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EDITED IN COLLABORATION  
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VOLUME 36c

1981

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VERLAG DER ZEITSCHRIFT FÜR NATURFORSCHUNG  
TÜBINGEN

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# Chromophore Content and Molar Absorptivity of Phytochrome in the P<sub>r</sub> Form

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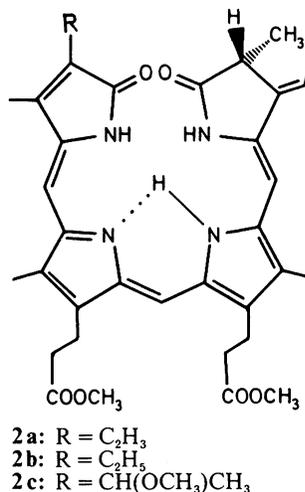
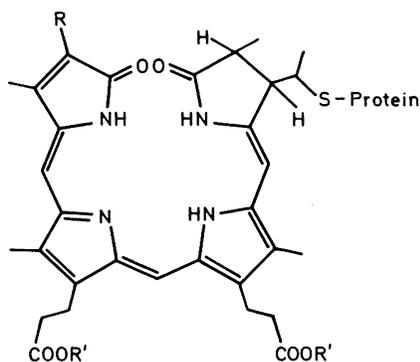
Z. Naturforsch. **36 c**, 431–439 (1981); received February 20, 1981

Absorption, Spectroscopy, A-Dihydrobilindiones, Isophorcabalin, Phycobiliproteins, Phytochrome, Phytochromobilin-Peptides

Quantitative absorption spectra in the visible and UV region were recorded for denaturated phytochrome (P<sub>r</sub>) and phytochromobilin peptides in comparison with native P<sub>r</sub>. The method was tested with C-phycoerythrin from *Spirulina platensis*. Based on known molar absorptivities for denaturated phycoerythrin and suitable model compounds, and on the ratio  $A_{\text{native}}^{620}/A_{\text{denaturated}}^{655-665} = 2.9$ ,  $\epsilon_{\text{native}}^{620}$  was determined to be  $102000 \text{ M}^{-1} \text{ cm}^{-1}$  for one phycoerythrin chromophore in native phycoerythrin. Likewise,  $\epsilon_{\text{native}}^{360}$  was calculated to be  $19000 \text{ M}^{-1} \text{ cm}^{-1}$ . The corresponding ratios for P<sub>r</sub> were  $A_{\text{native}}/A_{\text{denaturated}} = 3.4$  and  $A_{\text{native}}/A_{\text{denaturated}} = 3.7$ ; this yielded  $\epsilon_{\text{native}}^{665} = 109000$  to  $118000$  and  $\epsilon_{\text{native}}^{360} = 36000 \text{ M}^{-1} \text{ cm}^{-1}$  for phytochromobilin in native P<sub>r</sub>. This value corresponds to one phytochromobilin per small phytochrome (60000 D) if data from the literature are corrected for the content of colorless proteins. The latter has been assessed from (i) the purity index ( $A_{280}/A_{665}$ ) and (ii) the contribution of the phytochromobilin chromophore at 280 nm as derived from model compounds.

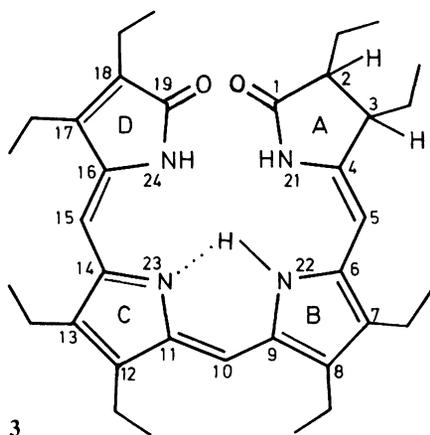
Phytochrome, the light receptor for most photomorphoses in higher plants (reviews: [1–3]), is a biliprotein. A characteristic feature of plant biliproteins (phytochrome, phycocyanins, phycoerythrins) are covalently linked bilin chromophores the spectral properties of which are changed upon cleavage from the protein [4–6]. This is due to the presence of a thioether bridge between chromophore and protein. Cleavage yields an ethylidene double bond instead of a single bond which leads to an additional contribution to the conjugated system of the chromophore [4–6]. The thioether (structure **1a**) was proven in phytochrome by absorption spectroscopy and degradation experiments [7, 8]. The same structure **1a** was derived from <sup>1</sup>H-NMR spectroscopy of a phytochromobilin peptide [9]. The chemical structure (**2a**) of free phytochromobilin has recently been elucidated [10] by its cleavage, isolation, and comparison with the authentic compound obtained by total synthesis [11]. Phytochromobilin differs from phycocyanobilin **1b** and **2b** [5, 6] only in a substituent of ring D: the former is the 18-vinyl, the latter the 18-ethyl compound.

