

## Tocopherol and Plastoquinone Synthesis in Spinach Chloroplasts Subfractions<sup>1</sup>

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Subfractions isolated from intact purified spinach chloroplasts are able to prenylate the aromatic moiety of  $\alpha$ -tocopherol and plastoquinone-9 precursors. The biosynthesis of  $\alpha$ -tocopherol and plastoquinone-9 is a compartmentalized process. The chloroplast envelope membranes are the only site of the enzymatic prenylation in  $\alpha$ -tocopherol synthesis whereas the thylakoid membrane is also involved in the prenylation and methylation sequence of plastoquinone-9 biosynthesis. A very active kinase which forms phytyl-PP is localized in the stroma. Phytyl but not geranylgeraniol is the polyprenol precursor of the side chain of  $\alpha$ -tocopherol in spinach chloroplasts.

$\alpha$ -Tocopherol ( $\alpha$ T)<sup>2</sup> plays an important role as a constituent of the chloroplast membranes and this compound has been shown to be synthesized within the chloroplast (1). The two methylation steps involved in  $\alpha$ T synthesis are specifically localized at the envelope membrane (2). Plastoquinone-9 (PQ-9, see Appendix) has not only a function as electron carrier between the two photosystems which are localized in the thylakoids, but is also a constituent of the chloroplast envelope (Douce and Soll, unpublished data). These findings strongly suggest that the envelope is a carrier of other enzymes involved in PQ-9 and  $\alpha$ T synthesis, e.g., the enzymatic prenylation of homogentisic acid, either with a C<sub>45</sub>-polyprenol in the case of PQ-9 or a C<sub>20</sub>-terpenol in the case of  $\alpha$ T synthesis. Homogentisate is an intermediate of the shikimate pathway which also occurs within the chloroplast (3).

The chloroplast envelope is a continuous boundary of two osmiophilic membranes, and galactolipid synthesis is located in this chloroplast compartment (4). The present

paper indicates that the envelope membrane is the main site of the enzymatic prenylation and methylation involved in  $\alpha$ T and PQ-9 synthesis.

### MATERIALS AND METHODS

#### *Radiochemicals*

[Me-<sup>14</sup>C]SAM (sp act, 50 mCi/mmol) was purchased from Amersham Buchler, Braunschweig, West Germany. [<sup>3</sup>H]Homogentisic acid (sp act, 8 Ci/mmol) was labeled by the tritium exchange service of NEN Dreieichenhain and purified prior to use on the system I. [ $\gamma$ -<sup>32</sup>P]ATP in 2 M LiCl<sub>2</sub> (sp act, 0.30  $\mu$ Ci/nmol) was a gift from Prof. Dr. H. Strotmann, Botanisches Institut, Tierärztliche Hochschule, Hannover, West Germany.

#### *Chemical Synthesis*

The geranylgeranyl- and phytyl-substituted methylquinols and quinones (see Appendix) were prepared from the corresponding methylquinol with *trans*-geranylinalool and isophytol according to Ref. (1). Me-6-SQ and Me-6-SQH<sub>2</sub> (see Appendix) were synthesized from natural solanesol and the corresponding quinol by a procedure similar to that described previous by (1). Polyprenylpyrophosphates were prepared by the method of Joo *et al.* (5). Geranylgeraniol was synthesized in a modified Horner Wittig reaction from triethylphosphonoacetate and *trans*-farnesylacetone according to Refs. (6, 7). The structure was verified by mass spectroscopy and tlc on system III and IV by comparison with an authentic sample of geranylgera-

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<sup>2</sup> Abbreviations used:  $\alpha$ T,  $\alpha$ -tocopherol; SAM, S-adenosylmethionine, PQ-9, plastoquinone-9; tlc, thin-layer chromatography; b.r., boiling range.

niol, which is a gift from Dr. W. Hoffmann, BASF, Ludwigshafen, West Germany. The geranylgeraniol provided is a mixture of 70% *E* and 30% *Z*. *trans*-Farnesylacetone was a gift from Dr. F. Weber, Hoffmann-La Roche, Basel, Switzerland.

