Various Metallopheophorbides as Substrates for Chlorophyll Synthetase

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Chlorophyllide, Zincpheophorbide *a*, Copperpheophorbide *a*, Cobaltpheophorbide *a*, Nickelpheophorbide *a*

Pheophorbide *a* was prepared from a mixture of chlorophylls *a* and *b* by differential extraction with HCl and saponification. The insertion of the following metal ions was investigated: Mg, Zn, Co, Cu, Ni. In the enzyme test with chlorophyll synthetase, the metallopheophorbides fall into two categories: the Mg- and Zn-complexes are good substrates, the Co-, Cu- and Ni-complexes are neither substrates nor competitive inhibitors for the enzyme reaction. This corresponds to two categories of complex structures: Mg- and Zn-porphyrins prefer pentacoordinate square-pyramidal structures, Co-, Cu- and Ni-porphyrins prefer tetracoordinate square-planar structures. A model for substrate binding to chlorophyll synthetase is proposed.

Introduction

The last step of chlorophyll biosynthesis is the prenylation of chlorophyllide with either geranylgeranyl diphosphate or phytyl diphosphate [1]. The activity of chlorophyll synthetase – the enzyme which catalyzes this step – was at first detected in dark-grown ("etiolated") seedlings [1a, 2] but was later also described in green plants [3, 4]. Chlorophyll synthetase activity does not depend on light whereas the previous biosynthetic step, hydrogenation of protochlorophyllide to chlorophyllide requires continuous irradiation in Angiosperms. Etiolated Angiosperm seedlings accumulate protochlorophyllide in the dark. Protochlorophyllide is neither a substrate nor a competitive inhibitor for chlorophyll synthetase. Broken etioplasts prepared from dark-grown oat seedlings which contain protochlorophyllide and chlorophyll synthetase have therefore been incubated with various exogenous chlorophyllides in order to test the substrate specificity of the enzyme [5]. It was found in these experiments that structural variations of substituents at ring A and the isocyclic ring are tolerated by the enzyme. Removal of the central magnesium ion, *i.e.* formation of pheophorbide led to nearly complete loss of acceptance as a substrate [5].

Abbreviations: Hepes, 2-[4-(2-hydroxyethyl)-1-piperazino]ethane sulfonic acid; HPLC, high performance liquid chromatography; MS, mass spectrum; TLC, thin-layer chromatography.

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Since chemical reactions of chlorophyllides, *e.g.* modifications of the side chains, result easily in a loss of the central magnesium, we investigated the insertion of several metal ions into pheophorbide *a* and the possible acceptance of the resulting metal complexes as substrates or competitive inhibitors of chlorophyll synthetase.

Materials and Methods

Thin-layer chromatography (TLC) was performed on silica gel RP8 F_{254} plates (Merck) with methanol/acetone/water (64:20:16, v:v:v). For preparative separations by column chromatography, silica gel C-18 (reverse phase, 55–105 µ 125 Å, Waters) was used with the elution solvent 65% acetone containing 2 mM Hepes-KOH and 1 mM $Na_2S_2O_4$. Electronic absorption spectra were measured with a Lambda 2 spectrophotometer (Perkin Elmer). ^{1}H NMR spectra were recorded on a 360 MHz instrument (Bruker). FAB mass spectra were obtained with a CH7a instrument (Varian MAT).

All operations were carried out under dim light. The solvents were saturated with argon before use. Oxygen was removed from all reaction vessels with argon or nitrogen.

Preparation of pheophorbide a (**1**)

Green leaves of spinach (*Spinacea oleracea* L.) or pea (*Pisum sativum* L.) were used for isolation of a mixture of chlorophylls *a* + *b* according to Iriyama *et al.* [6]. Briefly, 1 kg of leaves were frozen in liquid nitrogen, pulverized and extracted several times with acetone (total volume 4.5 l). The crude

extract was cleared by filtration and mixed with dioxane (270 ml). Water was added slowly to this mixture at 0 °C until the absorption band of chlorophyll at 660 nm disappeared from the supernatant (usually 800–900 ml H_2O were needed). The precipitate which consisted of mainly chlorophylls *a* + *b* was left in the suspension at 0 °C for 90 min. It was then removed by fast filtration at 0 °C with a precooled glass frit. The precipitate was dissolved in a few ml diethyl ether. A typical yield was 340 µmol chlorophyll *a* and 120 µmol chlorophyll *b* (61% of the chlorophylls in the crude extract).