The spectroscopic properties of biliprotein chromophores are profoundly altered by non-covalent interactions with the native apoprotein, which im-



Reprint requests to Prof. Dr. W. Rüdiger.

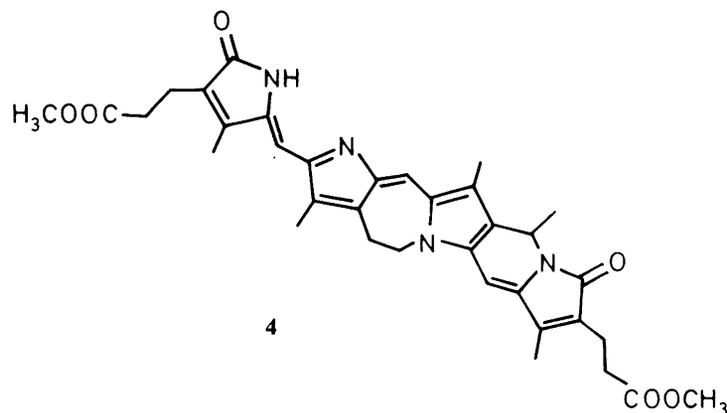
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pedes the determination of molar absorptivities, and the number of chromophores. The chromophore content of phycocyanins [12], phycoerythrocyanin [13] and phycoerythrins [14, 15] has been determined by quantitative absorption spectroscopy of the denatured biliproteins and comparison with the known extinction coefficients of free bilins. We describe here a similar approach for phytochrome from *Avena sativa* which included calculation of the extinction coefficient of native phytochrome. The method was tested with C-phycocyanin from *Spirulina platensis*.

### Experimental

Small phytochrome (60000 D) was isolated from 3.5 day old etiolated oat seedlings [16] according to previously described procedures [10]. The purity index  $A_{280}/A_{665}$  of the fractions used in this study was 1.5 or lower.



C-phycocyanin was isolated from *Spirulina platensis* as previously described [17, 18]. The purity index  $A_{620}/A_{280}$  was  $\cong 5$ . Spectra of this phycocyanin preparation were recorded in 10 mM Tris/HCl, pH 7.4, containing 10 mM KCl, 1 mM EDTA, 1 mM  $\text{NaN}_3$ . 2,3,7,8,12,13,17,18-Octaethyl-2,3-dihydrobilindion (3) [19] and isophorocabilin (4) [20] were prepared by known procedures.

Quantitative denaturation with acidic urea was performed in Thunberg cuvettes under nitrogen as described earlier [7]. By this method, bleaching of the chromophore during denaturation was avoided. For quantitative proteolysis, phytochrome and phycocyanin, respectively, were dissolved in 10 mM Tris/HCl buffer. Formic acid was added to a final concentration of 5% and then 1 mg lyophilized pepsin (Merck, Darmstadt) per 10 mg total protein content of the solution. Preliminary experiments had shown that proteolysis is completed within less than 30 min under these conditions.

To study the effect of solvent and urea on the UV-vis absorption spectra, stock solutions ( $\sim 29 \mu\text{M}$  of the models 2c and 3) in methanolic sulfuric or hydrochloric acid (1% v/v) were diluted with defined mixtures of methanolic sulfuric acid and a solution of 8 M aqueous urea which was acidified with HCl to pH 1.5, or with mixtures of methanolic aqueous acid.

UV-vis spectra were recorded on a DMR 22 (Zeiss, Oberkochen) or PE 320 (Perkin Elmer Bodenseewerk, Konstanz) spectrophotometer.

