Chlorophyll Biosynthesis: Various Chlorophyllides as Exogenous Substrates for Chlorophyll Synthetase

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Dedicated to Professor Dr. H. Merxmüller *on the Occasion of His 60th Birthday*

Chlorophyllides a and b, Protochlorophyllide, Bacteriochlorophyllide a, 3-Acetyl-3-devinylchlorophyllide a, Pyrochlorophyllide a, Pheophorbide a

The esterification of various chlorophyllides with geranylgeranyl diphosphate was investigated as catalyzed by the enzyme chlorophyll synthetase. The enzyme source was an etioplast membrane fraction from etiolated oat seedlings (*Avena sativa* L.). The following chlorophyllides were prepared from the corresponding chlorophylls by the chlorophyllase reaction: chlorophyllide a (**2**) and b (**4**), bacteriochlorophyllide a (**5**), 3-acetyl-3-devinylchlorophyllide a (**6**), and pyrochlorophyllide a (**7**). The substrates were solubilized with cholate which reproducibly reduced the activity of chlorophyll synthetase by 40–50%. It was found that the following compounds were good substrates for chlorophyll synthetase: chlorophyllide a and b, 3-acetyl-3-devinylchlorophyllide a, and pyrochlorophyllide a. Only a poor or no reaction was found with protochlorophyllide, pheophorbide a, and bacteriochlorophyllide. This difference of reactivity was not due to distribution differences of the substrates between solution and pelletable membrane fraction. Furthermore, no interference between good and poor substrate was detected. Structural features necessary for chlorophyll synthetase substrates were discussed.

Introduction

The last steps of chlorophyll a (Chl a) biosynthesis are the photoconversion of Protochlide (**1**) to Chlide a (**2**) and the subsequent esterification to Chl a. The latter reaction is catalyzed by the recently detected enzyme chlorophyll synthetase [1]. This enzyme needs – besides Chlide a – tetraprenyl alcohols plus ATP or the diphosphate derivatives of tetraprenyl alcohols as substrates. The best substrate is geranylgeranyl diphosphate (GGPP) which leads to Chl a_{GG} (**2a**). This is then stepwise hydrogenated via Chl a_{H_2GG} and Chl a_{H_4GG} to Chl a_P (**2b**) *in vitro* [2] and *in vivo* [3].

A problem of previous experiments with chlorophyll synthetase was the limited source of the second substrate, Chlide a (**2**). This substrate is barely water-soluble. Solvents (*e. g.* acetone) or detergents (*e. g.* triton X 100) which can solubilize **2** destroy the activity to chlorophyll synthetase [1]. Therefore no exogenous **2** was applied. The only substrate was **2** formed by photoconversion of endogenous Protochlide (**1**) in the etioplast membrane. Thus the substrate source was limited with regard to substrate nature and amount. Furthermore, chlorophyll synthetase activity could only be determined in illuminated membranes which left the question open whether the enzyme had been activated by light or not.

We describe here experiments with exogenous chlorophyllides in which we adopted the system of Griffiths [4] for solubilization of Protochlide with cholate. This enabled us to vary the substrate concentration and to test the following compounds as possible substrates: pheophorbide a (**3**), chlorophyllide b (**4**), bacteriochlorophyllide a (**5**), 3-acetyl-3-devinylchlorophyllide a (**6**), and pyrochlorophyllide a (**7**). Furthermore the experiments were carried out with etioplast membranes without any illumination.

Abbreviations: Chl, chlorophyll; Chlide, chlorophyllide; Protochl, protochlorophyll; Protochlide, protochlorophyllide; GGPP, geranylgeranyl diphosphate; subscripts for chlorophylls and pheophytins containing GG, geranylgeraniol; H_2GG, dihydrogeranylgeraniol; H_4GG, tetrahydrogeranylgeraniol; P, phytol.

