyanobacterium [204].

too, they differ spectroscopically, chemically and functionally, due to the different environments of their chromophores (see Section 3.4) and their correspondingly different chromophore-protein interactions. For example, APC-B absorbs at 670 nm[148b], the s-chromophore of C-PC at 590 nm[36,140,142]; in spite of their absorption difference of 80 nm ($\triangleq$2020 cm^{-1}), however, they possess the identical chromophore *(1a)*. Chemical differences are evidenced, *e.g.*, by a different stability towards reduction[129] or unfolding[125], spectroscopically several absorption bands can be resolved, especially at low temperatures[125,136,139], in the fluorescence[139,143] and CD-spectra[144,147]. These differential effects are physiologically essential (see Section 5.1) for an optimal energy-transfer and they were investigated for the first time in some detail by fluorescence-measurements[140]. The site-specific chromophore interactions are obviously already fixed in the sub-units, since the CD- and absorption spectra are unchanged during aggregation to the ($\alpha\beta$)-monomers. In a C-PE the spectrum was identical with the sum of the subunit spectra[144]. In contrast, further aggregation of the monomers causes shifts to longer wavelengths of the visible bands. An additional long-wavelength band appears in the difference[148,154], low-temperature[136,137] and CD-spectra[145-147,152]. Its displacement is small in PC and PE (10—15 nm$\triangleq$250—450 cm^{-1}), but rather pronounced in APC. Monomeric APC absorbs at 620 nm, the trimer at 650—670 nm, at the same time the absorption is almost doubled[149].

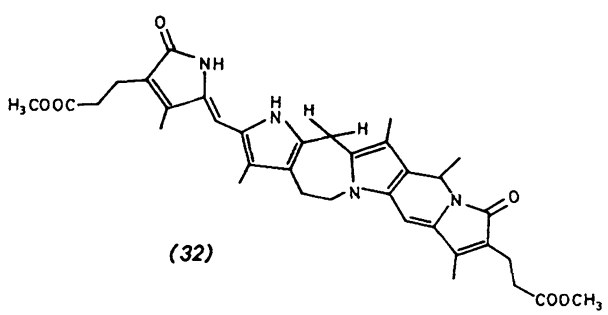
Recently, evidence has also accumulated for chromophore-chromophore interactions which have been related to displacements of absorption maxima. S-shaped CD-bands were observed in C-PE[144], B-PE[31], K-PC[34] and an allophycocyanin[152]. This type of band may be caused by exciton splitting, but a decision is difficult if several chromophores are present. The long-wavelength shift of the absorption-band[147] of the trimeric APC $(\alpha\beta)_3$[149] was recently interpreted as a chromophore-chromophore interaction of "intermediate strength" (=CD-inactive) between different monomers in the aggregate[150].

3.4. Increased Fluorescence and Chemical Stability

The second conspicuous feature of native biliproteins is their photochemical behavior. Phycobiliproteins fluoresce with quantum yields near to one (see [142]), phytochrome has quantum yields of 0.13 and 0.17 for the photoreversible transformation between the two forms[17]. Free bilindiones and denaturated biliproteins show only a very low fluorescence[96,119,130,132,155-157] and phosphorescence[156,158]; the "natural" *all-syn,Z*-bilindiones[66,105,159-162] and even the distinctly more reactive A-dihydrobilindiones[66,105] are also photochemically rather inert. The major pathway of deexcitation of free bile pigments or denaturated biliproteins is therefore radiationless deactivation. Two mechanisms are plausible: vibration-induced deactivation[163] and proton-transfer or even only perturbations of hydrogen-bond potential-curves[164]. Both are able to cooperate in a complex manner, since, *e.g.*, an intramolecular hydrogen bond is able to hinder torsional movements, but at the same time can open a new channel of radiationsless deactivation (see [163,164]).

Both mechanisms have been investigated on partial-structures[159] and—hitherto less systematically—on integral bile pigments[96,155-157]. Both were recognized as essential, but a quantitative estimation is difficult, since chemical manipulations will generally influence both factors. Thus, *e.g.*, the *N*-methylation of a dipyrromethenone will inhibit intermolecular proton transfer by suppression of dimerization in unpolar solvents[156], but will at the same time restrict the conformative mobility. Similar reasoning applies to *N*-protonation[96,155]. The influence of viscosity which essentially decreases vibrational relaxations has hitherto mainly[158] been investigated at lower temperatures[96,132,155]. Bilindiones[96,132,155] and especially conformatively more rigid pigments like isophorcabilin *(31)* show a moderate fluorescence at 77 K[155c].

In this respect, the rather strong fluorescence of isophorcarubin *(32)* is noteworthy[165]. The ring-A,B fragment of *(32)* is identical to that of a common 10,23-dihydrobilindione ("bilirubin"), and its fluorescence should therefore only be weak[86,166]. The C,D-fragment is rather rigid, however, and is incapable of allowing intramolecular H-transfer, so the fluorescence has thus been related to the latter[165]. Hitherto, no direct approach has been tested to quantitatively separate the contributions of proton-transfer processes; one possibility would be a systematic investigation of ^{2}H-isotope effects[167].



(32)

In biliproteins the radiationless deactivation is strongly decreased. The vibrational relaxation can be effectively decreased by a rigid fixation of the chromophores. Indications of this are the decreased bandwidths of the absorption spectra[66,125] which is also evident in the fluorescence-excitation-spectra[139-143], the large negative temperature coefficient of the fluorescence[17,139], and the small phonon-coupling to the protein[138].

An effective instrument for repressing proton-transfer-reactions would be the transfer of chromophores into a hydrophobic environment, *e.g.* into the interior of proteins. An extended conformation of the molecule would further inhibit intramolecular transfer. The lack of typical reactions of bilindiones, *e.g.* the formation of Zn-complexes[168] in biliproteins, could be evidence of this[22,169] as well as the quenching of fluorescence with benzoquinone[170,322].

On the other hand, there are several recent results which put the chromophores into a hydrophilic or at least water accessible position near the surface of the biliproteins: Thus there are reports of redox-reactions of chromophores at electrode-surfaces[171], in solution[99,100,129,172] and at membranes[173], of the reversible addition of thioles and dithionite (or rather sulfoxylate[174]) to the central methine bridge[129,172], of chromophore-chromophore interactions during the aggregation of APC[151], of low-temperature pho-

tochemistry related to proton transfer[138] and of an $H_2{}^{18}O$-exchange of *both* lactam oxygens at C-1 and C-19[175]. In the case of the reaction of PC, APC and PE with dithionite, it could be demonstrated that this reagent is in thermodynamic equilibrium with the chromophores. At least in this case, the decreased reactivity of the chromophores in the native pigment is thus determined thermodynamically and not kinetically[129]. Possibly, the conformational changes are one direct cause of the increased stability.