### Thin-Layer Chromatography Systems

Thin-layer chromatography system I: silica gel with benzene/MeOH/acetic acid, 45/8/4; tlc system II: silica gel with propan-2-ol/NH<sub>3</sub>/H<sub>2</sub>O, 6/3/1; tlc system III: silica gel with petroleum ether (b.r. 60–80°C)/Et<sub>2</sub>O, 1/1; tlc system IV: silica gel impregnated with 10% AgNO<sub>3</sub> in acetonitrile, solvent petroleum ether (b.r. 60–80°C)/acetone, 3/2; tlc system V: silica gel with petroleum ether (b.r. 60–80°C)/Et<sub>2</sub>O, 10/1, tlc system VI: cellulose impregnated with 7% paraffin in petrol (b.r. 100–140°C), solvent acetone/H<sub>2</sub>O, 85/15; tlc system VII: same cellulose plates as VI but solvent acetone/MeOH, 2/1. All thin-layer plates were pre-coated and purchased from Schleicher und Schüll (Germany).

All substances were cochromatographed with authentic samples and detected by either the uv quench at 254 nm or by spraying with 20% molybdotophosphoric acid in MeOH and heating at 120°C for 5 min.

### Isolation of Broken Chloroplasts

Broken chloroplasts were prepared from intact chloroplasts isolated as described in (8). The chloroplast pellet resulting from centrifugation of medium B was suspended with a very small amount of distilled water (1 ml/5–10 mg chlorophyll) to shock them hypotonically. After 10 min the suspension was adjusted with medium C to a final chlorophyll concentration of not less than 1 mg/ml. Media B and C were as reported in Ref. (8).

### Isolation of Chloroplast Subfractions

Chloroplast compartments were isolated by fractionation of intact chloroplasts on a biphasic dextran gradient (9) followed by gentle osmotic shock and separation of envelope membranes and thylakoids on a sucrose gradient (10). Thylakoids were washed twice in medium C prior to use. The top phase of the membrane fractionating gradient (10) was used as chloroplast extract (stroma). The ratio between the different amounts of protein is: stroma, about 50% of chloroplast protein, envelope, about 1–2%, and thylakoid, about 50% of total chloroplast protein (11). Protein determinations were done by the method of Lowry.

### Reaction Mixture

The complete reaction mixture contained the following if not otherwise defined:

(a) *Experiments with broken chloroplasts.* Chloroplast suspension in medium C, pH 7.6 (quantitated prior to use); 20 μM [<sup>3</sup>H]homogentisic acid; 2.5 mM NaHCO<sub>3</sub>; 100 μM C<sub>20</sub>-prenyl-PP; or 100 μM C<sub>20</sub>-prenyl-OH plus 100 μM ATP. Reactions were initiated by addition of polyprenyl-PP or the corresponding alcohol and were incubated at 20°C for 80 min. Light intensity in experiments with illuminated chloroplasts was 0.1 J/cm<sup>2</sup> s. Aliquots (100 μl) were taken at different times. The reaction was stopped with a mixture of MeOH/CHCl<sub>3</sub> (500/250 μl) to give a monophasic solution. After 2 h 250 μl CHCl<sub>3</sub> and 250 μl H<sub>2</sub>O were added. The mixture was shaken vigorously (12). The organic layer containing the prenylquinones was evaporated to dryness and subjected to chromatography.

(b) *Experiments with chloroplast fragments.* In experiments on Me-6-prenyl-QH<sub>2</sub> synthesis the reaction mixtures were: chloroplast subfraction protein in medium C, pH 7.6; 20 μM [<sup>3</sup>H]homogentisic acid; 100 μM C<sub>20</sub>-prenyl-PP; or 100 μM C<sub>20</sub>-prenyl-OH plus 100 μM ATP.