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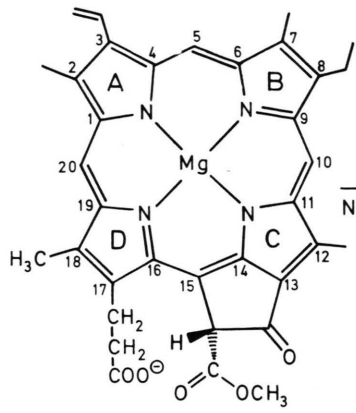
Materials and Methods

Preparation of Protochlide (**1**): The shoots (120 g fresh weight) of 7.5 days old etiolated oat seedlings (*Avena sativa* L. var. Parzival, Baywa, München) were harvested in dim-green safelight and frozen in liquid nitrogen for 2 minutes. The frozen tissue was then homogenized (Waring blender) and exhaus-



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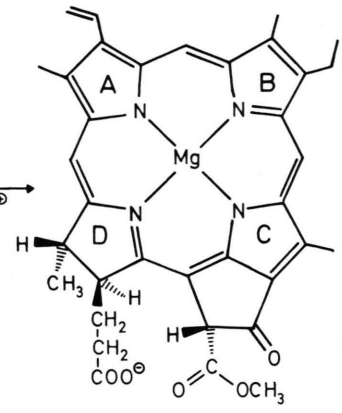

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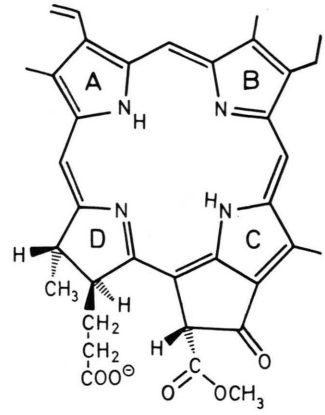

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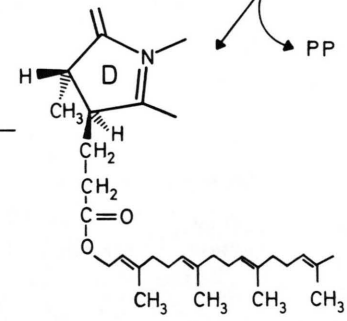

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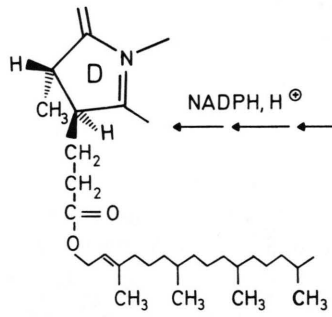

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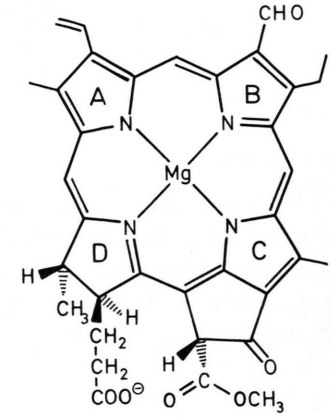

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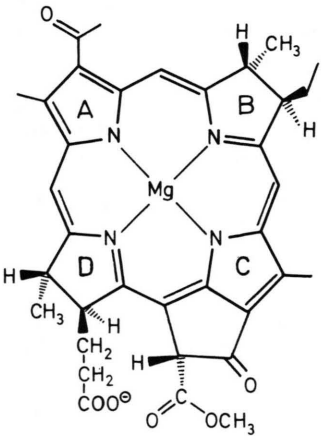

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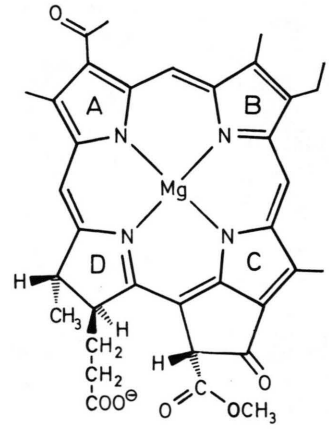

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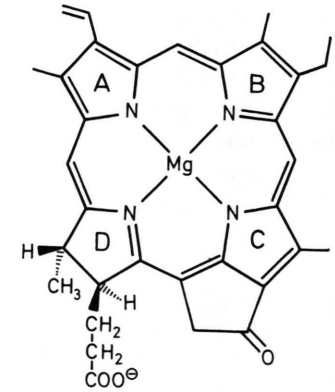

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tively extracted with acetone (total volume 500 ml). The acetone solution was concentrated in vacuo to about 250 ml and mixed with 100 ml diethyl ether. Then water was added until the phases separated. The diethyl ether phase was washed with water (2–3 times 100 ml) and diluted with 100 ml petroleum benzene (b. p. 40–60 °C). Extraction into methanol/NH_3 and reextraction into diethyl ether were performed according to Griffiths [4].