Perhaps the quantum yield of fluorescence is the most sensitive parameter of the state of phycobiliproteins. During controlled protein unfolding it already clearly decreases before the absorption- or CD-spectra noticeably change[144,176]. Under partial denaturating conditions, the photochemical behavior of phycobiliproteins changes in a complementary fashion. In presence of allylthiourea[178], the photochemical stability of PC is decreased[177]. In presence of 0.5 M guanidinium chloride[179] or on diminution of the pH-value to 3.8[180], solutions of PC and APC become photoreversibly-photochromic. The latter result is especially of interest with regard to the phycochromes[10-12] and possibly to phytochrome, too, because they show similar difference-spectra.

4. Proteins

Phycobiliproteins represent a period of evolution of about 3.5 billion years and they exist in procaryotes as well as in eucaryotes, in very different biotopes. They are thus of interest for comparative investigations which currently culminate in a complete sequencing of two PC-molecules[62,63]. The most important data of biliproteins that have been characterized spectroscopically and by their origin, are summarized in Table 3. The phycobiliproteins are globular and soluble in water, those isolated from red algae and cyanobacteria show pronounced aggregation. Analysis of the N-terminal sequences, immunochemical investigations and hybridization experiments indicate a common phylogenetic tree for these pigments (Fig. 2). The closest relationship exists between corresponding subunits of one type of protein from different organisms; a more distant relationship exists between corresponding subunits of different types of pigments and between the different subunits of the same pigment. Also the pigments of red algae and cyanobacteria seem to be more closely related with each other than than with the pigments of cryptophytes.

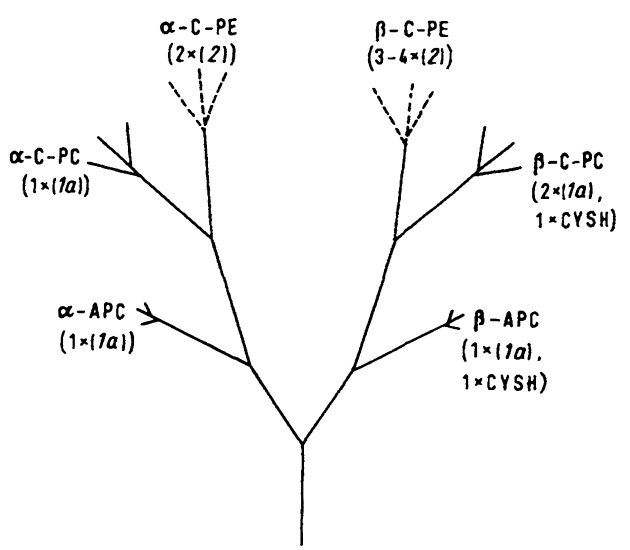


Fig. 2. Phylogenetic tree of biliproteins and their α- and β-subunits from cyanobacteria. C-PC = Phycocyanin, C-PE = phycoerythrin, APC = allophycocyanin (after [205]).

The protein moiety of phytochrome is distinctly different from that of the phycobiliproteins. It is considerably larger, it probably possesses a dumbbell-shaped structure, and P_{fr} in particular has a tendency to associate with membranes. Immunochemical and spectroscopical investigations demonstrate the distinct similarity of phytochrome(s) from different organisms. So far there is no direct indication of a phylogenetic relationship with the phycobiliproteins. The similar structure of the chromophores, their similar binding to the apoprotein, comparable non-covalent interactions, and finally the occurrence of phycochromes are indirect indications thereof, but they don't exclude a converging development.

4.1. Phycobiliproteins: Monomers

The most data available on primary structures are those for phycocyanins. Aside from the determination of N-terminal sequences (see[15,205,206]) and of chromophore binding regions[62-65,207-211] of several organisms, the sequences of PC isolated from *Mastigocladus laminosus*[62], a thermophilic cyanobacterium, and from *Cyanidium caldarium*[63], a monocellular thermoacidophilic red alga, have recently been established. The complete analyses of the β-chain of PC isolated from *Synechococcus spec.* 6301 (formerly called *Anacystis nidulans*)[64], a monocellular mesophilic cyanobacterium, and of the β-chain of APC from *M. laminosus* have also been carried out[205]. The homologies of the respective α- or β-chains of the two complete structures are remarkable (80 or 78%) considering the different taxonomic positions and biotopes of the organisms.

However both are thermophilic organisms and the sequence, known up to 80%, of the marine cyanobacterium *Agmenellum quadruplicatum* shows only a homology of about 30% (*L. Fox,* private communication). A high homology is also found on comparing the PC β-chains with that of *S. spec.* The α- and β-chains of PC from each of the two organisms show much lower homologies (25% in PC from *M. laminosus*)[205]. The relationship between the same subunits of pigments of a different spectral-type is also obvious in the primary structures. Thus, the β-chains of PC and APC of *M. laminosus* are homologous to 25%, and the relationship becomes even more distinct on comparing the secondary structures (as calculated by the method of *Chou* and *Fassman*)[205].

The homology is not uniform, but particularly high in the chromophore regions. An earlier survey indicated a very high homology of the N-terminal regions within the corresponding subunits of pigments of different origin and of different spectral-type[15]. The low homology of these regions of PC and APC of *M. laminosus* found by the "Zürich-group", is, however, the first example of an exception to this rule, or it indicates an exceptional position of APC. The homology of the chromophore binding regions is supported by comparison of sequences of chromopeptides isolated from different phycoerythrins[209] and cyanins[62-65,207,210,211]. Thus, five dif-

ferent amino acid sequences have been identified in the region of the binding cysteins in PE from *Pseudanabaena* W1173, containing five chromophores. PE from *Phormidium persicum,* containing six chromophores, possesses six different binding regions, five of which correspond unequivocally to the five regions of the first named pigment[209]. The sequences of peptides characteristic of the individual chromophores can be correlated with the spectra of the chromophores in these pigments[139]. Hitherto, little was known about the phylogenetic origin of the increasing number of chromophores on the individual peptide chains on going from APC to PC to PE. Interestingly, however, a comparison of PC and APC from *M. laminosus* shows that the additional chromophore binding site on the β-chain of PC is formed by insertion of a short peptide in the APC sequence[205].