The preparation of pheophorbide *a* (**1**) was performed according to Hynninen and Lötjönen [7]. The mixture of chlorophylls *a* + *b* (300 µmol) was dissolved in 500 ml ice-cold diethyl ether. Ice-cold 30% aqueous HCl (148 ml) was added to the solution. Within 1 h under occasional shaking, pheophorbide *a* accumulated in the lower (HCl) phase whereas pheophytin *b* remained in the upper diethyl ether phase. After separation of the phases, the diethyl ether phase was reextracted three times with 30% HCl (40 ml each). The combined HCl phases were diluted in a separatory funnel with the same volume (ca. 350 ml) ice-water. Ice-cold diethyl ether (350 ml) was added. The pH value was increased by dropwise addition of ice-cold 25% aqueous NH_3 until all pheophorbide *a* was transferred into the diethyl ether phase. The pigment was reextracted into 16% aqueous HCl and from this phase into fresh diethyl ether after dilution of the lower phase with ice-water. The diethyl ether phase was then washed with water until it was acid-free, and dried with Na_2SO_4. The yield of pheophorbide *a* (**1**) was 174 µmol (78%). Analysis by HPLC (on a reverse phase C-18 column with 70% acetone) revealed minor amounts of pheophorbide *b* (less than 2%) as the only contamination.

Preparation of metallopheophorbides a

Various methods were applied for preparation of various metallopheophorbides.

Cu complex [see ref. 8]: Pheophorbide *a* (4.5 µmol) was dissolved in 3 ml glacial acetic acid and refluxed with 2 g Cu(II)acetate. After evaporation of the solvent, the residue was dissolved in acetone/diethyl ether. The diethyl ether phase was washed several times with water in order to remove the acetone, traces of acetic acid and excess of Cu salt. Yield 2.3 µmol (51%) blue-green pigment, λ_{max} in $CHCl_3$ (relative absorption coefficients) = 400 (0.92), 424 (1.0), 507 (0.09), 656 (0.80) nm. TLC revealed 4 components with R_F = 0.29, 0.33, 0.36 and 0.40.

Ni complex: Pheophorbide *a* (4.5 µmol) was dissolved in 2 ml dimethylformamide and heated under stirring with the saturated solution of $NiCl_2 \cdot 6\,H_2O$ in 1 ml dimethylformamide to 70–90 °C until no further spectral change occurred (*ca.* 5 h). Workup with diethyl ether/water as usual yielded 2.0 µmol (44%) green pigment. λ_{max} in $CHCl_3$ (relative absorption coefficients) = 395 (1.02), 421 (1.0), 653 (1.04) nm. TLC revealed 1 main product (≈ 90%) with R_F = 0.28 and 3 byproducts with R_F = 0.24, 0.43 and 0.51.

Co complex: Pheophorbide *a* (4.5 µmol) was dissolved in 2 ml dimethylformamide and heated to 60 °C under stirring. The saturated solution of $CoCl_2 \cdot 6\,H_2O$ in 1 ml dimethylformamide was added and the mixture heated to 60 °C for further 10 min. Workup with diethyl ether/water yielded 1.9 µmol (42%) green pigment. λ_{max} in $CHCl_3$ (relative absorption coefficients) = 405 (1.0), 641 (0.56) nm. TLC revealed 4 products with R_F = 0, 0.20, 0.25 and 0.31.

Zn complex: Pheophorbide *a* (52 µmol) was dissolved in 10 ml CH_2Cl_2 and heated to 35 °C. The saturated solution of $Zn(OAc)_2 \cdot 2\,H_2O$ in 3 ml methanol was added. The mixture was refluxed for 10 min, cooled and poured into diethyl ether. Workup with diethyl ether/water yielded 51 µmol (98%) green pigment. λ_{max} in $CHCl_3$ (relative absorption coefficients) = 427 (1.0), 571 (0.09), 613 (0.17), 659 (0.76) nm. TLC revealed 3 products with R_F = 0.39, 0.45 and 0.53.