Preparation of Chlide a (**2**) *and Chlide b* (**4**): Leaves from *Heracleum lanatum* (70 g fresh weight) were frozen in liquid nitrogen and exhaustively extracted with acetone (total volume 1000 ml). The solid residue (acetone powder) was dried at room temperature under air (14 g dry weight) and used for extraction of chlorophyllase (see below). The acetone solution was mixed with diethylether, washed with water, dried over sodium sulfate and evaporated. The residue was dissolved in petroleum benzene (b. p. 40–60 °C). Chlorophylls were isolated by chromatography on a powder sugar column according to Hager [5] with petroleum benzene containing 0–1% *n*-propanol. Yield 10 mg Chl a (**2b**), 3.5 mg Chl b (**4b**).

For the chlorophyllase reaction, 1 g acetone powder of *Heracleum lanatum* (see above) were extracted with 15 ml 0.02 M citrate buffer (pH 7.2) containing 0.5 M NaCl and 0.001 NaN_3 according to Bacon and Holden [6]. The chlorophyllase solution was mixed with 2.2 mg of the respective chlorophyll (**2b** or **4b**) in 15 ml acetone and incubated 2.5 h at 30 °C in the dark. The reaction was stopped by addition of 40 ml acetone. The pigments were then extracted into ethyl acetate. This solution was evaporated in vacuo and the residue dissolved in diethyl ether/petroleum benzene (1:1, v/v). The chlorophyllide (**2** or **4**) was purified according to Griffiths [4]. Yield 10–25%.

Further substrates: Pheophorbide a (**3**) was prepared from Chlide a by demetallation with hydrochloric acid [3]. Bacteriochlorophyll a (from Rhodopseudomonas spheroides), 3-acetyl-3-devinylchlorophyll a obtained from bacteriochlorophyll a by dehydrogenation with dichlorodicyanobenzoquinone [7] and pyrochlorophyll obtained from Chl a by decarboxymethylation in pyridine [8], were gifts from Dr. H. Scheer, München. The hydrolysis catalyzed by chlorophyllase (see above) yielded the corresponding substrates bacteriochlorophyllide a (**5**) 3-acetyl-3-devinyl-chlorophyllide a (**6**) and pyrochlorophyllide a (**7**) (yield 10–20%), respectively.

Chlorophyll synthetase test: Broken etioplasts were prepared from 7.5 days old, etiolated oat seedlings as previously described [1] but using ultrasonic power (1 × 5 sec) in 0.05 M Hepes buffer (pH 7.2) for lysis of etioplasts. Cofactors were added as previously described [1] but omitting NADPH if not otherwise stated. The respective chlorophyllide substrate in the diethyl ether stock solution was, after addition of the same volume petroleum benzene and 0.1vol. 90% methanol containing 0.1% sodium cholate (Sigma, München), transferred into aqueous cholate-containing solution according to Redlinger and Apel [9]. A ratio of 6 nmol chlorophyllide: 1 mg cholate: 1 ml incubation volume was maintained in all experiments. GGPP was added in 18–25 fold molar excess over the exogenous chlorophyllide substrate. The mixture was incubated in the dark (without illumination!) for 60 min at 28 °C. Chlorophyll pigments were extracted, demetalled to pheophytins and analyzed by HPLC as previously described [1]. For calculation of pigment concentrations, spectra were run in diethylether. The following molar extinction coefficients ($1 \cdot mmol^{-1} \cdot cm^{-1}$) were calculated from data of French [10]: Protochlorophyll(ide) $\varepsilon_{624} = 35.6$; protopheophytin/protopheophorbide $\varepsilon_{565} = 22.0$; Chl(ide) a $\varepsilon_{662} = 90.2$; pheophytin a $\varepsilon_{667} = 55.5$; Chl(ide) b $\varepsilon_{644} = 56.3$; pheophytin b $\varepsilon_{655} = 37.3$.