Studies on the protein moieties of the biliproteins complement the data on the chromophore binding mode as derived from investigations of chromophores (see Section 2). With only one exception[213] all of the known "chromopeptides" contain cysteine[35,62 65,205 216]. The early proposition[212], that this amino acid takes part in chromophore binding was first verified for a B-PE peptide from *Porphyridium cruentum*[208], and it was later recognized that cysteine functions as the binding amino acid for *all* chromophores in several C-phycocyanins and phycoerythrins[62 63,209,211,216]. These results are supported by a comparison of the number of bonded cysteines and the number of chromophores in pigments of several red algae and cyanobacteria[217]. It follows, together with the proof of the thioether bond to ring A of the chromophores [Section 2.2, formulas *(1), (2)*], that the blue *(1a)* and red *(2)* chromophores are bound to the protein *via* cysteine residues. The yellow urobilinoid chromophores of B-PE and R-PE are supposed to have even a second cysteine-chromophore bond to ring D[22,217].

Pigments of cryptophytes have hitherto been the subject of fewer investigations; the finding of a single, free cysteine-SH in one chromopeptide is indicative of a special binding position[215].

There is less agreement on other and possibly additional chromophore protein bonds. In particular, the linking of serine with one of the propionic acid side chains is plausible[22,209,213,215]. There are no such indications from the sequenced phycocyanins and allophycocyanins, but such a connection is discussed as being the only binding site of a K-PC[215] and of a R-PE[22], and it was identified in addition to the cysteine bond in one C-PE chromophore[209]. The problem is complicated by the possibility of both artifactual hydrolysis of esters and the formation of new bonds during protein degradation (see [22]). *Crespi* and *Smith*[214] postulated a lactim-ester binding to aspartate to account for the easy splitting of the thioether bond, but investigations of models show that this activation is not necessary[54c,58,218]. Also, a binding to glutamate is reported for a cryptophytan chromopeptide[215].

Only little is known so far about the secondary- and tertiary structures. Although PC and PE in particular can be readily isolated and crystallize well, there exists as yet no high-resolution X-ray analyses[32,193,194,219,220]. This may be explained by an unfavorable packing, leading to partial obliteration of reflexes due to interference from neighboring molecules[221]. The amino acid sequences and the solubilities are typical for globular proteins, with APC containing a higher proportion of hydrophobic amino acids than PC and PE. The crystal packing[32,193,194] and the investigations in solution[222] show an oblong shape (2.5—3.5 nm diameter, 5—6 nm in length) of the (αβ)-monomer of PC; a size which has also been deduced for other biliproteins[223 225] from electron-microscopical measurements. According to circular dichroism measurements on several pigments, the α-helix content is about 60% in the α- and 40% in the β-chain[144,147]. The secondary structure of PC and APC of *M. laminosus* has been estimated from the sequence. Here, too, the α- and β-chains show differences, and there are indications of a different flexibility of the peptide backbone in the environment of the different chromophores[205].

As the second essential method, the immunochemistry confirms the relationships between the phycobiliproteins. The pigments of the same spectroscopic type, APC[148b,149,187,227 229] or PC[32,72,148b,149,187,227 229] or PE[198,227,228,230,231] are closely related immunochemically irrespective of their origin from the procaryotic cyanobacteria and the eucaryotic red algae. In contrast, the pigments of the different spectroscopic types do not undergo cross reactions, even if they are produced from the same organism. On this basis, R-PC[72] and phycoerythrocyanin[32,230], which contain blue chromophores *(1a)* as well as red chromophores *(2)*, were classified as phycocyanins, and APC-B as a true allophycocyanin[148b]. The subunits of individual C-PE[231] and also those of APC[149]—which are difficult to differentiate by other methods—are immunochemically not related, in contrast to the close relationship of corresponding subunits of pigments from other different organisms.

The cryptophytan-pigments, too, assume an immunochemically special position. First investigations[227] revealed no relationship with pigments from cyanobacteria and red algae; this has been supported for PC[228], whereas PC as well as PE from two different cryptophytes cross-react with PE from the red alga *Prophyridium cruentum,* but not with C-PE[198]. Accordingly, both pigments of cryptophytes seem to be related to PE from red algae.

Until now no defined immunological determinants are known. The missing cross-reaction of APC and PC has been taken as an indication of the chromophore not being a determinant, since both contain *(1a)*[231]. The different spectra of PC and APC, however, point to different states of this chromophore in the native pigments which may lead to significant differences in immunochemically relevant criteria, *e.g.* conformation and charge. There are also indications of the chromophore being accessible from the outside (Section 3.4). Immunochemical methods are principally suitable for a numeric taxonomy. With C-PE, isolated from seven different types of cyanobacteria, it could be demonstrated by a quantitative study that there are determinants specific of the species, as well as determinants specific of the spectral type, and that the results depend upon the method used (phage test or precipitation test)[230].

4.2. Phycobiliproteins: Quaternary Structure

The quaternary structure and higher aggregates have been investigated more thoroughly than the secondary and tertiary structures. They are decisive for the biological function of the phycobiliproteins. Most of the pigments possess an

$(\alpha,\beta)_n$-structure, with n-varying between one and higher than 12 depending on their state of aggregation and their origin *(vide infra)* (Table 3). In some pigments, yet a third subunit has been found, as in B-PE and R-PE $(\alpha_6\beta_6\gamma)$[153,193,194], in APC I $(\alpha_3\beta_3\gamma)$[182] and in PC from *Chroomonas spec.* with an $\alpha\alpha'\beta_2$ structure[189].

The molecular weights vary between 9200[189] and 20500[232] for the α-subunits and between 16000 and 23500 for the β-chain, with the α-subunits of the cryptophytan biliproteins at the lower limits. The γ-chains are considerably heavier. The subunits are classified by definition according to their molecular weights ($\alpha < \beta$). In the case of a different number of chromophores, the β-subunit always contains more than the α-subunit. An alternative classification is possible immunochemically. It has been proven especially useful with APC[149], which has subunits carrying one chromophore each and which may have very similar molecular weights[1].

The subunits can be separated preparatively by ion-exchange chromatography, after being partially[139,196] or completely[62-64,147,149,223,234] unfolded. The separately renaturated subunits preferentially aggregate to dimers. In a mixture, the native pigments $(\alpha\beta)_n$ can be reconstituted in good yields. Smooth hybridization in 40—60% yield has been found[233] with complementary C-PC subunits isolated from monocellular and filamentous cyanobacteria, respectively; and there are even APC hybrids from the subunits of cyanobacteria and the red alga, *Cyanidium caldarium*[149]. A limiting factor for the yields is certainly the sensitivity of the chromophore in the denaturated pigments.

The controlled denaturation of monomer pigments has been investigated in the case of PC[125] and PE[144]. In each case sequential effects could be observed. The fluorescence decreases first[144,176], followed by absorption changes of the chromophores (in C-PC in a stepwise fashion[125]), and finally the protein structure (observed by CD) melts[144].

Corresponding to the strong coupling between chromophore and protein the quantum yield of fluorescence and the absorption spectra are the most sensitive indicators of the state of the protein (see Sections 3.3 and 3.4), *e.g.* partial proteolysis or denaturation.