The mixture was separated by column chromatography. Pure products were obtained from the first and the last fraction. The most polar fraction proved to be *13²-hydroxy-zinc-pheophorbide a* (**3a**). λ_{max} in $CHCl_3$ (relative absorption coefficients) = 428 (1.0), 570 (0.08), 613 (0.15), 660 (0.78) nm. MS: *m/z* 670 (59%, M^+), 653 (100%), 611 (33%, $M^+ - COOCH_3$) for ^{64}Zn. The unpolar fraction was *zinc-pheophorbide a* (**3**). λ_{max} in $CHCl_3$ (relative absorption coefficients) = 427 (1.0), 567 (0.08), 611 (0.16), 659 (0.80) nm. MS: *m/z* 654 (100%, M^+), 595 (18%, $M^+ - COOCH_3$), 577 (18%) for ^{64}Zn.

Mg-complex: a) according to Strell and Urumov [9]: Pheophorbide *a* (3 μmol) was dissolved in 5 ml dimethylsulfoxide and heated with 350 mg $Mg(OAc)_2 \cdot 4\ H_2O$ to 190 °C for 10 min. The solution was then cooled under a stream of N_2. After workup with diethyl ether/water, a green pigment (1.25 μmol, 42%) was obtained. TLC revealed 1 main green product, pyrochlorophyllide (**5**) ($R_F = 0.48$) and some pyropheophorbide, (**4,** $R_F = 0.27$), which was generated on the thin-layer plate. **5** was identified by its UV/Vis and the ^1H NMR spectra. λ_{max} in diethyl ether (relative absorption coefficients) 428 (1.0), 535 (0.04), 578 (0.05), 615 (0.11), 660 (0.68) nm.

^1H NMR (ppm in d_5-pyridine): 10.00 (s, 10-H), 9.72 (s, 5-H), 8.26 (dd, 3^1-H_X), 6.33 (d, 3^2-H_B, $J_{BX} = 17$ Hz), 6.01 (d, 3^2-H_A, $J_{AX} = 12$ Hz), 5.52, 5.25 (2 × d, AB-spin system of 13^2-H, $J_{AB} = 20$ Hz), 4.62 (m, 18-H), 4.44 (m, 17-H), 3.80, 3.42, 3.24 (3 × s, 2-, 7-, and 12-CH_3).

5 was then demetallated by acidification. The product was identified as a mixture of pyropheophorbide (**4**) and traces of 13^2-hydroxypyropheophorbide (**4a**) by TLC and mass spectrometry: *m/z* 551 (44%, **4a,** $M^+ + 1$), 535 (100%, **4,** $M^+ + 1$), 534 (83%, **4,** M^+), 523 (35%).

b) According to Zass *et al.* [10]. To pheophorbide *a* (10 μmol) dissolved in 2 ml CH_2Cl_2 (distilled over CaH_2), 0.8 mmol 2,6-di-*t*-butyl-4-methylphenoxymagnesium iodid in 1 ml CH_2Cl_2/diethyl ether [10] were added and the mixture stirred for 5 min. After workup with phosphate buffer (pH 6)/diethyl ether a mixture of brown and green pigments was obtained. The excess of 2,6-di-*t*-butyl-4-methylphenol was extracted with hexane. The pigments were separated by column chromatography. Chlorophyllide *a* was eluted ahead of all by-products. It was compared with authentic chlorophyllide *a* prepared from etiolated oat seedlings after flash irradiation according to [22]. λ_{max} in diethyl ether (relative absorption coefficients) = 428 (1.0), 531 (0.05), 575 (0.07), 615 (0.14), 660 (0.78) nm. The demetallated product was investigated by TLC on silica gel (Kieselgel 60, Merck) with the solvent toluene/ethyl acetate/*n*-propanol/ethanol (16:4:1:1, v:v:v:v) according to Endo *et al.* [28]. Besides pheophorbide *a* (**1,** $R_F = 0.23$, *ca.* 40%) the 13^2hydroxy derivative (**1a**) is present (2 epimers, $R_F = 0.18$ and 0.08, *ca.* 60%). MS: *m/z* 630 (59%, **1a,** M^+), 614 (35%, **1,** M^+), 577 (74%), 551 (90%), 549 (100%), 537 (72%).