### Results and Discussion

An essential step in our previous determination of the chromophore content in phycoerythrins [15] was

the quantitative denaturation and proteolytic digestion of native phycoerythrins. This allowed the correlation of extinction coefficients between the native state and the denatured state or the chromopeptide, respectively. Such investigations have not yet been performed with phycocyanins. We used here C-phycocyanin from the cyanobacterium *Spirulina platensis* because quantitative spectral data [21] and data of quantitative denaturation at neutral pH [18] are available for this biliprotein. The data of Table I show that quantitative denaturation with acid urea and quantitative proteolytic digestion with pepsin lead to the same qualitative and quantitative changes of the spectrum of native C-phycocyanin. This corresponds with the situation in C-phycoerythrin [15]. On the basis of previously published  $\epsilon$  values for the denatured state [12], the  $\epsilon$  values per chromophore for the native state are calculated to be 98 400–102 000 at the long-wavelength maximum (Table I). This is in reasonable agreement with the  $\epsilon$  value of 107 000 calculated from recently published amino acid analytical data on this C-phycocyanin [21], and the value of 99 000 determined from denaturation experiments by a similar approach [18].

The 2,3-dihydrobilindion **3** has the same chromophore as phycocyanin (**1b**) [18, 26]. It is soluble in organic solvents, but the cation is also soluble in

methanolic solutions containing up to 80% water. This has been used to study quantitatively the effect of solvent and urea on the UV-vis-spectrum of the 2,3-dihydrobilindion chromophore. The data (Table II) show a moderate increase of the long-wavelength band absorption if the methanol is replaced to 50% by water, with no further change up to 75% water. These values are essentially independent on the presence or absence of urea. Similar data have been obtained for phycocyanobilin peptides and phytochromobilin peptides. The latter finding contrasts to the remarkable effect of urea on the CD-spectra of **1a** [22]. A single chromophore of the 2,3-dihydrobilin type has a molar absorptivity between 34 000 (methanolic sulfuric acid) and 41 000 (= 50% water). The values for the chromopeptides are distinctly smaller (29 100 and 32 000, respectively). This could be due either to residual influence of the peptide moiety or – more probably – to partial chromophore bleaching during the isolation procedure. We therefore used the higher value (35 500) determined for one phycocyanobilin in acid-urea denatured phycocyanin [12] for calculations of Table I. The solvent effect is similar on the near UV-band as on the visible band. The ratio of the two  $\epsilon$ -values, a sensitive parameter for chromophore conformation, remains unchanged. It should be noted, however, that measurements in the near UV, especially in the

Table I. Spectral data of native, denatured and proteolytically degraded C-phycocyanin from *Spirulina platensis*. Spectra of native C-phycocyanin in 10 mM Tris/HCl buffer pH 7.4 were compared with spectra after quantitative denaturation in 8 M urea at pH 2.0, or after quantitative digestion with pepsin at pH 2.0. All data were calculated under the assumption of 3 phycocyanobilin chromophores per protein monomer ( $\alpha/\beta$ ) and the  $\epsilon$  values given in [12].  $n$  = number of independent measurements.

	$\lambda_{\max}^1$ long-wavelength maximum	$\lambda_{\max}^2$ short-wavelength maximum
$\epsilon$ per chromophore denatured state <sup>a</sup> [12] (M <sup>-1</sup> cm <sup>-1</sup> )	662.5 nm: 35 500	352 nm: 34 700
ratio $A_{\text{native}}/A_{\text{denatured}}$ <sup>a</sup>	620/665 nm: 2.9 ± 0.2 ( $n = 4$ )	360/355 nm: 0.55 ± 0.03 ( $n = 4$ )
ratio $A_{\text{native}}/A_{\text{degraded}}$ <sup>b</sup>	620/655 nm: 2.9 ± 0.1 ( $n = 2$ )	n. d. <sup>c</sup>
$\epsilon_{\text{native}}$ per chromophore calculated from denaturation (M <sup>-1</sup> cm <sup>-1</sup> )	620 nm: 102 000 ± 7000	360 nm: 19 000 ± 1000
$\epsilon_{\text{native}}$ per chromophore calculated from proteolysis (M <sup>-1</sup> cm <sup>-1</sup> )	620 nm: 102 000 ± 3500	n. d. <sup>c</sup>
$\epsilon_{\text{native}}$ per chromophore from Kufer and Scheer [18] (M <sup>-1</sup> cm <sup>-1</sup> )	620 nm: 99 000	360 nm: 16 000
$\epsilon_{\text{native}}$ per chromophore calculated from data of Boussiba and Richmond [21] (M <sup>-1</sup> cm <sup>-1</sup> )	620 nm: 107 000	

<sup>a</sup> In 8 M urea at pH 2.0.

<sup>b</sup> After complete digestion with pepsin at pH 2.0.

<sup>c</sup> n. d. = not determined because of high background absorption in this spectral region.