The thermodynamics of the unfolding of proteins has been investigated on phycocyanins from several biotopes. Starting with undefined aggregates, $\Delta G^{0'}$ for pigments from meso-, psychro- and halophilic organisms is in the range of 10—22 kJ/mol[135] for complete unfolding by 8 M urea. These values are considerably lower than for globular proteins of similar size and free of disulfide bonds[235,236]. Comparable values have been found only for pigments of thermophilic organisms[135]. A possible factor is the energy necessary for "stretching" the chromophores of the native pigments[129] (see Section 3.2). The refolding kinetics of PC from *Spirulina platensis* is multiphasic, with a rapid first phase ($\tau_{1/2} = 110$ msec) accessible by fluorescence as well as by absorption measurements[237].

Phycobiliproteins from cyanobacteria and red algae show (with exception of the already complex-monomers APC-I, B-PE and R-PE (Table 3)) a distinct aggregation, which was mainly investigated systematically by *Berns et al.*[23] on C-PC. Starting from monomers, preferentially tri- and hexamers are formed[238]. These are the basic building blocks both for the crystalline pigments[32,194,219] as well as for phycobilisomes[223-225], as the light harvesting superstructures of these organisms[5]. Dependent on the conditions of isolation, higher aggregates[23,239] and hetero aggregates[223,224,240] have also been found, which represent more or less intact fragments of phycobilisomes[5] and even aggregates thereof[225]. Electron microscopic investigations by *Mörschel et al.*[248] revealed that the hexamers of PC are made up of two torus-shaped $(\alpha,\beta)_3$-trimers, one on top of the other, and that in B-PE the γ-chain occupies the central cavity of the cylinder that is formed.

In the pH range close to the isoelectric point (≈ 5.3) the equilibrium is mainly shifted in favor of the hexamer. In dilute solutions at pH = 6 to pH = 5.4, an equilibrium between monomer and hexamer has been established for several phycocyanins with an equilibrium constant of about 10^{30}, in favor of the hexamer[239,241].

Also, trimer-hexamer equilibria[242] and—at higher pH values—monomer-tetramer[243] and monomer-dimer[244] equilibria have been studied by ultracentrifugation. At low concentrations, C-PE exists in a monomer-dimer equilibrium[139]. The aggregation is favored at elevated temperatures[238], at higher ionic strengths[245], by arenes[246], and by low concentrations of guanidinium chloride[247]; it is decreased by chaotropic salts[245] and H/D exchange[239,249]. These results, which indicate a high degree of hydrophobic interactions in aggregate formation, have been compared with the formation of detergent micelles[238]. The role of ordered water structures was recently studied in association experiments with tetraalkylammonium salts[250]. Since cyanobacteria also occur in extreme biotopes, the phycobiliproteins are suitable objects for the study of such adaptations. C-PC from a psychrophilic organism[251] possesses similar aggregation properties as the pigments from mesophilic organisms, whereas phycocyanins from halophilic[252] and thermophilic[253] organisms are clearly different. The molecular causes of these adaptions have also been investigated by calorimetric measurements of the protein folding[135] and sequencing studies[62].

In contrast to the pigments of the red algae and of the cyanobacteria, cryptophytan biliproteins show no significant aggregation. This is reflected in the absence of phycobilisomes in cryptophytes.

4.3. Phytochrome

Although ubiquituous in green plants, phytochrome has been purified from only a few species sufficiently enough to allow the chemical characterization of the protein.

Monomeric phytochrome from oats and rye has a molecular weight of about 120000[200,201] and it probably has a dumbbell shape[203,204] (for reviews see [1,14,18,21]). The hitherto best characterized pigment from oats is readily cleaved by endogenous proteases (at the incision?), to yield a still photochemically active fragment with a molecular weight of 60000[255-257]. This fragment is generally referred to as "small" phytochrome; for some time it was regarded as the native monomer. This partial degradation changes the absorption spectra, the photochemical quantum yield[262], the energy transfer from tryptophan to the chromophore[263], and the immunological properties[264]. "Large" phytochrome

forms aggregates, whose dimeric structure is confirmed by gel-filtration[203,256,258], sedimentation[201,254,255], and electron microscopy[254]. Additional forms of P_{fr} of even higher molecular weight have been reported, whose nature is still unknown[256]. Phytochrome contains about 35% hydrophobic amino acids and about 27 cysteines[201,203] including the chromophore-binding one. In addition, 4% sugar has been found in "small" phytochrome[111]; the "large" pigment contains one phosphate group[201,202].

There are conflicting results regarding the symmetry of the "large" phytochrome. Dimeric "large" phytochrome appears in the electron microscope as "tetramer" (9×9 nm)[254]. The molecular weight[255,257] of "small" phytochrome would also indicate two domains of roughly the same size to be present in "large" phytochrome. Two research groups have compared the peptides of the trypsin digests of "large" and "small" pytochrome from oats. *Stoker et al.*[259,260] have concluded a high degree of symmetry from the similar peptide maps, whereas *Kidd et al.*[261] arrived at the opposite conclusion on the basis of significant differences when using a different labeling technique. The available data also point to an asymmetric chromophore distribution, *i. e.* only one half contains a chromophore (see [14,18,21]). Antiserum against "large" phytochrome produces spurs with "small" phytochrome but not *vice versa*, which also indicates an asymmetry of the determinant regions[264].

The phytochromes from different sources are spectroscopically indistiguishable. Immunochemical experiments, too, reveal a close relationship between the hitherto investigated phytochromes from oat, rye, corn, barley, pea[203,258,264]. The amino acid analyses as well as the N-terminal sequences are different for "large" phytochrome from rye and oats, but this is possibly due to the purification procedures used[201,203].

For a better understanding of the function (see Section 5.3), protein-chemical differences between the two forms are essential. Following the works of *Tobin* and *Briggs*[191], who in 1973 questioned a greater part of the formerly reported differences, greatly improved isolation methods[200,265,267] and new analytical techniques led to more promising results. Thus, indications of a preferential interaction between P_{fr} and cholesterol[268] were obtained, and *Smith* discovered a preferential assoziation of P_{fr} with dextran blue[269] which may also be useful for the purification of phytochrome by sorption in the P_{fr}-form on dextran blue-agarose and subsequent desorption after irradiation with far-red light[269a]. P_{fr} isolated after *in vivo* transformation, contains a larger fraction of high molecular weight components (≥ 400000)[256]. In presence of bivalent ions, it binds relatively unspecifically to particulate fractions ("pelletability", see [270,271]). P_r and P_{fr} are immunochemically indistinguishable[272], and isoelectric focussing also gives identical results[113]. Significant differences were found, however, on titration of readily accessible amino acids[113]. P_{fr} contains one accessible cysteine and one histidine more than P_r, and the modification of two tyrosines inhibits the photochemical reactions.