Chlorophyll synthetase test

The enzyme test was performed with the fraction of purified prolamellar bodies prepared from 7 days old etiolated oat seedlings according to [11, 12]. In the standard assay, a membrane amount containing 0.45 nmol protochlorophyllide was used per sample. The chlorophyllide derivative was dissolved in a small volume of diethyl ether. This solution was added to the solution of 0.05% Brij-Wl and thoroughly mixed by vortexing. After removal of the diethyl ether in a stream of nitrogen, the pigment/detergent solution was used as substrate as described previously [5] but omitting

Table I. Molar extinction coefficients of metallopheophorbides used for calculation of esterification values. All values are given as ε_{mM} [$l \cdot mmol^{-1} \cdot cm^{-1}$].

	80% acetone			*n*-hexane			ref.
	λ_{max} determ.	λ_{max} ref.	ε_{mM} ref.	λ_{max} determ.	λ_{max} ref.	ε_{mM} ref.	
Protochlorophyllide	624	626	30.4	–	–	–	[13]
Mg-pheide (=Chlide) *a*	664	664	76.8	660	662	90.2*	[13]
Zn-pheide *a*	658	659	77.3	654	659	90.3*	[14]
Co-pheide *a*	646	662	29.5**	644	662	29.5**	[15]
Cu-pheide *a*	654	649	58.4	648	652	58.5*	[8]
Ni-pheide *a*	648	649	31.6**	646	649	31.6**	[15]
Pheide *a*	664	665	51.9	666	667	58.0*	[16]
Pyrochlide *a*	664	663	64.0***	660	660	80.0*	[26]

* In diethyl ether.
** In glacial acetic acid.
*** In dimethylformamide.

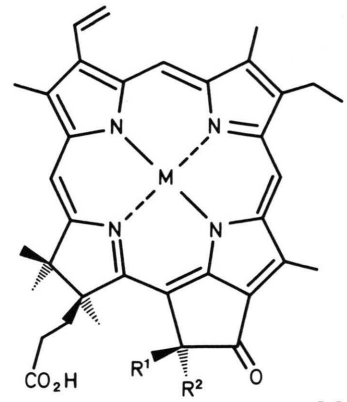
cholate. Geranylgeranyl diphosphate (80 nmol per sample) was added as aqueous solution. The mixture was incubated for 45 min at 25 °C. The enzyme reaction was then stopped by addition of acetone. The esterified pigment was extracted into *n*-hexane as previously described [2]. The molar extinction coefficients given in Table I were used for calculation of esterification values.

Results and Discussion

The aim of the present paper was the comparison of several metal complexes of pheophorbide in the chlorophyll synthetase reaction. In order to avoid variations in the side chains of the macrocyclus which might interfere with the enzyme reaction [5] we used only pheophorbide *a* (**1**) as educt. We found the most convenient method to prepare this educt from a mixture of chlorophylls *a* and *b* [6] by differential extraction from a diethyl ether solution into aq. HCl according to Hynninen and Lötjönen [7]: The *a*-type pigment is extracted into HCl and saponified to pheophorbide *a* whereas the *b*-type pigment remains as pheophytin *b* in the diethyl ether phase.

No general method for insertion of several metal ions into porphyrins exists [17]. Essential criteria for a good insertion reaction are among others the solubility of the porphyrin and of the metal salt in the desired solvent, acidity of the solvent and stability of the porphyrin under the reaction conditions [17]. The reactions described in the present paper turned out to meet these requirements: they were selected from previous collections of experimental procedures [17–19]. The poor stability of pheophorbide turned out to be a particular problem; in agreement with previous papers [20, 21] we found several reaction products instead of only one main product. The products of the zinc insertion reaction were separated by column chromatography. Pure pigments were obtained only from the most polar and the most unpolar fraction. The UV/Vis absorption spectra of these pigments were nearly identical; the absorption peaks of the polar compound are only slightly (*ca.* 1–2 nm) bathochromically shifted compared with the unpolar compound. According to the λ_{max} values, migration in TLC and mass spectra (see Materials and Methods), the most polar fraction is assumed to be 13^2-hydroxy-zinc-pheophorbide *a* (**3a**) and the unpolar fraction the unaltered zinc-pheophorbide *a* (**3**). In spite of working under argon gas, the reaction products must have arisen from reaction with oxygen from residual air. The tendency of the zinc complex to form these allomerization products is apparently very high.