Further molar extinction coefficient were taken from [7]: 3-acetyl-3-devinylchlorophyll(ide) a $\varepsilon_{676} = 65.2$; 3-acetyl-3-devinyl-pheophytin $\varepsilon_{682} = 39.1$; bacteriochl(ide) a $\varepsilon_{770} = 96.0$; bacteriopheophytin a $\varepsilon_{750} = 60.5$.

Pigment fractions isolated by HPLC were hydrolyzed with methanolic KOH [1]. The alcohol was analyzed by gas chromatography on a OV 101 capillary column (25 m), carrier 0.8 atü He, splitless, injector temperature 130 °C, column temperature 180 °C.

Results

The low solubility of Chlide in aqueous buffers did not allow the direct application of the previously used [1] buffer system for exogenous Chlide and similar substrates. Griffiths [4] and Redlinger and Apel [9] solubilized Protochlide with cholate. Chlide a (**2**) and similar substrates can be solubilized with

cholate as well. We therefore investigated at first the influence of cholate upon the esterification of endogenous Chlide a (*i. e.* the pigment present in the illuminated etioplast membrane [1]) (Table I). In accordance with the data of Griffiths [4], cholate did not significantly reduce the phototransformation of Protochlide to Chlide. Esterification of endogenous Chlide was, however, inhibited by cholate. Interestingly, the relative inhibition of esterification was of the same order of magnitude (40–60%) with exogenous GGPP as without exogenous GGPP (*i. e.* only with endogenous tetraprenyl precursors of the membranes) although the absolute yields of esterified pigment were very much different. The nature of inhibition of esterification by cholate is presently been investigated. The residual chlorophyll synthetase activity is constant under defined conditions and sufficient to investigated the substrate specificity of the enzymic reaction.

The esterification of exogenous Chlide a was then demonstrated in a series of experiments in which increased amounts of substrate were applied in the dark (Fig. 1). In these experiments, etioplast membranes were used which contained Protochlide (8 nmol per sample). This pigment was still present as the free acid at the end of incubation. Because the membranes did not contain any Chlide no esterified Chl could be detected at the end of the incubation if no exogenous Chlide was applied. Increased amounts of exogenous Chlide a then caused increased amounts of esterified Chl a far beyond the normal pigment content of the membrane. Esterification was 35–50% of exogenous Chlide a at low substrate concentrations and 50–60% at high substrate concentrations. This value was even higher than the esterification of endogenous Chlide produced by illumination of the membrane (see Table I).

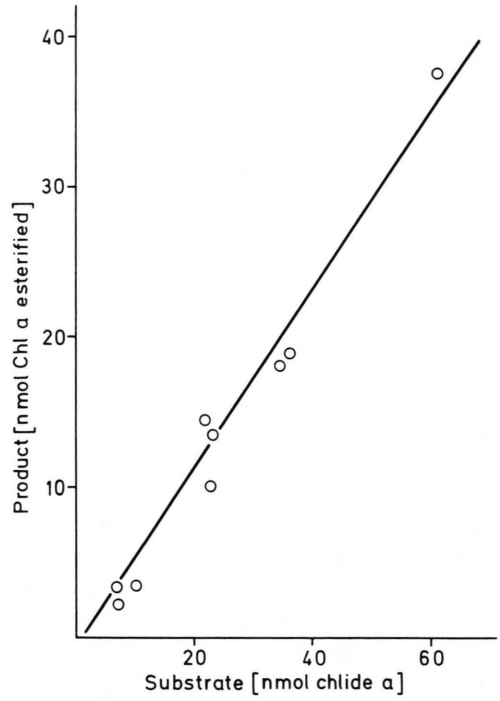

Fig. 1. Esterification of exogenous Chlide a with exogenous GGPP in an etioplast membrane fraction in the dark. The amount of Protochl(ide) in the membrane was 8 ± 2 nmol per sample. Saturating amounts of GGPP were applied (molar ratio Chlide a: GGPP = 1:20). The esterification was 50–60% of exogenous Chlide a but only 35–50% for low substrate concentrations.

