Phytochemistry, 1970, Vol. 9, pp. 2311 to 2313. Pergamon Press. Printed in England.

SHORT COMMUNICATION

STUDIES ON THE ESTERIFICATION OF CHLOROPHYLLIDES

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(*Received* 5 *March* 1970)

Abstract—Methyl chlorophyllide *a*, but not chlorophyllide *a*, was esterified with phytol (or geranylgeraniol) in the presence of chlorophyllase from *Chlorella protothecoides*. No detectable esterifying activity was found when the pyrophosphate derivatives of phytol and geranylgeraniol were used.

THE SUGGESTION by Granick[1] that "the mechanism of phytylation is probably via a phytol pyrophosphate rather than by the hydrolytic enzyme, chlorophyllase" has confused the generally accepted view that chlorophyllase catalyses chlorophyll *a* formation by esterifying chlorophyllide *a* with phytol.[2-4] Chiba *et al.*, using chlorophyllase from *Chlorella protothecoides*, were only able to demonstrate the phytylation of methyl chlorophyllide *a* but not of chlorophyllide *a* and have proposed a scheme to account for this result,[5] whilst Holden was not able to phytylate chlorophyllide with a chlorophyllase preparation from sugar beet leaves.[6] In contrast to this, Shimizu and Tamaki claimed to be able to convert chlorophyllide to chlorophyll and phaeophorbide to phaeophytin in the presence of phytol and a chlorophyllase preparation from tobacco leaves.[7]

Aliquots (2 ml) of a chlorophyllase preparation, similar to that described by Chiba *et al.*[5] and having identical hydrolytic characteristics, were used in the preparation of reaction mixtures containing 1·4 ml of methyl chlorophyllide *a* in acetone (absorbance = 23·2/ml at 663 nm) and either 2 ml of 0·1 M phytol or geranylgeraniol in acetone and 0·4 ml of 0·2 M borate phosphate buffer, pH 7·0, or 0·4 ml of phytyl pyrophosphate or geranylgeranyl pyrophosphate (prepared by the method of Upper and West[8] and purified by the method of Anderson and Porter)[9] in 0·2 M borate phosphate buffer, pH 7·0, and 2 ml of acetone. In addition a similiar reaction mixture containing an equimolar solution (0·1 M in all) of phytol and geranylgeraniol was prepared. The final acetone concentration in all reaction mixtures was 58·6%. Aliquots (1·6 ml) were taken from each reaction mixture at zero time and after 30 min of incubation, each diluted with an equal volume of acetone and extracted twice with diethyl ether (5 ml in total). The ethereal extracts were washed, dried (Na_2SO_4) and evaporated in a stream of nitrogen. Portions ($\frac{1}{10}$) of the samples were applied as spots in cyclohexane to a

[1] S. GRANICK, *Biochemistry of Chloroplasts* (edited by T. W. GOODWIN), Vol. II, p. 401, Academic Press, New York (1967).
[2] M. HOLDEN, *Biochem. J.* **78**, 359 (1961).
[3] S. SHIMIZU and E. TAMAKI, *Botan. Mag.* **75**, 462 (1962).
[4] P. BÖGER, *Phytochem.* **4**, 435 (1965).
[5] Y. CHIBA, I. AIGA, M. IDEMORI, Y. SATOH, K. MATSUSHITA and T. SASA, *Plant Cell Physiol. Tokyo* **8**, 623 (1967).
[6] M. HOLDEN, *Photochem. Photobiol.* **2**, 175 (1963).
[7] S. SHIMIZU and E. TAMAKI, *Arch. Biochem. Biophys.* **102**, 152 (1963).
[8] C. D. UPPER and C. A. WEST, *J. Biol. Chem.* **242**, 3285 (1967).
[9] D. G. ANDERSON and J. W. PORTER, *Arch. Biochem. Biophys.* **97**, 509 (1962).

thin layer (400 μ thick, 40 × 20 cm) of cellulose MN 300 and chromatographed in the longer dimension according to the method of Schneider.[10,11] A tracing of this plate, together with representative densitometric scans (Joyce Loebl Chromoscan, TLC attachment) of each chromatogram, is shown in Fig. 1. Material co-chromatographic with chlorophyll *a* was observed and confirms the results obtained by Chiba *et al.*[5] with respect to phytol. In addition a chlorophyll-like compound was detected in the reaction mixtures containing geranylgeraniol. No evidence for the formation of chlorophylls from pyrophosphate precursors was obtained. Chlorophyllide *a* was formed from methyl chlorophyllide *a* during 30 min incubation and methyl phaeophorbide *a* also appeared as a breakdown product. All manipulations

