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PHYTOL SYNTHESIS FROM GERANYLGERANIOL IN SPINACH CHLOROPLASTS

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SUMMARY: The reduction of /2-^{14}C/-geranylgeranylpyrophosphate to phytylpyrophosphate is shown for the first time in chloroplasts. The esterification of exogenous /2-^{14}C/-geranylgeranylpyrophosphate with endogenous chlorophyllide and the stepwise reduction of the pigment bound geranylgeraniol to phytol was also proved for spinach chloroplasts for the first time.

INTRODUCTION: The two diterpenes geranylgeraniol (GG) and phytol (P) play an important role in the chloroplasts of higher plants through their function as intermediates in the synthesis of components such as chlorophyll (Chl), phylloquinone and tocopherol (T). A stepwise 2 electron reduction of Chl a esterified with GG (Chl a_{GG}) to Chl a was demonstrated for different etiolated seedlings and etioplasts systems (1 - 4). GG was shown to be the precursor of phytol in whole leaves (5). The reduction of GG or GG-pyrophosphate (GGPP) to P or phytylpyrophosphate (PPP) could not yet be demonstrated in isolated chloroplasts. This reaction however is essential for the synthesis of α-tocopherol (αT) in chloroplasts (6). The enzyme which catalyses the incorporation of PPP in αT precursors is strongly specific for PPP and does not use GGPP (6). Here we report for the first time the reduction of GG → P and of Chl a_{GG} → Chl a for intact purified spinach chloroplasts.

MATERIAL AND METHODS: Radiochemicals: /2-^{14}C/-ethylbromacetate (spec. act. 4.7 mCi/mmol) was purchased from Amersham Buchler (Braunschweig). GG was a gift from Dr. W. Hoffmann (BASF, Ludwigshafen). All-trans farnesylacetone was a gift from Dr. F. Weber, Hoffmann-La Roche, Basel).

Synthesis of /2-^{14}C/-geranylgeraniol: This synthesis is a multistep synthesis, the intermediates were neither isolated nor purified inbetween.
a) Synthesis of /2-^{14}C/-triethylphosphonoacetate (7): 0.19 mmol triethylphosphite were heated with 0.175 mmol /2-^{14}C/-ethylbromacetate 5 h under reflux at 115 - 125° C.
b) Synthesis of /2-^{14}C/-3,7,11,15-tetramethyl-2,6,10,14-hexadecatetraene-1-acidethylester: The in a) formed intermediate was suspended in 200 µl of dimethoxyethane and 0.4 mmol NaH (50 % dispersion in oil) were added at -18° C over a period of 0.5 h. The solution was stirred for another 0.75 h, then 0.16 mmol all-trans farnesylacetone (6, 10,14-trimethyl-5,9,13-pentadecatri-

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ene-2-on) was added. It was heated at 60° C for 4.5 h and stirred for 15 h at room temperature. The solution was extracted with Et_2O and the Et_2O-phase evaporated.
c) Synthesis of /2-^{14}C/-GG: The intermediate ester was resolved in 500 µl Et_2O and 40 mg $LiAlH_4$ were added at -30° C over a period of 0.5 h. The solution was stirred for 1 h at room temperature. Saturated NH_4Cl solution was added and the alcohol extracted with Et_2O. /2-^{14}C/-GG was purified by TLC (System I) on silicagel plates. A solvent system of petroleum (b.p. 60-80° C) / Et_2O 1:1 (v/v) was used. The yield after purification on System I was 63 % (spec. act. 1.2 mCi/mmol). According to the radioscan the purity was 97 %. The synthesized GG is a mixture of 70 % E and 30 % Z (8, 9).

Synthesis of /2-^{14}C/-GGPP: /2-^{14}C/-GGPP was synthesized from the alcohol and triethylammoniumphosphate as tetraammoniumsalt as described in (10, 11).

Isolation of purified chloroplasts: Chloroplasts were isolated from field-grown spinach by standard methods (12). They were further purified on percoll gradient (13). To assure free passage of ATP and /2-^{14}C/-GGPP through the chloroplast envelope the intact purified chloroplasts were osmotically shocked in hypotonic buffer solution (HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfone acid) 10 mM pH 7.6; 4 mM $MgCl_2$).