Table II. Solvent effects on the molar absorptivities of bilipeptides and model compounds.

Compound	Solvent composition			$\epsilon \times 10^{-3}$ at $\lambda_{\max}^1$ (M <sup>-1</sup> cm <sup>-1</sup> )	$\epsilon \times 10^{-3}$ at $\lambda_{\max}^2$ (M <sup>-1</sup> cm <sup>-1</sup> )	$\epsilon_2/\epsilon_1$
	% (v/v) 1% methanolic H <sub>2</sub> SO <sub>4</sub>	% (v/v) 8 M urea, pH 1.5	% (v/v) 1% aqueous HCl			
Dihydrobilindion ( <b>3</b> )	100	—	—	34.0 <sup>a</sup>	37.2 <sup>a</sup>	1.10
	50	50	—	41.2	44.6	1.08
	25	75	—	39.9	44.1	1.10
	100	—	—	34.0 <sup>a</sup>	37.4 <sup>a</sup>	1.10
	75	—	25	36.4	41.2	1.13
	50	—	50	35.0	39.3	1.12
Methanol adduct ( <b>2c</b> )	100	—	—	33.9	36.5	1.08 <sup>d</sup>
	20	80	—	43.0	46.4	1.08 <sup>d</sup>
	10	90	—	42.9	46.3	1.08 <sup>d</sup>
PC-Peptid ( <b>1b</b> )	100	—	—	29.1	29.7	1.02
	25	75	—	32.0	32.6	1.02
	25	—	75	32.0 <sup>b</sup>	32.6 <sup>b</sup>	1.02
P <sub>r</sub> -Peptid ( <b>1a</b> )	100	—	—	29.2	36.4	1.25
	25	75	—	32.0	40.0	1.25
	25	—	75	32.0 <sup>c</sup>	40.0 <sup>c</sup>	1.25

<sup>a</sup> From ref [26], the other molar absorptivities have been determined with respect to these values.

<sup>b</sup> From ref [38], the other molar absorptivities have been determined with respect to these values.

<sup>c</sup> From ref [23], the other molar absorptivities have been determined with respect to these values.

<sup>d</sup> Average from the three different measurements.

Table III. Spectral data of native, denatured and proteolytically degraded phytochrome (P<sub>r</sub> form) from *Avena sativa* L. *n* = number of independent measurements.

	$\lambda_{\max}^1$	$\lambda_{\max}^2$
$\epsilon$ per chromophore in chromopeptide [23] <sup>a</sup>	658 nm: 32000	370 nm: 40000
ratio $A_{\text{native}}/A_{\text{denatured}}$ <sup>b</sup>	665/680 nm: $3.4 \pm 0.2$ ( <i>n</i> = 7)	380/375 nm: 0.9 ( <i>n</i> = 1)
ratio $A_{\text{native}}/A_{\text{degraded}}$ <sup>c</sup>	665/665 nm: $3.7 \pm 0.2$ ( <i>n</i> = 5)	n. d. <sup>d</sup>
$\epsilon_{\text{native}}$ per chromophore calculated from denaturation	665 nm: $109000 \pm 6000$	380 nm: 36000
$\epsilon_{\text{native}}$ per chromophore calculated from proteolysis	665 nm: $118000 \pm 6000$	n. d. <sup>d</sup>

<sup>a</sup> In 5% formic acid; the same values have been used for the chromopeptide in 0.01 N trifluoroacetic acid [9].

<sup>b</sup> In 8 M urea at pH 2.0.

<sup>c</sup> After complete digestion with pepsin at pH 2.0.

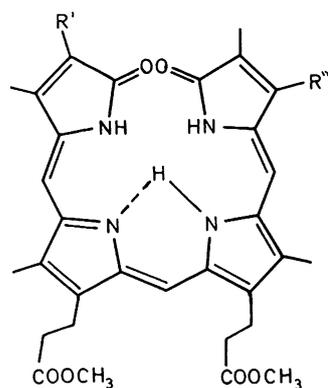
<sup>d</sup> n. d. = not determined because of high background absorption in this spectral region.

presence of large amounts of urea, are generally less reliable.

