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Methyl Chlorophyllide *a* as a Probable Intermediate in the Chlorophyll *a* Pathway[1]

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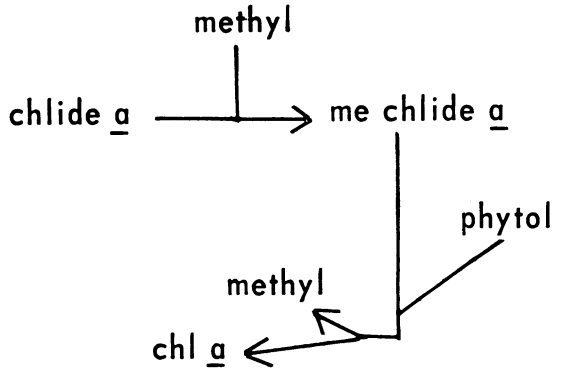
Although the enzyme chlorophyllase has been long known (13) only recently has evidence been presented for its probable function in chlorophyll *a* (chl *a*) biogenesis, namely, the demonstration that methyl chlorophyllide *a* (me chlide *a*) was transesterified with phytol in the presence of chlorophyllase yielding chl *a* (2). However, no evidence has been presented that would indicate the presence of me chlide *a* in green plants, a finding that would strongly support the above as the function of chlorophyllase in chl *a* biosynthesis.

This report describes the isolation and partial characterization of minor quantities of me chlide *a* from extracts of expanding wheat (*Triticum aestivum*) or passion flower leaves (*Passiflora* × *alatocaerulea*). This finding together with that described above suggests that the final steps in chl *a* biogenesis are



Approximately 5-day-old expanding leaves were ground in a mortar in the dark with reagent acetone. The extracted pigments in acetone were immediately transferred into diethyl ether. The diethyl ether solution was dried briefly over anhydrous Na_2SO_4, and then evaporated to dryness in a flash evaporator. The residue was redissolved in a minimal amount of fresh anhydrous diethyl ether for chromatography.

During the extraction procedure great care was exercised to avoid contamination of the pigment solution with short chain alcohols. [Since chlorophyllase has been shown to be present and active in pigment extracts such as the one described here (9), the presence of such contaminants would probably result in the production of small quantities of me chlide *a* during pigment extraction and subsequent work up.]

The me chlide *a* was isolated on sucrose (3) or commercial silica gel (Brinkman Instruments, MN Polygram Sil-S-HR) thin layer plates (10) or on sucrose columns [as described in detail elsewhere (12)] using synthetic me chlide *a* (5) as the chromatographic reference. Sucrose thin layer plates were developed using 0.5 % (v/v) isopropanol in petroleum ether (35°–60° fraction); silica gel plates with benzene-ethyl acetate-ethanol (80:20:2.5) (v/v). The thin layer R_F's found for me chlide *a* are presented in table I. To obtain the pure compound from the crude pigment extracts several chromatographic runs were necessary.

Alternatively, to assist in the verification of the porphyrin as methyl pheophorbide *a* (me ϕfbd *a*) the free base could be extracted and partially purified from an ethereal solution of crude pigments using 18 % (v/v) aqueous HCl. The porphyrins in HCl were immediately transferred into ether to avoid hydrolysis. The me ϕfbd *a* was purified on sucrose plates (0.5 % v/v) isopropanol in petroleum ether) or the commercial silica gel plates (benzene-ethyl acetate-ethanol 80:20:2.5) using synthetic me ϕfbd *a* [from pheophorbide *a* and diazomethane (3)] as the chromatographic reference. The R_F's of these compounds are also presented in table I.

The metalloporphyrin, resembling chlide *a*, that was isolated by column or thin layer chromatography

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Table I. *Thin Layer Chromatographic R_F Values of Methyl Chlorophyllide* a *and Its Free Base*

Compound	Silica gel[1]	Sucrose[2]
Methyl ϕfbd *a* (synthetic)	0.57	0.57
Methyl ϕfbd *a* (isolated from expanding leaves)	0.57	0.57
Methyl chlide *a* (synthetic)	0.84	0.16
Methyl chlide *a* (isolated) from expanding leaves)	0.84	0.16

[1] Developer—benzene, ethyl acetate, ethanol (80:20:2.5 v/v).

[2] Developer—petroleum ether, isopropanol (99.5:0.5 v/v).

of the crude pigment extract of wheat or passion flower leaves had visible absorption spectrum and absorption maxima identical with that of synthetic me chlide *a* (6) (table II), thin layer chromatographic R_F's identical with that of the synthetic material on silica gel and sucrose thin layer plates (table I), an HCl number of 16 (13) (that of methyl pheophorbide *a* is $\cong$ 16.0), and gave a positive phase test (1).

The suspected free base (me ϕfbd *a*, prepared by acid hydrolysis of the isolated me chlide *a* or extracted by 18% aq HCl (v/v) from the crude ethereal solution) had a visible spectrum identical with that of authentic me ϕfbd *a* (table II) and chromatographic R_F's on sucrose or silica gel chromatograms (see above) identical with that of synthetic me ϕfbd *a* (table I). When the free base was subjected to acid hydrolysis, the product, with a visible spectrum of ϕfbd *a*, had an R_F identical with that of authentic ϕfbd *a* on silica gel chromatograms [using benzene-ethyl acetate-ethanol (80:20:5.0 (v/v) (10)]. Too little of the material was available to perform a methanol determination of the hydrolysate.

Table II. *Visible Absorption Maxima of Methyl Chlorophyllide* a *and Its Free Base in Ether*

Compound	λmax	Ratio[1]
	nm	
Methyl ϕfbd *a* (synthetic)	408,667	2.04
Methyl ϕfbd *a* (isolated from expanding leaves)	408,667	2.05
Pheophytin *a* (11)	408.5,667	2.07
Methyl chlide *a* (synthetic)	430,662	1.30
Methyl chlide *a* (isolated from expanding leaves)	430,662	1.31
Chlorophyll *a* (11)	430,662	1.31

[1] Ratio of Soret absorption maximum to that of the red maximum as recorded on a Cary Model 14 spectrophotometer.

Table III. *Total Pheophytin* a *to Isolated Methyl Pheophorbide* a *From Expanding Wheat Leaves as Determined by Visible Spectrophotometry*

Samples were taken from 100 wheat plants.

Days grown	Wt ratio
5[1]	218.5
7	795.0
9	...[2]
11	...[2]

[1] Youngest age of plants for which a workable size could be obtained.

[2] No methyl ϕfbd *a* detectable.

When primary wheat leaves of various ages were examined for relative me chlide *a* content (extracted as me ϕfbd *a* for these studies), it was found that mature leaves yielded no detectable me ϕfbd *a* (table III). The maximum accumulation of the compound appeared at 5 days. The maximum rate of chlorophyll *a* biogenesis appears at 5 to 7 days in these leaves (7, 8). These findings further support the hypothesis that me chlide *a* is a chl *a* precursor.

During the course of this study, no methyl chlorophyllide *b* or its free base were discernible.

Presently, radiotracer experiments using *in vitro* systems are being performed in an attempt to determine the probable biological methyl donor and role of chlorophyllase in the methylation reaction. These results should specify the precise function of chlorophyllase in chlorophyll *a* biogenesis.

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