Structures



	M	R^1	R^2
1 pheophorbide *a*	2H	H	CO_2CH_3
2 chlorophyllide *a*	Mg	H	CO_2CH_3
3 zinc-pheophorbide *a*	Zn	H	CO_2CH_3
4 pyropheophorbide *a*	2H	H	H
5 pyrochlorophylide *a*	Mg	H	H

1a–5a are the corresponding 13^2-hydroxy derivatives, $R^1 = OH$.

The mixtures of insertion products with Cu, Co and Ni were not investigated further when it turned out that they did not react with chlorophyll synthetase (see below). According to earlier literature [20, 25] and our experience with Mg- and Zn-derivatives, the main by-products should be oxidation products at the 13^2-position (hydroxy-, acetoxy-) under the applied reaction conditions. This would not hamper the enzyme reaction (see below). Further modifications of the macrocyclus (*e.g.* lactone formation) should be detectable in the UV/Vis spectra. Such compounds were not present as major products in our reaction mixture.

Since the large-scale separation of chlorophyll derivatives *a* and *b* was best achieved by HCl extraction which implied demetallation, we tried also to reinsert magnesium into pheophorbide *a*. The reaction with magnesium acetate in dimethylsulfoxide according to Strell and Urumov [9] required high temperature (about 190 °C) for metal inser-

tion. At this temperature, the 13^2-carbomethoxy group is already cleaved off. We obtained pyrochlorophyllide *a* (**5**) under these conditions in good yield. Identification of **5** was achieved with several methods. Thin-layer chromatography of the reaction mixture revealed only one green spot which was different from authentic chlorophyllide *a* (**2**). We could not find any **2** in the reaction mixture. The absorption spectrum (Fig. 1) of the product revealed λ_{max} values identical with those of authentic **2**. However, the absorbance ratio $A_{660}:A_{428}$ was smaller (0.68) than for authentic **2** (0.78, Fig. 1). The final identification was achieved by mass and ^{1}H NMR spectroscopy.

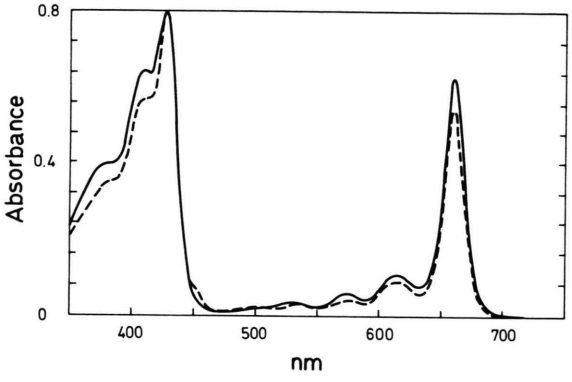


Fig. 1. Electronic spectra of chlorophyllide *a* (——) and pyrochlorophyllide *a* (– – –) in diethyl ether.

In the ^{1}H NMR spectrum of **5**, the 13^2-H singlet expected for **2** is replaced by 2 doublets ($J_{AB} = 20$ Hz) at 5.52 and 5.25 ppm which represent the AB-spin system of the 13^2-methylene protons of **5**. The relatively large difference in the chemical shift of these protons seems to be due to the presence of the free 17^3-carboxy group. Another typical feature for the pyrocompound is the missing singlet for the methoxy-group at C-13^3; only the singlets for the methyl-groups at C-2, C-7 and C-12 are found in the range between 3.2 and 4 ppm in the spectrum of **5**.