In experiments on Me-6-SQH<sub>2</sub> biosynthesis the concentrations were: chloroplast subfraction protein in medium C, pH 7.6; 20 μM [<sup>3</sup>H]homogentisic acid; 80 μM solanesyl-PP; or 80 μM solanesol plus 80 μM ATP.

The reaction mixture with Me-6-SQH<sub>2</sub> as precursor of PQH<sub>2</sub>-9 was: chloroplast subfraction protein in medium C, pH 7.6; 70 μM [Me-<sup>14</sup>C]SAM; 100 μM Me-6-SQH<sub>2</sub> used immediately after reduction of the corresponding quinone (1). Reactions were initiated either by addition of the polyprenyl component or by SAM and incubated at 20°C for 80 min in the dark. Aliquots (100 μl) were taken at different times. The reaction was stopped and the lipids extracted as described above.

### Chromatography of Labeled Products

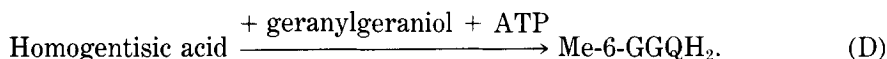
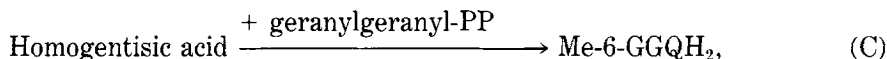
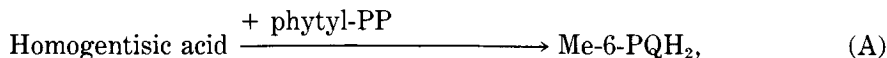
Unlabeled carrier substances (about 25 μg) identical to the expected labeled products were added to the extraction solution. All prenylquinols were oxidized by air for better purification before chromatography. Me-phytyl- and Me-geranylgeranyl-quinones were chromatographed on system V and rechromatographed on system VI. Me-6-SQ and PQ-9 were first chromatographed on system V and rechromatographed on system VII. Phytyl-PP was chromatographed on system II. For further details of the chromatography see (1, 13). The radioactive areas were scraped off the plates, dissolved in 2 ml MeOH and were counted in 5 ml scintillation mixture (Quickzint, W. Zinsser GmbH, Frankfurt, West Germany) using a Packard liquid scintillation counter.

## RESULTS

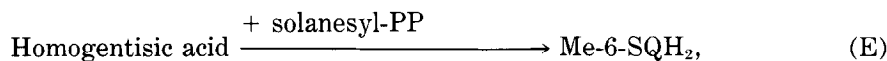
The prenylation of homogentisate involved in αT and PQ-9 synthesis was examined with broken chloroplasts and

chloroplast subfractions, isolated from intact purified chloroplasts. Furthermore the methylation of PQ-9 precursors is shown. The following reactions were tested.

### Tocopherol Synthesis



### Plastoquinone Synthesis



### Tocopherol Synthesis

Figure 1 clearly indicates that the envelope membranes are able to catalyze the enzymatic prenylation of homogentisate with phytyl-PP to give Me-6-PQH<sub>2</sub> (reaction A). When stromal and membrane fractions were combined the rate of Me-6-PQH<sub>2</sub> is not stimulated. The findings show that the enzymes of prenylation are bound to the envelope membrane. The incorporation rate was 26 pmol/h × mg envelope protein. In marked contrast to the envelope fraction, the prenylation activity was negligible in the thylakoid and stromal fraction (Fig. 1).

When phytol and ATP (reaction B) are used instead of phytyl-PP the rate of synthesis of Me-6-PQH<sub>2</sub> is equal to that of phytyl-PP alone but for this reaction stroma protein is necessary (Fig. 2). In additional experiments with phytol and [ $\gamma$ -<sup>32</sup>P]ATP a kinase was found to be located in the stromal fraction. Thus, when 100  $\mu$ M phytol and 1  $\mu$ M ATP (sp act 0.30  $\mu$ Ci/nmol) in a solution of 3.8 mg stroma protein in medium C were incubated as described under Material and Methods, the conversion rate of phytol to phytyl-PP was 1.5 pmol/h × mg stroma protein. Envelope or thylakoid membranes were not able to perform this reaction.