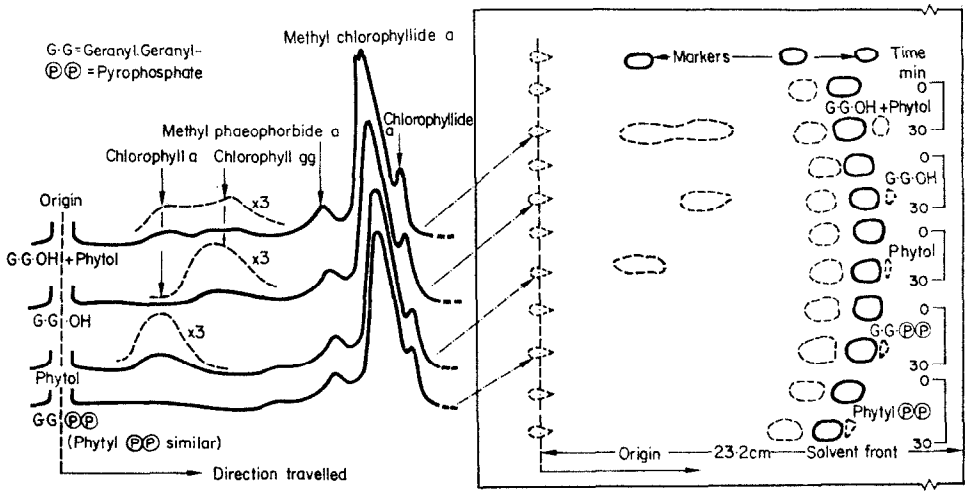

FIG. 1. TRACING AND DENSITOMETRIC SCANS OF TLC OF THE PRODUCTS OF ENZYMIC ESTERIFICATION AND HYDROLYSIS OF METHYL CHLOROPHYLLIDE *a*.

were carried out in a darkened room to avoid photodecomposition but the high levels of methyl phaeophorbide *a* (12·6%) in relation to the low levels of chlorophyll *a* (5·5%) and chlorophyll *gg* (4·4%) may be ascribed to the effect of the adsorbent during chromatography. Those fractions containing chlorophyll-like compounds were chromatographed as bands, eluted with ether, and the extract blown to dryness in a stream of nitrogen. The total absorbance of each component was determined in diethyl ether at 663 nm and these values are shown in Table 1. The visible spectra of the two chlorophyll-like compounds were indistinguishable from that of chlorophyll *a*. The solutions containing chlorophyll-like material were blown to dryness in nitrogen, redissolved in CCl_4 and i.r. spectra were recorded on a Perkin Elmer 257 spectrophotometer using microcells. Although hampered by small quantities of material the spectra corresponded to that of authentic chlorophyll *a* but no distinction due to the contribution made by the esterifying moiety could be made. The chlorophyll-like samples were saponified and the ether-extractable material subjected to GLC in a Pye 104 Chromatogram (Model 4) and a 7 ft column (packed with 3% SE-30 on 100/120 mesh diatomite) operated isothermally at 190° with a carrier gas flow rate of 120 ml/min. A peak with a retention time of 13·6 min corresponding to that of authentic geranylgeraniol was obtained

[10] H. J. A. W. SCHNEIDER, *J. Chromatog.* **21**, 448 (1966).
[11] H. J. A. W. SCHNEIDER, *Phytochem.* **7**, 885 (1968).

TABLE 1. TOTAL ABSORBANCIES AT 663 nm OF THE PRODUCTS OF ENZYMIC ESTERIFICATION AND HYDROLYSIS OF METHYL CHLOROPHYLLIDE *a*

	Methyl chlorophyllide *a*	Chlorophyllide *a*	Methyl phaeophorbide *a*	Chlorophyll *a*	Chlorophyll *gg*
Phytol incubation	16·8	1·71	2·12	0·92	—
Geranylgeraniol incubation	16·2	1·67	1·91	—	0·71

from the material running ahead of chlorophyll *a* (R_f 0·28), presumably chlorophyll *a* with a geranylgeranyl side-chain and tentatively called chlorophyll *gg*. The ratio of the peaks from the saponification of chlorophyll-like material from equimolar incubation was 1–0·7 (chlorophyll *a*–chlorophyll *gg*).

A parallel experiment using chlorophyllide *a* instead of methyl chlorophyllide *a* failed to show any phytylation. This is in agreement with the findings of Chiba *et al.*[5] for this chlorophyllase from *C. protothecoides*.

Further experiments at lower acetone concentrations of 25% and 5% using phytol or phytyl pyrophosphate with either methyl chlorophyllide *a* or chlorophyllide *a* and in the presence and absence of chlorophyllase also failed to demonstrate *in vitro* phytylation. It would appear, therefore, that a high concentration of acetone is necessary for the esterification to proceed *in vitro*.

In conclusion, no *in vitro* evidence for phytylation by phytyl pyrophosphate, as suggested by Granick, was found under the conditions employed. The presence in nature of a chlorophyll with a geranylgeranyl side-chain has never been reported and raises questions about the relationship between esterification and the saturation of the phytyl side-chain. Could the former precede the latter *in vivo* or is the *in vitro* formation of chlorophyll *gg* only due to the high concentration of geranylgeraniol in the reaction mixture? Further experiments are needed to resolve this problem.

Acknowledgement—The gift of two samples of geranylgeraniol from F. Hoffmann La Roche & Co., Basle, Switzerland, is gratefully acknowledged.