4.4. Phycochromes

Originally, the term phycochromes was coined—in analogy to phytochrome—for the light sensory pigments in cyanobacteria (for a recent survey see [10]). Today, however, all potentially photoreversibly-photochromic pigments of these organisms are called phycochromes, irrespective of any known function (see [10]). To characterize the function of these photoreceptors, which in no case is as yet certain, *Bogorad*[1] proposed the term adaptochromes, in accord with the most important and obvious effect of light-regulation, the chromatic adaption (see Section 5.3). Already at an early stage, phycochromes were related to phycobiliproteins. *Scheibe*[273] succeeded for the first time in the enrichment of a fraction having photoreversibly-photochromic properties. Subsequently, the *Björns* characterized four such pigments as fractions of phycobiliproteins from different algae[274,275] which were called phycochromes a, b, c and d. Of these, phycochrome c is of particular interest, as its light-induced difference spectrum is similar to the action spectra of chromatic adaption of several cyanobacteria and to the photomorphogenesis of *Nostoc*. The phycochromes have been enriched by isoelectric focussing; in the case of phycochrome b an almost complete separation was possible from the photochemically inactive light-harvesting phycobiliproteins present in large excess. So far, only one of the proteins has been characterized in detail. It is suggested to be identical with the α-subunit of phycoerythrocyanin[275a].

A fascinating aspect was revealed by two research groups during recent investigations on partially denaturated "common" biliproteins. Treatment of PC and APC from *Tolipothrix tenuis* with 0.5 M guanidinium chloride[179] gave them photoreversibly-photochromic properties characteristic of phycochrome a or c[174], and likewise APC from *Fremyella diplosiphon* obtained the photochromic properties of phycochrome c, on lowering the pH to 3.8[180]. By this treatment the chromophores seem to be sufficiently decoupled from the protein such that the fluorescence is already decreased, but the radiationless deactivation is not yet prominent. The quantum yields of the photoreactions (about 10%) are comparable to those of phytochrome. Independent of the proof of the biological relevance of phycochromes these results throw new light on the physicochemical interactions between chromophore and protein, and on the possible phylogenetic relations between phycobiliproteins and phytochrome.

5. Biological Functions

5.1. Phycobiliproteins

Phycobiliproteins are antenna or light-harvesting pigments of cyanobacteria, red algae, and cryptophytes. The absorption bands of the most abundant pigments of this group, the phycoerythrins (PE) and phycocyanins (PC), are found in the green to orange spectral range. The light-harvesting pigments of green plants, chlorophyll a and b, absorb only slightly in this range, thus guaranteeing biliprotein producing organisms an ecological advantage in deep water and under a canopy of green plant.

The organization of biliproteins and the mechanism of energy transfer is very similar in cyanobacteria and red algae, but the cryptophytes differ considerably. In the former, the phycobiliproteins are localized in particles visible by electron microscopy, called phycobilisomes, which are located at the outer surface of the thylacoid membranes[5,224,276]. The phycobilisomes of different species have distinctly different sizes

and fine-structures, the ones most thoroughly investigated to date being those from the two red algae, *Porphyridium cruentum*[5,279] and *Rhodella violacea*[223,224] (see Fig. 3), and from the cyanobacterium *Synechococcus sp.* 6301 *(Anacystis nidulans)*[278]. Besides the light-harvesting pigments proper, *e.g.* PC and PE, they contain small amounts of several different allophycocyanins[148b,182], which are essential for the transfer of energy to chlorophyll. In addition, small amounts of colorless proteins have been described which are possibly important for the organization of phycobilisomes[223,278,280].

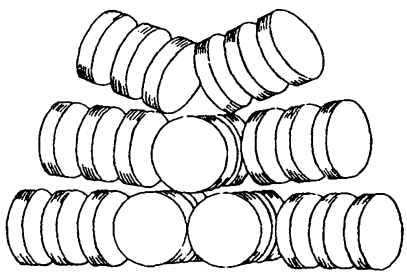


Fig. 3. Model of a phycobilisome of the monocellular red algae, *Rhodella violacea*. The "core" is made up from three APC hexamers, the rods fixed to it ("tripartite units") consist of one PC hexamer and two B-PE monomers, in this order when looking from the core (after *E. Mörschel, W. Wehrmeyer*, Ber. Dtsch. Bot. Ges. 92, 393 (1979)).

Within the phycobilisomes the different pigments are densely packed in such order, that the absorption maximum increases from the "outside" to the "interior" of the phycobilisome. *Gantt et al.*[5] developed a model for phycobilisomes from *Porphyridium cruentum*, which reminds one of an onion cut in half: layers composed of PE surround inner layers of PC, which surrounds an APC-core. This arrangement was derived from dissociation experiments in buffers of low ionic strength, whereby the pigments were released sequentially[5]. It was convincingly proved by fluorescence spectroscopy[281] and immuno-electron-microscopy[281,282]. Using the latter method, APC could be shown to be localized on that side of the phycobilisome facing towards the membrane[282].

The architecture of phycobilisomes of the monocellular red alga *Rhodella violacea* (see Fig. 3) is basically similar but perhaps even more impressive, due to their flatness[223,224]. Six conspicuous rod-shaped stacks are attached to the APC-core[224]. These rods can be isolated by careful dissociation[223]. By electron-microscopic inspection, they appear as stacks of three small double-disks ("tripartite units"). The double-disks have the dimensions and fine structure of hexameric C-PC $(\alpha\beta)_6$ or of the fundamentally similar B-PE $(\alpha_6\beta_6\gamma)$. The former are supposed to be identical with the C-PC-hexamers which have been observed during the *in-vitro* association or during the crystallization (homoaggregates, see Section 4.3). A further dissociation of the rods and an analysis of the fragments revealed that two adjacent double-disks consist of PE, the final one of PC, *i.e.* they form a heteroaggregate of two different biliproteins[223]. Such heteroaggregates of PC and PE were recently also isolated from *P. cruentum*-phycobilisomes and from cyanobacteria[240,283a,284]. They show an excellent energy-transfer from PE to PC (reversible dissociation, see [283a]). A reconstitution of phycobilisomes starting with the isolated biliproteins is not (yet) possible. Perhaps the aforementioned colorless proteins are necessary as structural elements[280]. In support of this, phycobilisomes partially dissociated into a crude APC fraction and a PC-PE-complex can be reconstituted[283b]. Since the tripartite units of *R. violacea* contain no colorless proteins, these rather seem to take part in the APC-PC-coupling or in the membrane-binding[223]. A first experimental indication of this is the recent identification of a heavy, colorless protein (molecular weight about 80000), in both isolated phycobilisomes and thylacoid membranes freed of biliproteins[283b].