Reaction mixture: The complete reaction contained, if not otherwise defined: 10 mM HEPES pH 7.6; 4 mM $MgCl_2$; 2 mM $MnCl_2$; 0.1 mM NaF; 0.5 mM ATP; 10 mM $NaHCO_3$; 0.5 mM /2-^{14}C/-GGPP; the final volume of 1 ml contained not less than 3 mg of chlorophyll. The chloroplast suspension was illuminated (0.1 J/cm^2 sec) and slowly stirred at 20° C in the water bath.

Chromatography of products: Aliquots (300 µl) were taken at different times. The reaction was stopped and the lipids extracted in a MeOH/$CHCl_3$ mixture according to (14). For better purification Chl were converted to phaeophytins (Phae) and the terpenoid-PP were hydrolized to the corresponding alcohols in the extraction mixture by acidification with HCl (final conc. 1 N) at 37° for 0.5 h (10). Unlabeled P was added as carrier and the products were purified on System I. The isoprenoids were rechromatographed on silicagel plates (impregnated with 10 % $AgNO_3$ in EtOH / acetonitrile 9 : 1 (v/v). A solvent system of n-hexan / di-isopropylether / ethylacetate 2 : 1 : 1 (v/v/v) was used (System II). Phae was saponified by 0.5 M KOH in MeOH 0.5 h under reflux. The reaction was carried out under N_2, pyrogallol, GG and P were added. The reaction mixture was extracted with Et_2O, washed free of alkali and the terpenoid alcohols were chromatographed on System II. The terpenoid alcohols were visualized by spraying with 20 % molybdatophosphoric acid in MeOH and heating at 120° C. The radioactive areas were scraped off the plates, dissolved in 2 ml MeOH and were counted in 5 ml scintillation mixture (Hydroluma, Baker, Groß Gerau, West Germany) using a Packard liquid scintillation counter.

RESULTS: Reduction of free GGPP: When purified spinach chloroplasts were incubated in the presence of /2-^{14}C/-GGPP labeled phytol could be detected as product (see Fig. 1).

Reduction of Chl a_{GG}: The synthesis of Chl a_{GG} from IPP was shown in a recombined system of thylakoids and soluble chloroplast proteins for spinach (14).

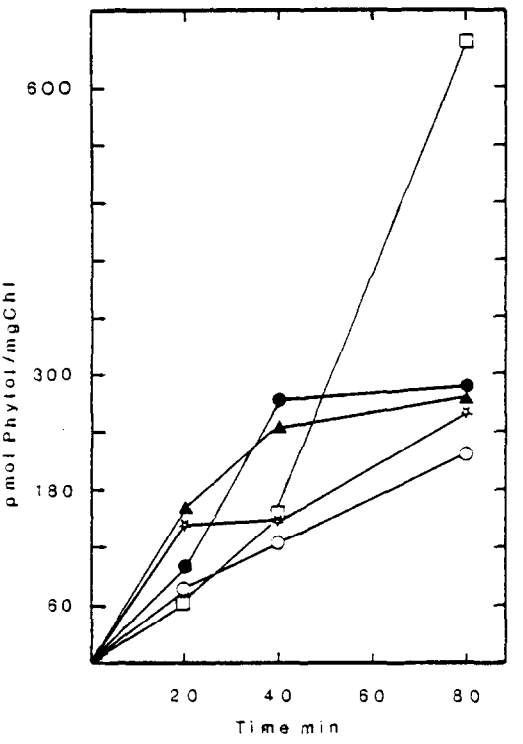

Figure 1: Time course incorporation of /2-^{14}C/-GGPP in phytol by spinach chloroplasts. ▲ NADPH (3 mM); ● NADPH (3 mM) + $FADH_2$ (0.1 mM); ○ $FADH_2$ (0.1 mM) + dithiothreitol (2 mM); ✱ NADPH (3 mM) + FAD (0.1 mM); □ NADPH (3 mM) + $FADH_2$ (0.1 mM) + dithiothreitol (2 mM) + thioredoxin (80 µg). For further details see Material and Methods.