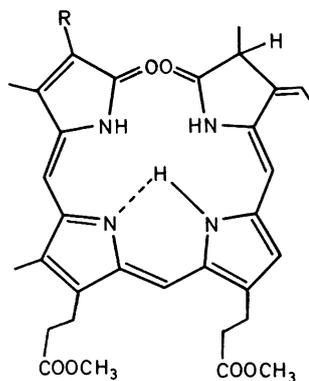
The same approach applied to oat phytochrome (Table III) gives similar  $\epsilon$  values for phytochromobilin and phycocyanobilin in the native state at the long-wavelength maximum ( $\lambda_{\max}^1$ ) but a higher  $\epsilon$  value for phytochromobilin at the short-wavelength maximum ( $\lambda_{\max}^2$ ) (Table III compared with Table I). This difference at  $\lambda_{\max}^2$  could be due to different interactions between chromophore and peptide residue in P<sub>r</sub> and phycocyanin or – more probably – to the presence of the 18-vinyl group in phyto-

chromobilin (**1a**, **2a**) instead of the 18-ethyl group in phycocyanobilin (**1b**, **2b**). The replacement of the 18-ethyl by a vinyl substituent in model compounds (see Table IV) increases the molar absorptivity of the UV band by approximately 15% in the free base, by 28% in the cation form. This has been discussed already by Stoll and Gray [24] (compounds **5–13**) and can also be derived from the data of Gossauer *et al.* [11, 25] for compounds **2a**, **b** and **14**, **15**.

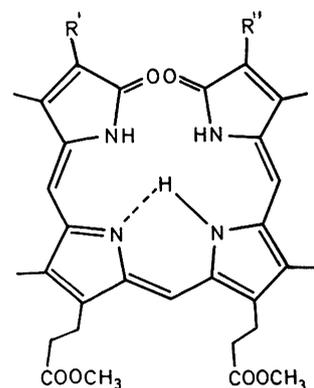
A high value for the molar absorptivity of phytochromobilin at  $\lambda_{\max}^1$  in the native state was determined here (109000–118000, Table III). An even



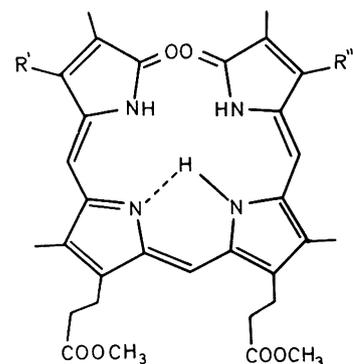
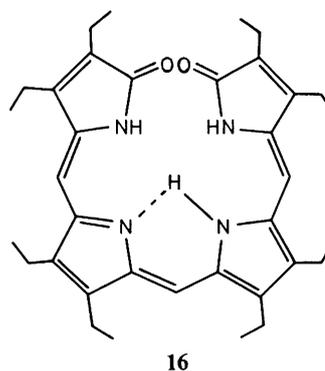
- 5: R' = R'' = C<sub>2</sub>H<sub>5</sub>  
 8: R' = C<sub>2</sub>H<sub>3</sub>  
    R'' = C<sub>2</sub>H<sub>5</sub>  
 11: R' = C<sub>2</sub>H<sub>5</sub>  
     R'' = C<sub>2</sub>H<sub>3</sub>



- 14: R = C<sub>2</sub>H<sub>5</sub>  
 15: R = C<sub>2</sub>H<sub>3</sub>



- 6: R' = R'' = C<sub>2</sub>H<sub>5</sub>  
 9: R' = C<sub>2</sub>H<sub>3</sub>  
    R'' = C<sub>2</sub>H<sub>5</sub>  
 10: R' = C<sub>2</sub>H<sub>3</sub>



- 7: R' = R'' = C<sub>2</sub>H<sub>5</sub>  
 12: R' = C<sub>2</sub>H<sub>3</sub>  
     R'' = C<sub>2</sub>H<sub>3</sub>  
 13: R' = R'' = C<sub>2</sub>H<sub>3</sub>

higher molar absorptivity (150 000) was suggested earlier by Burke *et al.* [27] on the basis of direct comparison of the phytochrome intermediate P<sub>B1</sub> with biliverdin IX $\alpha$ . Considering the variations of  $\epsilon$  within the rather different chromophores, this value is in fair agreement with our data. The similar value for phycocyanobilin at  $\lambda_{\text{max}}^1$  in the native state (102 000, Table I) points to similar chromophore conformations in P<sub>r</sub> and phycocyanin. In this sense, more extended chromophore conformations in the native state and more closed chromophore conformations in the denatured state have been discussed for P<sub>r</sub> [27–29] as well as for phycocyanin [17].