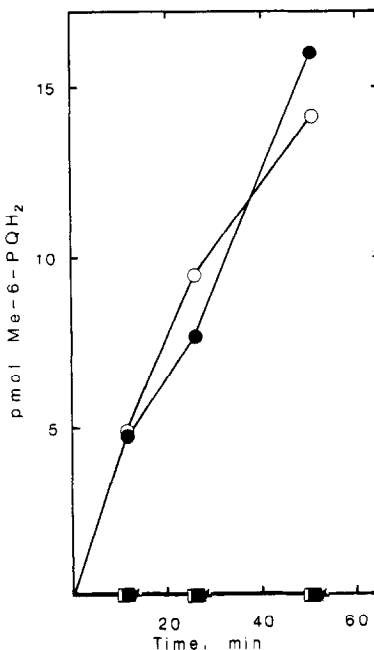


FIG. 1. Me-6-PQH<sub>2</sub> synthesis from [<sup>3</sup>H]homogentisate and phytyl-PP by different chloroplast subfractions and their recombined systems. For further details see Material and Methods. Incubation mixture plus: (○), +0.7 mg envelope protein; (●), +0.7 mg envelope protein + 0.9 mg stroma protein; (□), +11.4 mg thylakoid protein; (■), +5.7 mg thylakoid protein + 0.9 mg stroma protein; (★), +1.3 mg stroma protein.

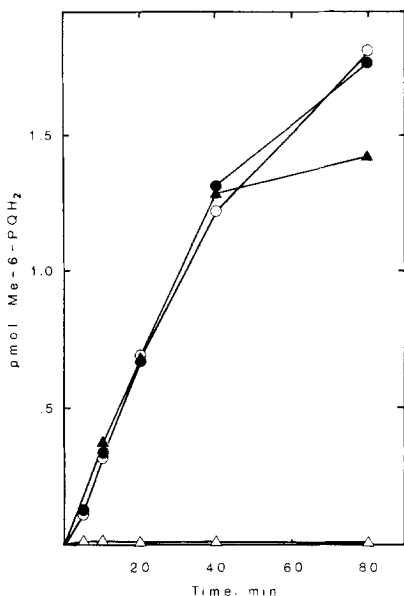


FIG. 2. Me-6-PQH<sub>2</sub> synthesis from [<sup>3</sup>H]homogentisate with phytyl-PP and phytol + ATP, respectively, by different chloroplast subfractions and their recombined systems. For further details see Material and Methods. Incubation mixture plus: (○), phytyl-PP + 0.54 mg envelope protein; (●), phytyl-PP + 0.54 mg envelope protein; + 0.5 mg stroma protein; (△), phytol + ATP + 0.54 mg envelope protein; (▲), phytol + ATP + 0.54 mg envelope protein + 0.5 mg stroma protein.

Both the prenylation and methylation were demonstrated either with broken chloroplasts (see Material and Methods) or with chloroplast subfractions. The prenyltransferase activity described above uses only phytyl-PP as precursor, but not geranylgeranyl-PP. In contrast, the methyltransferase shows a 3:1 preference for the geranylgeranyl-substituted derivative in the reaction Me-6-prenyl-QH<sub>2</sub> → 2,3-Me<sub>2</sub>-prenyl-QH<sub>2</sub> (for details see (2, 13)). When broken chloroplasts were incubated with [<sup>3</sup>H]homogentisate without exogenous poly-prenyl-PP only Me-6-PQH<sub>2</sub> (4.6 pmol/h/mg chlorophyll) and not Me-6-GGQH<sub>2</sub> was found. This result was supported by further experiments. When broken chloroplasts were incubated with [<sup>3</sup>H]homogentisate and geranylgeranyl-PP or geranylgeraniol plus ATP in the light and in the dark no increase in labeled Me-6-PQH<sub>2</sub> was found. This indicates that under the present condi-