A stepwise reduction to Chl a which is proved in etiolated systems could not be demonstrated for this recombinated system (personal communication of Prof. Dr. R. Douce). As shown in Fig. 2 the reduction of Chl a_{GG} can occur with good yields also in the green system. The reduction is very quick. Labeled phytol could already be detected after 5 min (data not shown). Exogenously NADPH effects in a higher yield in the chlorophyll bound terpenoids DHGG (dihydro-GG) and THGG (tetrahydro-GG) (compare Fig. 2a and b). This is in good agreement with the results in ref. (3). The determination of DHGG and THGG was done by their characteristics on thinlayers chromatograms (compare 1, 3, 15).

DISCUSSION: The results show for the first time that the last step in P biosynthesis is also performed in the chloroplast. Until now only indirect evidence was given for this reaction by the incorporation of various precur-

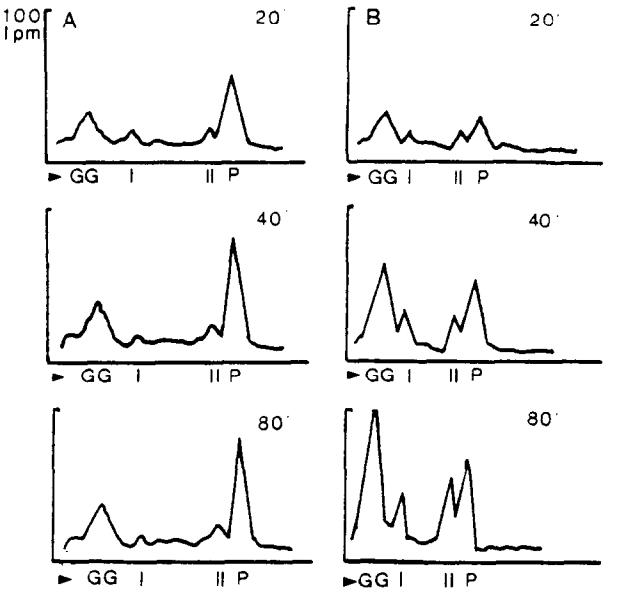

Figure 2: Radioscan of the time course incorporation (20, 40, 80 min) of /2-^{14}C/-GGPP in Chl a by purified spinach chloroplasts. Chloroplasts were incubated as described in Material and Methods. The radioscan shows the rechromatography of the terpenoids after saponification on System II. Incubation mixture plus: A) $FADH_2$ (0.1 mM) + dithiothreitol (2 mM); B) NADPH (3 mM) + FAD (0.1 mM); I DHGG; II THGG; ► Start of TLC.

sors in P e.g. acetate (5), mevalonic acid (16), IPP (14, 17), NADPH (18). The reduction is very important in αT synthesis since the prenylating enzyme is specific for P and does not use GG as substrate (6). GG and GGPP synthesis is achieved in a recombinated system of chloroplast envelopes and chloroplast soluble protein from IPP (14). The synthesized GGPP is discharged in the lipid phase of the envelope (14). The enzymes which catalyses the formation of αT in spinach chloroplasts are bound to the envelope (6, 19). So it seems very likely that the enzymes which catalyse the reduction are also organized at this membrane. It is not yet clear whether they are bound to the membrane or just get organized at the envelope during time of their activity as those are for the GGPP synthesis. The hydrogenation system seems to be very sensitive to external influences (personal communication of Prof. Dr. R. Douce, (3). We used a very dense chloroplast suspension (> 3 mg Chl/ml). The danger of desintegration of enzyme systems or dilution of cofactors is much lower. The concentration of endogenous NADPH is high enough to sustain a good conversion rate as can be seen from experiments with FADH (Fig. 1, 2). Regulatory proteins (e.g. thioredoxin) that are known to link light to enzyme activity seem to have only a small effect on the conversion rate. This pro-

blem has to be studied in more detail, because the synthesis of αT is light dependent (20) while the reaction from IPP to yield GGPP (14) and from PPP to αT are light independent (6, 19).