The molar extinction coefficient of native P<sub>r</sub> has been determined for small oat phytochrome (60 000 D) to be  $\epsilon^{665 \text{ nm}} = 76 000$  [30] and for large rye phytochrome (per 120 000 D) to be  $\epsilon^{665} = 70 000$  [31]. Both values are considerably smaller than those determined for one native chromophore in the

Table IV. Absorption maxima and molar absorptivities of phytochromobilin and model pigments.

Compound	State	Solvent	$\lambda_{\max}$ Vis	and	$(\epsilon \times 10^{-3})$ nUV	$\epsilon_{280} \times 10^{-3}$	$A_{\text{nUV}}/A_{\text{vis}}$	$A_{280}/A_{\text{vis}}$	Ref.
Mesobiliverdin IX $\alpha^a$ ( <b>5</b> )	free base	CHCl <sub>3</sub>	631 (15.8)		369 (54.7)	n. d.	3.46	n. d.	[24]
Mesobiliverdin III $\alpha^a$ ( <b>6</b> )	free base	CHCl <sub>3</sub>	631 (15.8)		369 (55.3)	n. d.	3.50	n. d.	
Mesobiliverdin XIII $\alpha^a$ ( <b>7</b> )	free base	CHCl <sub>3</sub>	631.5 (15.5)		368 (53.2)	n. d.	3.43	n. d.	
3 <sup>1</sup> ,3 <sup>2</sup> -Dihydrobiliverdin IX $\alpha^a$ ( <b>8</b> )	free base	CHCl <sub>3</sub>	649 (14.9)		376 (57.9)	n. d.	3.89	n. d.	[24]
2 <sup>1</sup> ,2 <sup>2</sup> -Dihydrobiliverdin III $\alpha^a$ ( <b>9</b> )	free base	CHCl <sub>3</sub>	648 (14.7)		375.5 (60.4)	n. d.	4.12	n. d.	
Biliverdin III $\alpha^a$ ( <b>10</b> )	free base	CHCl <sub>3</sub>	661.5 (14.2)		381 (68.0)	n. d.	4.77	n. d.	
18 <sup>1</sup> ,18 <sup>2</sup> -Dihydrobiliverdin IX $\alpha^a$ ( <b>11</b> )	free base	CHCl <sub>3</sub>	647 (15.0)		374 (48.4)	n. d.	3.23	n. d.	[24]
17 <sup>1</sup> ,17 <sup>2</sup> -Dihydrobiliverdin XIII $\alpha^a$ ( <b>12</b> )	free base	CHCl <sub>3</sub>	646 (15.2)		373 (46.8)	n. d.	3.07	n. d.	
Biliverdin XIII $\alpha^a$ ( <b>13</b> )	free base	CHCl <sub>3</sub>	648 (15.7)		378 (44.9)	n. d.	2.85	n. d.	
$\Delta$ 3,3 <sup>1</sup> - <i>E</i> -Phycocyanobilin <sup>a</sup> ( <b>14</b> )	free base	MeOH	600 (12.3)		362 (41.7)	n. d.	3.39	n. d.	[25]
$\Delta$ 3,3 <sup>1</sup> - <i>Z</i> -Phycocyanobilin <sup>a</sup> ( <b>2b</b> )	free base	MeOH	599 (20.9)		360 (57.5)	n. d.	2.75	n. d.	[11]
$\Delta$ 3,3 <sup>1</sup> - <i>E</i> -Phytochromobilin <sup>a</sup> ( <b>15</b> )	free base	MeOH	610 (17.8)		372 (57.5)	n. d.	3.23	n. d.	[11]
$\Delta$ 3,3 <sup>1</sup> - <i>Z</i> -Phytochromobilin <sup>a</sup> ( <b>2a</b> )	free base	MeOH	610 (13.5)		368 (41.7)	n. d.	3.09	n. d.	[11]
$\Delta$ 3,3 <sup>1</sup> - <i>E</i> -Phycocyanobilin <sup>a</sup> ( <b>14</b> )	cation	MeOH/2% HCl	690 (37.9)		374 (47.9)	n. d.	1.26	n. d.	[25]
$\Delta$ 3,3 <sup>1</sup> - <i>Z</i> -Phycocyanobilin <sup>a</sup> ( <b>2b</b> )	cation	MeOH/2% HCl	685 (37.2)		368 (46.8)	n. d.	1.26	n. d.	[11]
$\Delta$ 3,3 <sup>1</sup> - <i>E</i> -Phytochromobilin <sup>a</sup> ( <b>15</b> )	cation	MeOH/2% HCl	708 (38.0)		386 (64.6)	n. d.	1.70	n. d.	[11]
$\Delta$ 3,3 <sup>1</sup> - <i>Z</i> -Phytochromobilin <sup>a</sup> ( <b>2a</b> )	cation	MeOH/2% HCl	702 (24.5)		382 (38.0)	n. d.	1.55	n. d.	[11]
2,3,7,8,12,13,17,18-Octaethylbilindion ( <b>16</b> )	free base	MeOH	657 (15.6)		367 (51.2)	21.6	3.28	1.38	[26]
2,3,7,8,12,13,17,18-Octaethyl-2,3-dihydrobilindion ( <b>3</b> )	free base	MeOH	594 (17.6)		347 (39.4)	20.4	2.24	1.16	[26]
2,3,7,8,12,13,17,18-Octaethylbilindion ( <b>16</b> )	cation	MeOH/1% HCl	693 (31.2)		357 (55.9)	n. d.	1.79	n. d.	[26]
2,3,7,8,12,13,17,18-Octaethyl-2,3-dihydrobilindion ( <b>3</b> )	cation	MeOH/1% HCl	665 (34.0)		351 (36.5)	16.3	1.07	0.48	[26]
Isophorcabilin <sup>a</sup> ( <b>4</b> )	free base	MeOH	606 (60.0)		397 (15.2)	21.8	0.25	0.36	this paper
Isophorcabilin <sup>a</sup> ( <b>4</b> )	cation	MeOH/1% HCl	695 (105)		406 (18.9)	21.2	0.18	0.20	this paper