Sharper TLC separations than with the Mg complexes are achieved with the metal-free pheophorbides. After demetallation of **5**, traces of a more polar product were obtained besides the expected pyropheophorbide **4**. The mass spectrum indicated the presence of pyropheophorbide but revealed also a by-product which contains one more oxygen than the expected pyropheophorbide; we assume that it is the 13^2-hydroxy derivative **4a**. This must have been formed before decarboxymethylation: the pyro-compound is not hydroxylated even in the presence of oxygen. No indication for the presence of pheophorbide or 13^2-hydroxypheophorbide was obtained by TLC or mass spectrometry. The decarboxymethylation must have been quantitative.

The preparation of chlorophyllide *a* (**2**) from pheophorbide *a* (**1**) was successful with 2,6-di-*t*-butyl-4-methyl-phenoxy-magnesium iodid according to [10]. The product was purified by column chromatography and identified by UV/Vis spectroscopy, TLC and mass spectroscopy as a mixture of chlorophyllide *a* (**2**, 40%) and its 13^2-hydroxy-derivative (**2a**, 60%). Hydroxylation at C-13^2 occurs apparently also under these reaction conditions. Other green and brown by-products were not investigated further.

The low solubility of metallopheophorbides in water demanded addition of a detergent for application of these substrates to the enzyme preparation. We found the polyoxyethylene ether detergent Brij-W 1 superior to the previously used cholate [5]. The enzyme reaction was performed with each metallopheophorbide at several concentrations. It had been shown previously [22] that the enzyme reaction is inhibited by lack of release of the product into the membrane lipid phase. Since the capacity of the membrane preparation is limited for the uptake of the product, saturation curves are expected for the enzyme reaction. Furthermore, substrate inhibition with "natural" chlorophyllide (isolated from etiolated oat seedlings after flash irradiation [22]) has been observed at higher substrate concentrations [22] so that optimum curves are expected when the amount of product is plotted against the amount of applied substrate.

The typical optimum curve for "natural" chlorophyllide *a* (Mg pheophorbide *a*, **2**) is shown in Fig. 2. The values for esterification can deviate considerably for different enzyme preparations. Within one enzyme preparation, however, the shape of the optimum curve can easily be detected. Chlorophyllide *a* added in small amounts (up to about 0.5 nmol per sample) was nearly completely esterified. The optimum amount of esterified pigment was obtained by addition of 1–2 nmol chlorophyllide; the esterification is about 30–50% of

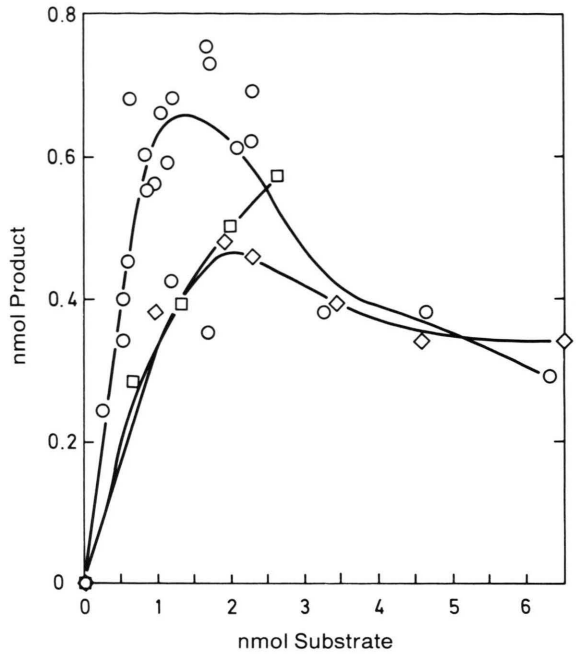


Fig. 2. Esterification of exogenous chlorophyllide *a*, isolated from oat seedlings (○—○) or produced by Mg insertion into pheophorbide *a* (□—□) and pyrochlorophyllide *a* (◇—◇) with exogenous geranylgeranyldiphosphate in a prolamellar body fraction from etiolated oat seedlings. The amount of protochlorophyllide was 0.45 nmol per sample. The esterified pigment, determined after incubation for 45 min at 25 °C, was nearly 100% of the added substrate at low (0.5 nmol) but only 30–50% of the added substrate at medium (1–2 nmol) substrate concentrations. Product values are given for several membrane preparations. The optimum curve was drawn with consideration of product values from only one membrane preparation.