Chl a_{GG} is synthesized in spinach chloroplasts from IPP by a recombinated system of thylakoid membranes and soluble chloroplast proteins (14). A reduction of Chl a_{GG} in this green systems was not demonstrated, while a stepwise two electron reduction of Chl a_{GG} → Chl a_{DHGG} → Chl a_{THGG} → Chl a (Chl $a_{DHGG,\ THGG}$ = Chl a esterified with DHGG, THGG) for etiolated systems is now well established (1, 4, 15). The chlorophyll synthetase (2) which catalyzes the esterification of chlorophyllide with either GGPP or PPP shows a 2:1 specifity for GGPP in oat seedlings (2). This enzyme is different to chlorophyllase which needs organic solvents to be active. The results presented here demonstrate for the first time that also spinach chloroplasts are able to perform the reduction of Chl a_{GG} to Chl a. An effect of exogenously supplied NADPH is especially detectable in the increase of Chl a_{DHGG} and Chl a_{THGG} while the rate of incorporation in Chl a is constant. Label in Chl a is already detectable after 5 min while Chl a_{DHGG} and Chl a_{THGG} accumulate at a later state. These results are in good agreement with a very recent report on the hydrogenation of Chl a_{GG} by etiolated oat seedlings (3). These results indicate that also in fully developed chloroplasts the esterification of chlorophyllide with GGPP and subsequent reduction can take place as well as esterification with PPP. Which alcohol in vivo is esterified with chlorophyllide is not yet clear. A regulatory function of PPP in the chlorophyll synthesis is discussed (21, 22) as well as a stimulating effect of GGPP on an endogenous P (PP) pool (15). In vivo this regulation might be dependent on the redox status of the chloroplast and it seems possible that both isoprenoid alcohols are esterified to chlorophyllide.

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REFERENCES:

1. Schoch, S., Lempert, U. and Rüdiger, G. (1977) Z. Pflanzenphysiol. 83, 427 - 436.
2. Rüdiger, W., Benz, J. and Guthoff, C. (1980) Eur. J. Biochem. 109, 193 - 200.
3. Benz, J., Wolf, C. and Rüdiger, W. (1980) Plant Sci. Letters 19, 225-230.
4. Rüdiger, W., Benz, J., Lempert, U. and Steffens, D. (1976) Z. Pflanzenphysiol. 80, 131 - 143.
5. Costes, C. (1966) Phytochemistry 5, 311 - 324.
6. Soll, J., Kemmerling, M. and Schultz, G. (1980) Arch. Biochem. Biophys. 204, 544 - 550.

7. Arbusow, A. and Dumin, A. (1914) Zurnal Russkajo Fiziko-Chimiceskago Obcestva Pri Pretorsgradshom Univertete 46, 295 - 302.
8. Walter. W.M. (1967) J. Label. Compounds 3, 54 - 57.
9. Mayer,H. and Isler, O. (1971) Methods in Enzymology (Colowick, S.P. and Kaplan, N.O. eds.) 18 c, 491 - 547, Academic Press, New York.
10. Joo, C.N., Park, C.E. and Kates, M. (1973) Can. J. Biochem. 51, 1527 - 1536.
11. Widmaier, R., Howe, J. and Heinstein, P. (1980) Arch. Biochem. Biophys. 200, 609 - 616.
12. Jensen, R.G. and Bassham, J.A. (1966) Proc. Nat. Acad. Sci. 56, 1095 - 1101.
13. Haas, R., Siebertz, H.P., Wrage, K. and Kleine, E. (1980) Planta 148, 238 - 244.
14. Block, M.A., Joyard, J. and Douce, R. (1980) Biochim. Biophys. Acta 631, 210 - 219.
15. Benz, J. (1980) Dissertation, Ludwigs-Maximilians-Universität München.
16. Wellburn, A.R., Stone, K.J. and Hemming, F.W. (1966) Biochem. J. 100, 23c - 25e.
17. Skilleter, D.N. and Keckwick, R.G.O. (1970) Phytochem. 9, 153 - 156.
18. Wellburn, A.R. (1968) Phytochem. 7, 1523 - 1528.
19. Soll, J., Douce, R. and Schultz, G. (1980) FEBS Letters 112, 293 - 246.
20. Lichtenthaler, H.K. (1973) Ber. Deutsch. Bot. Ges. 86, 313 - 329.
21. Gray, J.C. and Kekwick, R.G.O. (1973) Biochem. J. 133, 335 - 347.
22. Liljenberg, C. (1977) Physiol. Plant. 39, 101 - 105.