tions neither geranylgeranyl-PP nor Me-6-GGQH<sub>2</sub> is reduced during the test. The relative high prenylation rate without exogenous poly-prenol might be due to free phytol within the chloroplast. For the content of free phytol in leaves see Ref. (14, 15). In experiments with broken chloroplasts, [<sup>3</sup>H]homogentisate, and phytol-PP, neither Me-5-PQH<sub>2</sub> nor Me-3-PQH<sub>2</sub> but only Me-6-PQH<sub>2</sub> was detected as labeled product after rechromatography on system VI. A light effect was observed in the control (Fig. 3). This indicates that not enough ATP is synthesized in the dark, while under illuminated conditions the concentration of endogenous ATP (synthesized by photophosphorylation) is high enough to reconstitute the synthesis of phytyl-PP.

#### Plastoquinone Synthesis

The alcohol involved in PQ-9 synthesis is solanesol, a C<sub>45</sub>-poly-prenol (reactions E, F). The yield of Me-6-SQH<sub>2</sub> from homogentisate and solanesyl-PP was lower than for the corresponding Me-6-PQH<sub>2</sub> synthesis. In this case the prenylation is not only due to the

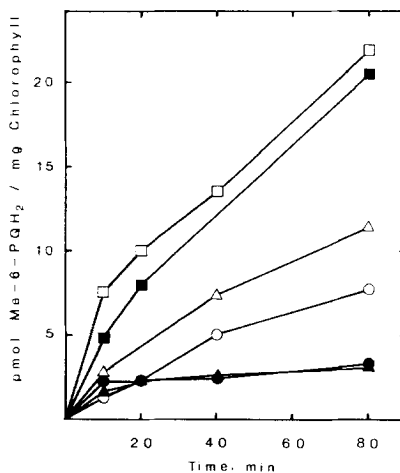


FIG. 3. Me-6-PQH<sub>2</sub> synthesis from [<sup>3</sup>H]homogentisate without adding poly-prenylalcohols, with phytyl-PP or with phytol + ATP by broken chloroplast in the light and in the dark. For further details see Material and Methods. Incubation mixture: (○), in the light without poly-prenylalcohols; (□), in the light + phytyl-PP; (△), in the light + phytol + ATP; (●), in the dark without poly-prenylalcohols; (■), in the dark + phytyl-PP; (▲), in the dark + phytol + ATP.

envelope but also to the thylakoid membranes (Fig. 4). The conversion rate was 1.2 pmol/h  $\times$  mg envelope protein and 0.013 pmol/h  $\times$  mg thylakoid protein. Stroma protein had no enzymatic activity involved in the prenylation reaction (Fig. 4). When a recombined system of envelope and stroma protein was used in experiments with [ $^3$ H]homogentisate and solanesyl-PP sometimes no incorporation in Me-6-SQH<sub>2</sub> was detected. This might be due to a phosphatase (Fig. 4). Under the present conditions as described under Material and Methods the chloroplast fragments and their recombinations were not able to form solanesyl-PP from solanesol and ATP.

Me-6-SQH<sub>2</sub> was used as precursor to locate the methyltransferase activity to yield PQH<sub>2</sub>-9 (reaction G). The envelope (10 pmol/h  $\times$  mg envelope protein) and also to a small extent the thylakoid membranes (0.35 pmol/h  $\times$  mg thylakoid protein) are able to catalyze the introduction of a methyl group from SAM into Me-6-SQH<sub>2</sub> to give PQH<sub>2</sub>-9 (Fig. 5). Stroma protein is again not involved. When stromal and membrane fractions (thylakoid and envelope membrane) were combined the rate of PQH<sub>2</sub>-9 synthesis was not stimulated (Fig. 5). These results are in good agreement with a previ-