Comparatively less is known about phycobilisomes of cyanobacteria, but the results indicate similar structural principles as in those of *R. violacea*. By electron microscopy, *Wildman* and *Bowen*[277] have identified phycobilisomes in all of the 27 tested cyanobacteria, in some of them with excellent resolution, but until recently the isolation was more difficult than in red algae. A complete separation from other pigments was achieved by immunochromatography, but the elution of the phycobilisomes purified in this manner was only possible under dissociating conditions[280]. Phycobilisomes have been isolated from *Nostoc* species[151a,284] from *Synechococcus spec.* 6031 *("Anacystis nidulans")*[278] and from other species[285,286]. The phycobilisomes of *S. spec.* 6301 appear in the electron microscope as aggregates of rods similar to those of *R. violacea* (Fig. 3). Here, too, APC seems to be located at the coupling position, since the energy transfer from PC to APC is interrupted by the dissociation[278]. Recently, the electron-microscopic characterization of phycobilisomes of two other cyanobacteria was achieved by using zwitterionic detergents which are reported to inhibit the aggregation during purification[225]. These investigations confirm the general structural principle of Figure 3, with variations in the number of the central APC-disks, the number and the length of the "branches"[278], and also by their arrangement in two (Fig. 3) or three dimensions (*P. cruentum*[279,282]). The phycobilisomes of chromatically adapting cyanobacteria show small but distinct differences in size and arrangement[287], as well as in the pattern of the colorless proteins[280]. The "branches" of *Tolypothrix tenuis* increase in length in green light[288]. In this process, not only additional PE-units seem to be added, but also part of the PC is removed.

Phycobilisomes are extraordinary efficient antennas which absorb light by a high effective cross-section, and transfer the excitation energy to the reaction-centers. This energy transfer has quantum yields up to 100%[142,143,289,290] and is only decoupled under starving conditions (light[201], N[292]). The transfer mainly occurs to photosystem II (PS II), as was originally concluded from the bichromatic action-spectra[3,4]. Heterocysts contain no PS II and have been regarded as being free of phycobilisomes[293] (see, however, [323]). Recently, *Katoh* and *Gantt*[294] isolated photosynthetic vesicles with the phycobilisomes still bound, showing PS II activity. This activity decreases in parallel with the dissociation of the phycobilisomes (induced by a decrease in ionic strength). The coupling between the phycobilisomes and the reaction centers is variable and depends on the physiological conditions[295]. For *Anabaena variabilis*, grown heterotrophically in darkness, however, an energy transfer to PS I was reported, and recently in PS I samples from *Chlorogloea fritschii*[296], fluorescence-spectroscopic evidence was obtained for the occurrence of APC. APC fluoresces similarly to certain chloro-

phylls[294], but direct support comes from the results cited in ref. [323].

One reason for the efficient energy transfer is the organization of the phycobilisomes. This ensures that the energetically favored "downhill" transport of excitation-energy from the "high-energy PE" to the "low-energy APC" is directed at the same time from the PE periphery to the APC-core of the phycobilisome. Possibly, a recently described "foot structure"[279] serves for further transfer into the membrane and for the fixation of the phycobilisomes[279]. A second factor is the intense absorptions of the biliproteins ($\approx 10^5$ $cm^2 \times mol^{-1}$) which cover almost the entire spectral region from about 500 to 670 nm (cf. [5]), combined with a high degree of fluorescence (= low radiationless deactivation) of the chromophores[142] and distances of 3.5—6 nm between the chromophores[139, 142, 297]. These distances are optimal in a disordered structure for the energy transfer between suitable chromophores by the Förster-mechanism, because they are below the critical radius. As has recently been described for C-PE, the quantum yield of fluorescence increases by aggregation of isolated subunits to the monomer[139]. It increases even more during aggregation of the monomers to tri- and hexamers[141], or to the quasi-hexamer B-PE[142]. At the same time the fluorescence-polarization[139, 142, 297] and the time constant of the energy transfer decreases[297].

Due to the rapid migration of energy and the greater probability of a "downhill"-transport of energy, the fluorescence in each aggregate[139] is emitted almost exclusively from the chromophores, absorbing at the longest wavelengths, which were thus classified as "f"-chromophores[140]. The "s"-chromophores absorbing at shorter wavelengths act as sensitizers, and only exceptionally show a detectable fluorescence[139]. During the gradual association of phycobilisomes, this transfer chain is extended successively to yield "Förster-cascades". Due to their architecture, each pair of neighboring pigments provides for an optimal overlap of the emission band of the donor and the absorption band of the acceptor (Table 4). This process was demonstrated for biliproteins with different chromophores[140, 142, 297], of heteroaggregates[223, 253], and of whole phycobilisomes[143, 289, 290]. In the intact phycobilisome, the result of this process is such, that the chromophores of APC, the biliprotein present in least amount, are the only ones to fluoresce, and all the remaining chromophores serve as sensitizers, with a transfer-probability of "at least 99%" in phycobilisomes of *P. cruentum*[290].

Table 4. Schematic representation of energy transfer within a phycobilisome like the one shown in Fig. 3 (only one tripartite unit and one APC are considered), and of the absorption and emission spectra of the phycobiliproteins (averaged absorption maxima, the emission maxima of "s"-chromophores have been estimated from the absorption maxima by assuming a Stokes-shift of 15 nm).

Pigment	Chromophore	Absorption [nm]	Fluorescence [nm]
P-BE	PUB [a]	500	515
	"s" *(2)*	545	560
	"f" *(2)*	570	585
C-PC	"s" *(1a)*	590	605
	"f" *(1a)*	620	635
APC	*(1a)*	650	665
Chl a_{II}		686	
		↓ photochemistry	

[a] PUB = phycourobilin chromophore.

Cryptophytes possess biliproteins as antenna-pigments, too, but they are distinct from those of cyanobacteria and red algae (for a recent review, see [298]). Cryptophytan biliproteins are localized on the inner side of the thylacoid membrane[299]. Hitherto, the search for phycobilisomes has met without success, although *Wehrmeyer et al.*[300] recently obtained electron-microscopic evidence of particulate structures which may contain biliproteins. The energy transfer in cryptophytes must also be different, since no APC has so far been detected, and generally they only contain either PC *or* PE. As in the biliproteins of cyanobacteria and of red algae[34, 297, 300], only the chromophore lowest in energy fluoresces. In accord with the exciton-coupling between the chromophores postulated by *Jung et al.*[34], *Kobayashi et al.*[297] reported an extremely fast transient (energy-transfer) (≤ 8 ps) in PC of *Chroomonas*. The transfer from K-PC to chlorophyll a by the Förster-mechanism appears unproblematical, especially in pigments like PC-645 from *Chroomonas*[34, 189, 300] and PC-641 from *Hemiselmis virescens*[33], absorbing and emitting at rather long wave-lengths. In contrast, the emission-band of PE is located in a region of minimal absorption of chlorophyll a, so that either only incomplete energy-transfer is possible, or another pigment has to be positioned between them[301]. Indeed, cryptophytes contain possible candidates, *e.g.* chlorophyll *c*.