<sup>a</sup> Dimethylester

n. d. = not determined

Table V. Molar extinction coefficients and purity index of phytochrome preparations in the P<sub>r</sub> form.

Plant source	Mol wt	Purity index (PI) ( $A_{280}/A_{665}$ ) of purest fraction	PI <sup>corr a</sup>	$\epsilon^{665} \cdot 10^{-3}$ [M <sup>-1</sup> cm <sup>-1</sup> ]	$\epsilon^{665} \cdot 10^{-3}$ calculated from Eqn. (5) for PI <sup>corr</sup>
Oat	60000	1.08 [30] <sup>b</sup>	0.94 (0.88)	76 [30]	—
Oat	60000	0.78 [32, 33] <sup>b</sup>	0.64 (0.58)		112
Rye	68000	0.8 [34]	0.66 (0.60)		123
Rye	120000	1.29 [31]	1.15 (1.09)	70 [31]	124
Oat	120000	1.2 [35]	1.06 (1.0)		135
Oat	120000	1.15 [36]	1.01 (0.95)		141

<sup>a</sup>  $A^{280}/A^{660}$  values for the chromophore of 0.14 (0.20) have been used for the correction of PI according to Eqn. (1). See discussion for the origin of these values.

<sup>b</sup>  $\epsilon^{662}$  calculated from Eqn. (4) based on the standard value 26 000 [30] yields 38 200 (40 000) M<sup>-1</sup> cm<sup>-1</sup>.

present paper. When considering these determinations one has to keep in mind that a tedious and long-lasting isolation procedure had to be used to obtain these P<sub>r</sub> preparations. During this procedure, all colourless proteins had to be removed from phytochrome on the one hand and denaturation of phytochrome leading to chromophore bleaching had to be avoided on the other hand. The purity of P<sub>r</sub> is normally given as purity index  $A^{280}/A^{665}$ . The small oat phytochrome preparation used for the determination of the molar extinction coefficient had a purity index of 1.08 [30]. It is however questionable whether this was a pure preparation because later preparations had lower values for the purity index (see Table V).

It is possible to estimate the extinction coefficients for these preparations by a series of corrections. The first takes into account the correction of the purity index (PI) by the chromophore absorption at 280 nm ( $A_{\text{chromophore}}^{280}$ ):

$$\frac{A_{\text{protein}}^{280}}{A^{660}} = \frac{A_{\text{total}}^{280}}{A^{660}} - \frac{A_{\text{chromophore}}^{280}}{A^{660}}$$

or

$$\text{PI}_{\text{corr}} = \text{PI} - \frac{A_{\text{chromophore}}^{280}}{A^{660}} \quad (1)$$

where the total absorption at 660 nm ( $A^{660}$ ) is due only to the chromophore.