the added substrate in this case. Addition of even higher amounts of substrate yielded less esterified pigment. The esterification of **2** produced by Mg insertion into **1** is somewhat lower than that of "natural" **2** isolated according to [22] (see Fig. 2). We attribute this difference to the presence of 13^2-hydroxychlorophyllide (**2a**) in the product of Mg insertion which makes up about 60% of the applied chlorophyllide mixture. Pyrochlorophyllide *a* (**5**) is a relatively good substrate for chlorophyll synthetase. As shown in Fig. 2, the percentage of esterification at low concentrations was about half as high as for chlorophyllide *a*. The esterification values for **5** determined here are higher than those which had been determined previously [5]. We found that **5** looses its central Mg very easily. The resulting pyropheophorbide **4** (like pheophorbide **1**) is no substrate for chlorophyll synthetase. It may be that some demetallation of pyrochlorophyllide had occurred in the early esterification experiments [5] so that the apparent value for the esterification was too low.

In Fig. 3, the concentration dependency of the esterification is compiled for several potential substrates. Neither the metal-free pheophorbide *a* (**1**) nor its Co-, Cu-, or Ni-complexes show significant esterification at any of the tested concentrations. However, the zinc-complexes **3** and **3a** are suitable substrates for chlorophyll synthetase (Fig. 3). The best substrate is zinc-pheophorbide *a* (**3**); it yields about the same amount of esterified pigment as chlorophyllide *a* (see Fig. 1) albeit only at higher substrate concentrations. Surprisingly the 13^2-hydroxy derivative **3a** is also a suitable substrate. The maximum amount of esterified pigment ob-

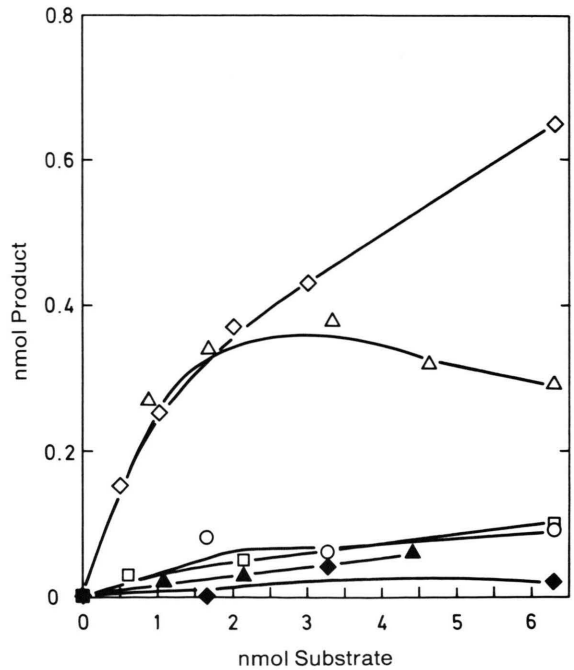


Fig. 3. Esterification of exogenous metallopheophorbides *a* with exogenous geranylgeranyldiphosphate in a prolamellar body fraction from etiolated oat seedlings. For conditions see Fig. 2. Substrates: ◇—◇ zincpheophorbide *a*, △—△ 13^2-hydroxy-zinc-pheophorbide, ○—○ copper-pheophorbide *a*, □—□ nickel-pheophorbide *a*, ▲—▲ cobalt-pheophorbide *a*, ◆—◆ pheophorbide *a*.

tained from **3a** is about half the maximum amount of esterified pigment after application of chlorophyllide (**2**).

We tested also the possible competitive inhibition with the natural substrate chlorophyllide *a* of those metallopheophorbides which are no substrate themselves. As shown in Table II, small amounts of chlorophyllide *a* are esterified in a normal manner when applied in a mixture with an excess of metal-free pheophorbide, of Co-, Cu-, or Ni-pheophorbide. The lack of competitive inhibition is most probably due to lacking affinity of pheophorbide and its Co-, Cu-, and Ni-complexes to the active center of the enzyme. Lack of binding to chlorophyll synthetase from etiolated oat seedlings has previously been deduced from similar experiments with pheophorbide *a* and bacteriochlorophyllide *a* [5].