According to the data summarized above, the morphology of the antennas and the structure of the biliproteins would seem to be closely related. Each biliprotein of the cyanobacteria covers only a comparably small portion of the spectrum. Only APC has properties favorable for energy transfer to chlorophyll; an energy transfer of PE to chlorophyll requires the aggregation of several pigments. This disadvantage is compensated for by the flawless architecture of the phycobilisomes, which enables a vectorial energy flux direct to the reaction-center of PS II. In the cryptophytan phycocyanins, such energy transfer-chains are already realized within the individual biliproteins. *Chroomonas*-PC, for example, contains chromophores absorbing in the region from 573 to 652 nm[32, 189], so a higher degree of organization would seem unnecessary. Finally, in the red algae both strategies are combined. They contain phycobilisomes, as well as the pigments R-PC, R-PE and B-PE, each of which contain different chromophores already joined to an energy transfer-chain of moderate length.

In addition to the well documented light-harvesting functions, other functions of phycobiliproteins are discussed. Under conditions of deficient N[11] and S[302], phycobiliproteins are degraded as protein-reserves (?). In cyanobacteria in particular they constitute a considerable fraction of the total protein, and basically they are of less importance for photosynthesis than the reaction centers. They also may have functions in light protection, since mutants free of biliproteins are more sensitive to light than the wild-types.

Finally, there is substantial evidence for phycobiliproteins, also having or having had functions in the electron transfer[303, 304]. A corresponding function *in vivo* has as yet not been detected, and may have been lost during evolution.

Electrodes covered with biliproteins show photopotentials[171,305]; moreover, PC catalyzes electron-transfer through synthetic lipid-membranes[173,304]. In both cases the action spectra and absorption spectra are similar. PC undergoes specific interactions with Fe^{3+}, but not with Fe^{2+}, and it shows an asymmetric effect on the electron transfer of charged synthetic membranes loaded asymmetrically with that pigment[306]. The redox potentials of free bile pigments have been calculated theoretically[95,307], and the chromophores of native biliproteins have been shown to be much more stable towards redox reagents than those of the denaturated pigments[35,129].

5.2. Phytochrome

The function of phytochrome is that of a light-sensory pigment of green plants. In the transformation from heterotrophic etiolated growth in higher plants, *e.g.* in seedlings below the surface, to autotrophic, photosynthetic growth, phytochrome mainly functions as a sensor of light as such, in the "high-energy reactions" phytochrome functions as a sensor of light-intensity, and finally due to the P_r and P_{fr} equilibrium being dependent on the spectral distribution of the light especially within the photosynthetically important red spectral region, it renders possible a "color-vision" in green plants. Whereas a large variety of physiological and some structural aspects of phytochrome have been investigated in considerable detail[9,14,16-18], knowledge of the mechanism of information-transfer and -transduction is as yet only fragmentary. The models are difficult to evaluate in this context and shall only be outlined here. For further information, the reader is referred to recent reviews[9,14,16b,17,18,21,303,309].

Phytochrome is synthesized in its physiologically inactive form, P_r; the beginning of each physiological reaction sequence is the photochemical transformation into P_{fr}. The formation of P_{fr} is fundamentally and nearly completely reversible by light $\geq$740 nm; this is not true, however, for all steps of the subsequent reaction-sequence. The *in vivo* decomposition of P_{fr} or P_r-receptor complexes is a simple example (cf. *e.g.* [14,18]). Depending on the duration of the reaction (τ_{rev}) until an irreversible step is reached, the physiological answer is reversible (or annulled) only for a certain time. Since the time (τ_{rev}) of escape from reversibility varies among the diverse physiological answers by several orders of magnitude, the phytochrome answers have been classified into modulation (large τ_{rev}-value = "reversible") and differentiation processes (small τ_{rev}-value = "irreversible") (for recent discussions, see [18,308]). A variety of biochemical and physiological results critically summarized by *Marmé*[309] have indicated, at least for the modulation reactions, reversible changes of membrane properties to be operative.

Based on the hitherto known differences between P_r and P_{fr} (see Section 4.4) and on the photochemistry of model-systems (see Section 3.3), several hypotheses have been proposed for the primary reactions. *Song et al.*[263] postulated a light-induced photoisomerization[40] by proton-transfer, by which a receptor site formerly covered by the chromophore becomes accessible. *Hunt* and *Pratt*[113] argue, that chemical modifications of amino acids newly accessible by this transformation should interfere with the photoreversibility. As this is not the case[113], they regard this result as support of the conformational change of the protein, discussed by *Smith*[9a] as a primary signal. Finally, a third hypothesis postulates a reversible redox-reaction between protein and chromophore with the formation of a new chromophore-protein bond[106]. Each of these hypotheses is able to explain modification of membrane-properties, directly by redox and/or protonation-deprotonation processes, and indirectly by conformational changes by uncovering receptor binding sites, but a differentiation is not yet possible.

5.3. Phycochromes and Phycobiliprotein Biosynthesis

The biosynthesis of the phycobiliprotein-chromophores follows fundamentally similar pathways as does the formation of the more thoroughly investigated mammalian bile pigments. δ-Aminolevulinic acid is condensed to a cyclic tetrapyrrole, most probably protoporphyrin, which is subsequently opened oxidatively resulting in formation of a bile pigment with loss of the former C-5 as carbon monoxide[311-313]. The ring-opening process is formally and mechanistically similar[314] to the heme-oxygenation[315], but chemical evidence would indicate that the ring-opening of a Mg-porphyrin *via* 7,8-dihydroporphinatomagnesium may also be possible[161]. It is also not clear, whether the apoprotein is bound to the chromophore after (and not before) the ring-opening, and whether it adds to the 3-ethylidene group of bilindione *(4)* (and not to the vinyl-groups of a precursor). A hint as to the alternatives, not put in brackets here, is the finding[312] that *Cyanidium caldarium* excretes *(4)* as well as addition-product(s) of *(4)* in the dark[54c,316]. Another indirect indication is the facile and reversible addition of nucleophiles to *(4)* and *(6)*[54c,58,59], and very recently *Troxler et al.* (private communication, 1980) have demonstrated the uptake of heme and its conversion into phycocyanin in cyanobacterial protoplasts.