The ratio  $A_{\text{chromophore}}^{280}/A^{660}$  can be estimated from the model compound **3**, where it amounts to 0.48 in the cation [26]. This agrees well with the data of Fry and Mumford [23] and Lagarias and Rapoport [9] for a phytochromobilin undecapeptide, containing one tyrosine ( $\epsilon^{280} \sim 1500$ ) as the only aromatic amino acid. Based on the molar absorptivities of tyrosine and the cation of **3** as a model for the

chromopeptide, PI = 0.52 has been calculated, vs. 0.54 found. **3** is thus a useful model for the phytochrome chromophore not only in the visible [26] but also in the UV spectral region.

In native P<sub>r</sub>,  $A^{660}$  is increased by a factor of 3.6 (see Table III). This would decrease the ratio  $A_{\text{chromophore}}^{280}/A^{660}$  to 0.14 assuming no change in  $\epsilon_{\text{chromophore}}^{280}$ . The latter assumption is supported by MO calculations (Schneider and Scheer, unpublished), and by the spectrum of **4** as a model for the native chromophore of P<sub>r</sub>. **4** is held in an extended conformation by the additional rings. Both show that, in contrast to the visible band, the absorption at 280 nm is rather unchanged by conformational changes and protonation (Table IV). For the cation of **4**, which has an absorption spectrum very similar to native **1a** and **1b**,  $A^{280}/A^{660} = 0.20$  has been determined.

Tobin and Briggs [31] calculated for the contribution of the chromophore to the total absorbance at 280 nm 38% for small and 23% for large phytochrome. This corresponds to ratio  $A_{\text{chromophore}}^{280}/A^{660} = 0.3$ , much higher than our value. Tobin and Briggs [31] had based their calculations on possibly impure phytochrome preparations as deduced from lower values for the purity index  $A_{280}/A_{660}$  than that of later phytochrome preparations (see Table V).

Under the assumption that the relative distribution and the state of protonation of the aromatic amino acids are the same in phytochrome and the other proteins the amount of protein impurities in different phytochrome preparations can be calculated. Standards for such calculations are the preparations for which  $\epsilon$  has been determined [30, 31]. For the calculation of molar absorptivity  $\epsilon$  of a preparation  $x$  this correction is combined with the correc-

tion of Eqn. (1) to:

$$\varepsilon = \frac{PI_{\text{corr}}^s}{PI_{\text{corr}}^x} \times \varepsilon_s, \quad (2)$$

whereby *s* signifies standards values.

A possible third correction takes into account the molecular weight (MW) differences between the standard (*s*) and the preparation (*x*).

$$\varepsilon'_x = \frac{MW_x}{MW_s} \cdot \varepsilon_s. \quad (3)$$

Eqs. (2) and (3) combine to

$$\varepsilon_x = \frac{PI_{\text{corr}}^s \times MW_x}{PI_{\text{corr}}^x \times MW_s} \cdot \varepsilon_s \quad (4)$$

which yields

$$\varepsilon_x = C \cdot \frac{MW_x}{PI_{\text{corr}}^x}. \quad (5)$$

With the data of Mumford and Jenner [30] as a standard and the PI-corrections of 0.14 and 0.20 as discussed above *C* is 1.19 and 1.11, respectively. The molar absorptivities for small phytochrome preparations obtained by these corrections are between 112000 and 126000 (Table V, row 6), which is within the limits of the extinction coefficient of one native phytychromobilin derived at above (Table

III). Small phytochrome thus contains one chromophore.

The same formalism can be applied to the chromophore(s) of large phytochrome because controlled proteolysis of large to small phytochrome does not significantly change the absorption spectrum [37]. The formal molar absorptivities derived at by this calculation from small phytochrome (Table V) indicate the presence of 1 chromophore per peptide chain (120000 D). The basis for this calculation is the assumption that the percentage of aromatic amino acids and/or their environment is similar in large and small phytochrome (see ref. [31]). The considerably lower value determined for large rye phytochrome ( $E_{665\text{nm}} = 70000$  per 120000 D [31]) would then be an indication that this assumption is not valid, or else that there is only one chromophore per dimer (240000 D), *i.e.* that large phytochrome consists of one peptide chain with and one peptide chain without chromophore.

#### Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft, Bonn-Bad Godesberg and the Fonds der chemischen Industrie. We thank Prof. C. Soeder (Jülich) for a gift of *Spirulina platensis*, Prof. H. H. Inhoffen (Braunschweig) for a gift of octaethylporphyrin, and Dr. W. Kufer (München) for isophorocabilin.

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