For an explanation of the different behavior of the Mg- and Zn-complexes on the one hand and the Co-, Cu-, and Ni-complexes on the other hand one has to consider the structures of the metal complexes. Cu(II), Co(II) and Ni(II) ions tend to form tetracoordinate square-planar porphyrin chelates; the addition of further ligands can only be observed with some porphyrins, the stability of such penta- or hexacoordinate complexes of these metal ions is low [19, 23]. Contrary to this situation, Mg(II) and Zn(II) ions prefer pentacoordinate square-pyramidal structures in their porphyrin complexes [19, 23]. Crystalline ethyl chlorophyllide *a* contains water as a further ligand; as a sterical consequence of the pentacoordinate structure, the Mg ion is slightly (+0.39 Å) shifted out of the porphyrin plane [24]. We assume that Cu-, Co-, and Ni-pheophorbides *a* exist as square-planar complexes without further axial ligands whereas Mg- and Zn-pheophorbides *a* contain an additional polar ligand (presumably water) under our experimental conditions (Fig. 4). This would explain the higher polarity of Mg- and Zn-pheophorbides in the TLC compared to Cu-, Co-, and Ni-pheophorbides (see Materials and Methods). In the enzyme-substrate complex, the axial ligand is most probably an amino acid side chain of chlorophyll synthetase. This assumption would compulsorily imply binding of only the Mg- and the Zn-complex and lack of binding of pheophorbide *a* and its Co-, Cu-, and Ni-complexes. We cannot yet predict which amino acid takes part in this presumptive substrate binding. It should be kept in mind, however, that oxygen ligands have a relatively high affinity to Mg and Zn ions and a relatively low affinity to Co, Cu and Ni ions. This

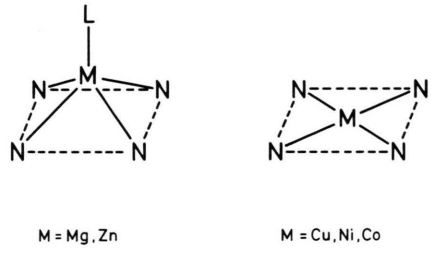


Fig. 4. Chelation of different metallopheophorbides. Only the Mg and Zn complexes bind to chlorophyll synthetase. Cu, Ni and Co complexes are tetracoordinate involving only the 4 nitrogens of the macrocyclus. Mg and Zn are pentacoordinate. The fifth ligand is water in crystalline ethylchlorophyllide [24]. We assume that the fifth ligand is offered by the active center of chlorophyll synthetase and contributes to substrate binding.

Table II. Lack of competitive inhibition of metallopheophorbides *a* in the chlorophyll synthetase reaction with chlorophyllide *a*.

Substrate added [nmol]	Substrate esterified (% of added substrate) [nmol]	Substrate mixture added [nmol]	Substrate esterified (% of added Mg substrate) [nmol]
0.50 Mg	0.38 (77%)		
0.55 Mg	0.45 (83%)		
1.03 Mg	0.56 (54%)		
1.57 H*	0 (0%)	1.57 H* + 0.55 Mg	0.46 (84%)
1.08 Co	0.03 (2%)	1.06 Co + 0.62 Mg	0.46 (74%)
1.69 Cu	0.03 (2%)	1.69 Cu + 0.51 Mg	0.39 (77%)
1.22 Ni	0.03 (3%)	1.22 Ni + 0.55 Mg	0.46 (84%)

* H: without central metal ion = pheophorbide *a* (**1**).

could explain the differences between these two groups of complexes even better than nitrogen ligands which usually have a higher affinity to Co, Cu, and Ni ions than to Mg and Zn ions.

It should be recalled here that substrate binding of chlorophyll synthetase depends on several structural requirements, *e.g.* the hydrogenated ring D and the unsaturated ring B [5]. We can add here requirements at the isocyclic ring: the carboxylic ester group at C-13^2 seems to contribute only marginally to substrate binding because the pyro-compound is a relatively good substrate. On the other hand, the active center must be large enough to allow the 13^2-hydroxy derivative to bind (see Fig. 3). Further modifications at side chains for production of new substrates for chlorophyll synthetase are presently under investigation.

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