The biliprotein-synthesis in most of the cyanobacteria and at least in some red algae is regulated by light. It is of special interest that the antenna-pigments adjust to the quality of light available ("chromatic adaption"). In prevailing red light, especially the blue phycocyanins are formed, whereas in green light, *e.g.* under a canopy of leaves or in deeper waters, the red phycoerythrins are preferentially formed[1,11,287,317]. This effect has been investigated mainly with *Tolypothrix tenuis*[12,318] and with *Freymella displosiphon*[319,320]. Based on the action spectra, the chromatic adaption has been explained in terms of photochromic receptor pigment-systems, functionally termed adaptochromes, or chromes. There are also photomorphogenetic effects in cyanobacteria and red algae which are connected with such receptors. Little is known about receptor pigments (see Section 4.4), and virtually nothing about their mechanism of action.

6. Concluding Remarks

For a long time phycobiliproteins have been the victims of "mammalian chauvism" and, owing to their covalent protein bonds, have been much less investigated than the other tetrapyrrolic pigments of photosynthesis, the chlorophylls. In contrast to the latter, however, the phycobiliproteins have

the great advantage of not being integrated into membranes. This property and the discovery of the central function of another biliprotein, phytochrome, during the development of plants, recently led to a boom in biliprotein research. No other photosynthetic light-harvesting system is as well investigated as the phycobilisomes, and—with the exception of rhodopsin—no light-sensory pigment is as well characterized as phytochrome.

In this report an attempt has been made to correlate the properties of the isolated chromophores with the function of the pigments *in vivo*. The correlation is somewhat subjective and partly fragmentary, but has been borne out for the most part in recent years. Some of the obvious gaps in the case of phytochrome are the structure of P_{fr} and the closely related questions regarding the nature of the primary signal and the role of specific interactions between phytochrome and certain receptor membranes and organelles. An advancement in this area should certainly stimulate further investigation of the related phycochromes as well.

Many of the numerous questions arising from the functionally and morphologically impressive model of the phycobilisomes will only be possible to answer by the collaboration of biophysicists and biochemists. Details on the chromophore-protein interactions and the interrelationships between the biliprotein-chromophores and the chlorophylls within the photosynthetic membrane are essential for an understanding of the energy transfer on a molecular basis. The increasing complexity of the phycobilisome structures raises questions as to their elements of organization, their biogenesis and its regulation. The answers will not only be of interest to the inquisitive, but may perhaps also contribute to our understanding of light-harvesting and information-transduction in general.

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COMMUNICATIONS

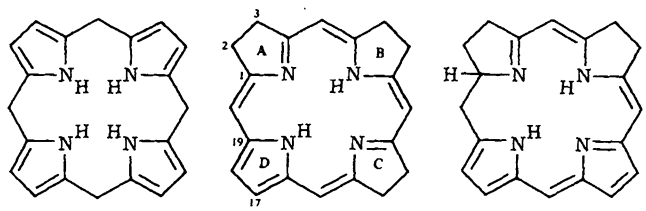
Communications are brief preliminary reports of research work in all areas of chemistry which, on account of its fundamental significance, novelty, or general applicability, should be of interest to a broad spectrum of chemists. Authors of communications are requested to state reasons of this kind justifying publication on submission of their manuscript. The same reasons should be clearly apparent from the manuscript. In cases where the editorial staff decide, after due consultation with independent referees, that these conditions are not met, manuscripts will be returned to the authors with the request to submit them for publication in a specialist journal catering for scientists working in the field concerned.

Interconversion of the Chromophore Systems of Porphyrinogen and 2,3,7,8,12,13-Hexahydroporphyrin[**]

By *Jon Eigill Johansen, Virginia Piermattie, Christof Angst, Eva Diener, Christoph Kratky,* and *Albert Eschenmoser*[*]

Dedicated to Professor Hans Herloff Inhoffen on the occasion of his 75th birthday

Our search for a non-oxidative isomerization of porphyrinogens *(1)* to the corphinoid ligand system of 2,3,7,8,12,13-hexahydroporphyrin *(2)* led at first to structures of type *(3)*[1a,b], in which the cyclic conjugation of the chromophore double bonds is broken, and not to *(2)*. We have now been able to convert a porphyrinogen into the ligand system *(2)*, about which only little is known so far[2]. This structure is of interest in relation to the problem of the origin of the corrin



(1) *(2)* *(3)*

structure[1b], and also to problems of contemporary corrin biosynthesis[3].

The tautomerization of octaethylporphyrinogen *(4)* under strict exclusion of oxygen produces nickel complexes of type *(3)*, as described earlier[1a]. This transformation proceeds faster, and gives different products, if instead of triethylamine the guanidine derivative 1,5,7-triazabicyclo[4.4.0]dec-5-ene (TBD)[4] is used as the base for tautomerization. Under the conditions shown in Scheme 1, *(4)* forms a mixture of nickel complexes, which apart from didehydrogenated components[5a] consists mainly of the diastereomers *(5)* (see Table 1). After anaerobic column chromatography on silica gel, a total of seven diastereomers were discernible by high pressure liquid chromatography (HPLC); three of them could be separated preparatively by HPLC on silica gel and crystallized, the major components being *tctcc-(5)*[5b] and *tctct-(5)*[5c]. The assignment of configuration for *tctct-(5)* followed from the molecular symmetry evident in the ^{1}H-NMR spectrum, as well as from the spontaneous didehydrogenation in air to the known[1a,d] Ni^{2+}-isobacteriochlorinate *tct-(6)* and a Ni^{2+}-bacteriochlorinate (*ttc-(7)*, Table 1). The corresponding didehydrogenation of *tctcc-(5)* gives as the main product *tct-(6)*[6] and a new (therefore not the *ttt*-configuration[1a,d]) isobacteriochlorinate, which must have the configuration *tcc-(6)*. X-ray structure analysis of *tctcc-(5)* (Fig. 1) again reveals the specific macro-ring deformation, which was previously

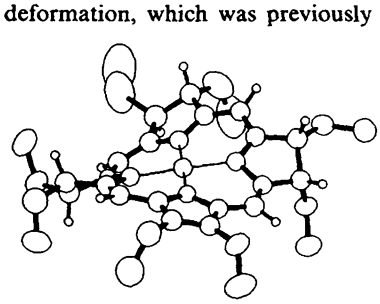


Fig. 1. Crystal structure of *tctcc-(5)*. Projection oblique to the ligand plane, with ring D in foreground. Positions of the hydrogen atoms are calculated, vibrational ellipsoids of non-hydrogen atoms with 50% probability (cf. also Table 1 and [1c], Fig. 3).

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