Table I. Influence of cholate (1 mg/ml) upon esterification of endogenous Chlide a in an illuminated etioplast membrane fraction (3 min white light, 3000 lx). Endogenous Protochlide (before illumination) 6.5–7 nmol, endogenous Chlide (after illumination), 6.0–6.5 nmol (with cholate) or 6.5–7.0 nmol (without cholate). Cofactors as in Materials and Methods plus 2 µmol NADPH.

	Chlorophyll a esterified	
	with cholate (1 mg/ml)	without cholate
without GGPP	0.5–0.7 nmol = 7–11%	1.0–1.5 nmol = 15–23%
with GGPP (20 fold excess)	2.3–2.6 nmol = 35–43%	4.4–4.9 nmol = 68–75%

The specificity of chlorophyll synthetase for various chlorophyllide pigments was then tested in a series of experiments with etioplast membranes in the dark (Table II). Chlide a (**2**) was defined as a good substrate (52% esterification) whereas nearly no esterification was observed with exogenous Protochlide (**1**). The small amount of Protochl (0.9%) was in the same order of magnitude as the endogenous esterified Protochl which was present in the membrane at the beginning of the experiment. It is not clear therefore whether Protochlide is at all a substrate for chlorophyll synthetase. Surprisingly, pheophorbide a (**3**) is a poor substrate (6% esterification). The central magnesium seems to be an essential factor of the substrate. Chlide b (**4**) was esterified to the same extent (53%) as Chlide a whereas bacteriochlorophyllide a (**5**) was a poor substrate (4% esterification). This is due to the

hydrogenated ring B of bacteriochlorophyllide because 3-acetyl-3-devinylchlorophyllide a (**6**), the corresponding product with the unsaturated ring B, is a good substrate again (52% esterification). Modification of side chains seems to have only little effect upon the substrate property for chlorophyll synthetase because pyrochlorophyllide a (**7**) (in which the carbomethoxyl group of the isocyclic ring of chlorophyllide a has been removed) is still a reasonable substrate (26% esterification).

Because we used GGPP as one substrate, some new Chl species (geranylgeranyl chlorophyllides) should be the products of esterification. We therefore investigated the esterified products by HPLC after removal of magnesium. We obtained 4 fractions of esterified pigments with each substrate. In the case of Chl a, the main compound had been identified as GG pigment with some H_2GG pigment and traces of H_4GG and phytyl pigment [1]. Because all of the chromatograms looked similar the distribution of the 4 alcohols in the esterified products must be about the same indicating a good esterification but less efficient hydrogenation in all cases as previously found for Chl a [2]. The main fractions were rechromatographed as single species and as a mixture (Figure 2). They were well separated from each other except pheophytin$_{GG}$ a and pyropheophytin$_{GG}$. The esterifying alcohol was identified in each of these pigments as geranylgeraniol by gas chromatography after alkaline hydrolysis.

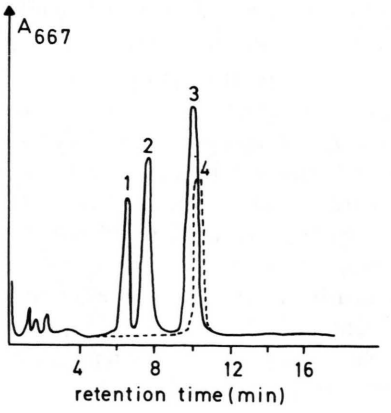

Fig. 2. High Performance Liquid Chromatogram of the main fractions of esterified pigments after removal of magnesium. 1 = 3-acetyl-3-devinyl-pheophytin a_{GG}, λ_{max} = 680 nm; 2 = pheophytin b_{GG}, λ_{max} = 655 nm; 3 = pheophytin a_{GG}, λ_{max} = 667 nm; 1 – 3 as mixture (——); 4 = pyropheophytin a_{GG}, λ_{max} = 667 nm (- - - -).

Table II. Esterification of various exogenous substrates with GGPP (20 fold excess) in an etioplast membrane fraction in the dark. The membrane fraction contained 8 nmol endogenous Protochlide per sample.

Substrate added		Substrate esterified	
	[nmol]	[nmol]	% esterification
Chlide a (**2**)	36.0	18.9	52
Protochlide (**1**)	42.7	0.4	0.9
Pheophorbide a (**3**)	27.6	1.6	6
Chlide b (**4**)	41.1	21.7	53
Bacteriochlide a (**5**)	20.5	0.7	4
Acetylchlide a (**6**)	41.0	21.5	52
Pyrochlide a (**7**)	10.8	3.0	26

Table III. Distribution of Chlide a (**2**) and pheophorbide a (**3**) between an etioplast membrane fraction and a cholate containing solution. Endogenous Protochl(ide) (**1**) of the membrane: 7–8 nmol.

	Incubation time [min]		
	7	15	30
Chlide a (**2**)			
in pellet (%)	87	89	94
in solution (%)	13	11	6
ratio **2**/endogenous **1**	2.8	3.5	3.1
pheophorbide a (**3**)			
in pellet (%)	94	n. d.	95
in solution (%)	6	n. d.	5
ratio **3**/endogenous **1**	4.2	–	4.1

Table IV. Esterification of exogenous Chlide a in a mixture with a) exogenous pheophorbide a and b) exogenous bacteriochlorophyllide a. Incubation with an etioplast membrane fraction and a 20 fold excess of GGPP in the dark. Conditions see legend of Fig. 1.

Substrate applied		Esterified	
	[nmol]	[nmol]	[%]
Chlide a	36	18.9	52
+ pheophorbide a	13	–	–
Chlide a	23	10.1	44
+ bacteriochlide a	10	0.75	7.5

The low esterification of pheophorbide a contrary to Chlide a caused the question whether the distribution of both pigments would be different between the (pelletable) membrane fraction and the (cholate containing) solution. Griffiths [4] had determined the distribution of Protochlide between such a membrane fraction and the cholate solution but did

not give a time dependency for the distribution. Our results show (Table III) that the bulk of Chlide a and pheophorbide a was found in the membrane fraction soon after the application. The difference in esterification is therefore not due to a distribution effect.

For a further comparison we mixed Chlide a (as a good substrate) with pheophorbide a or bacteriochlorophyllide a, respectively (as poor substrates). Analysis of the esterified products revealed (Table IV) that esterification of Chlide a was not inhibited by the poor substrates and that the low esterification of bacteriochlorophyllide a was not influenced by the presence of Chlide a.

Discussion

One important aspect of the present experiments was the esterification of Chlide *in the dark*. So far [1, 3, 11], esterification *in vivo* and *in vitro* had only been studied in illuminated membranes because the photoconversion of Protochlide to Chlide a was an obligatory precondition for those studies. We used here etioplast membranes which had not received light at any time. This was controlled by the presence of only Protochl(ide) with no trace of Chl-(ide) a in the membranes. The esterification of exogenous Chlide a with chlorophyll synthetase of these membranes (Fig. 1, Tables II and IV) means that *no* (direct or indirect) *light activation is necessary for this enzyme activity*. It can furthermore be concluded that the binding site for Chlide (at chlorophyll-synthetase) is different from the binding site for Protochlide (at protochlorophyllide-reductase [4] because the presence of Protochlide in the membrane did not inhibit the synthetase reaction.

Another aspect of the present work was the comparison of substrate specificity of chlorophyll synthetase and chlorophyllase. The latter enzyme has repeatedly been discussed as biosynthetic enzyme responsible for esterification of chlorophyllide (review see [12]). The previous observation that chlorophyll synthetase is sensitive against organic solvents and detergents (contrary to chlorophyllase [1]) was extended here to the mild detergent cholate (Table I). Interestingly, the esterification in the presence of cholate was even somewhat better with exogenous (Fig. 1) than with endogenous Chlide (Table I). It has already been pointed out [1] that chlorophyll synthetase acts only with tetraprenyl alcohols + ATP or the corresponding diphosphates, less efficiently with triprenyl alcohol and not with a diprenyl alcohol or other alcohols whereas chlorophyllase which does not use diphosphates has no preference as to the structure of the alcohol. Chlorophyll synthetase has a higher specificity than chlorophyllase for the second substrate, too. All substrates listed in Table II (except Protochlide) had been prepared by the action of chlorophyllase on the corresponding chlorophylls. The back reaction must therefore also be catalyzed by chlorophyllase with the proper substrate concentrations. Chlorophyll synthetase on the contrary shows nearly no reaction with bacteriochlorophyllide a and pheophorbide a. The biosynthesis of bacteriochlorophyll in photosynthetic bacteria is probably mediated by a chlorophyll synthetase of different specificity which has still to be detected. Rhodopseudomonas spheroides mutants which accumulate bacteriochlorophyllide and lack the esterification step [13] are possibly deficient in this enzyme. Pheophytin a which is probably present in the reaction center of photosystem I [14] is probably formed by demetallation of Chl a rather than by esterification of pheophorbide a (Table II). Interestingly, pheophorbide a shows about the same distribution between solution and membrane as Chlide a (Table III). We do not yet know anything about eventual receptor proteins and the distribution of these substrates within the membrane. But because pheophorbide a and bacteriochlorophyllide a do not interfere with the esterification of Chlide a (Table IV), the most simple explanation would be that the latter substrate is specifically bound to chlorophyll synthetase whereas the former both are not.

Within this model, structural features for the binding to chlorophyll synthetase can be deduced. Essential structural elements for the substrate are the central magnesium (results of substrate **3** versus **2**), the hydrogenated ring D (**1** versus **2**), and the unsaturated ring B (**5** versus **6**) whereas modification of side chains has nearly no effect upon the binding to chlorophyll synthetase (**2**, **4**, **6**, **7**). We consider **6** and **7** as artificial substrates which have only interest for comparative structural considerations. The reaction with chlorophyllide b (**4**) may be of physiological importance because it has been postulated that the oxidation of the 7-methyl side chain (leading from the chlorophyll a to the b series) occurs prior to the esterification reaction [15].

Acknowledgement

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[1] W. Rüdiger, J. Benz, and C. Guthoff, Eur. J. Biochem. **109,** 193–200 (1980).
[2] J. Benz, Ch. Wolf, and W. Rüdiger, Plant Sci. Letters **19,** 225–230 (1980).
[3] S. Schoch, U. Lempert, and W. Rüdiger, Z. Pflanzenphysiol. **83,** 427–436 (1977).
[4] W. T. Griffiths, Biochem. J. **174,** 681–692 (1978).
[5] A. Hager, Planta **48,** 552–621 (1957).
[6] M. F. Bacon and M. Holden, Phytochemistry **9,** 115–125 (1970).
[7] J. R. L. Smith and M. Calvin, J. Amer. Chem. Soc. **88,** 4500–4506 (1966).
[8] F. C. Pennington, H. H. Strain, W. A. Svec, and J. J. Katz, J. Amer. Chem. Soc. **86,** 1416–1426 (1964).
[9] T. E. Redlinger and K. Apel, Arch. Biochem. Biophys. **200,** 253–260 (1980).
[10] C. S. French, Handbuch der Pflanzenphysiologie, (W. Ruhland, ed.), **Bd. 5/1,** 252–297 (1960).
[11] H. Kasemir and G. Prehm, Planta **132,** 291–295 (1976).
[12] L. Bogorad, Chemistry and Biochemistry of Plant Pigments, (T. W. Goodwin, ed.), 2nd edn. **Vol. 1,** pp. 64–148, Academic Press, London 1976.
[13] A. E. Brown, F. A. Eiserling, and J. Lascelles, Plant Physiol. **50,** 743–746 (1972).
[14] L. Anderson and H. Egneus, Physiol. Plant. **47,** 11–14 (1979).
[15] H. Oelze-Karow and H. Mohr, Photochem. Photobiol. **27,** 189–193 (1978).