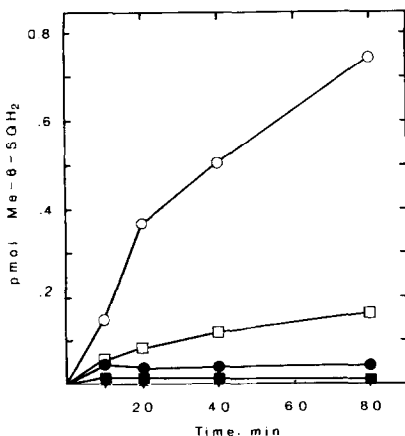


FIG. 4. Me-6-SQH<sub>2</sub> synthesis from [ $^3$ H]homogentisate and solanesyl-PP by different chloroplast subfractions and their recombined systems. For further details see Material and Methods. Incubation mixture plus: (○), 0.65 mg envelope protein; (●), 0.65 mg envelope protein + 0.66 mg stroma protein; (□), 5.8 mg thylakoid protein; (■), 2.9 mg thylakoid protein + 0.66 mg stroma protein; (★), 0.66 mg stroma protein.

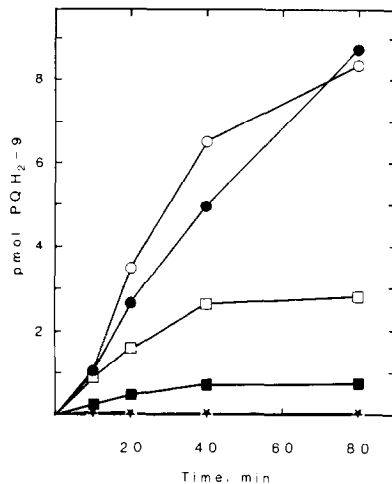


FIG. 5. PQH<sub>2</sub>-9 synthesis from Me-6-SQH<sub>2</sub> and [ $^{14}$ C]SAM by different chloroplast subfractions and their recombined systems. For further details see Material and Methods. Incubation mixture plus: (○), 0.88 mg envelope protein; (●), 0.88 mg envelope protein + 1.4 mg stroma protein; (□), 9.2 mg thylakoid protein; (■), 4.6 mg thylakoid protein + 1.4 mg stroma protein; (★), 1.4 mg stroma protein.

ous report in which the methylation of  $\alpha$ T precursors is localized at the envelope membrane (2). A contamination of thylakoids by envelope membrane was not likely. They were washed twice prior to use and there was no contamination detectable in experiments on tocopherol synthesis.

## DISCUSSION

The enzymes involved in  $\alpha$ -tocopherol synthesis are placed in the chloroplast envelope. The prenyltransferase and the methyltransferase activity are bound tightly to the envelope membrane. Furthermore the synthesis of geranylgeraniol and geranylgeranyl-PP from isopentenyl-PP is performed by a recombined system of envelope or thylakoids plus chloroplast stroma (soluble protein) (12). The compartmentation of the synthesis of homogentisate from hydroxyphenylpyruvate is not yet clarified in spinach chloroplasts. Phytol should be originated from geranylgeraniol or its pyrophosphate by the reduction of three double bonds (16, 17). This reaction could not yet be demonstrated in chloroplasts and

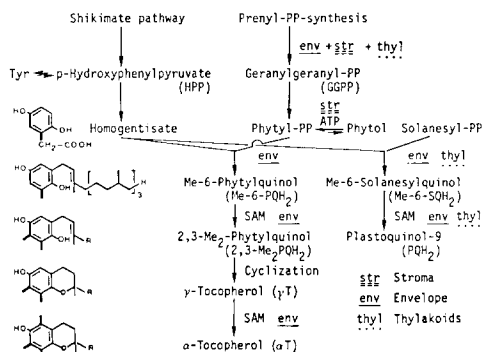


FIG. 6. Biosynthesis of prenylquinones in spinach chloroplasts.

chloroplast subfractions. Furthermore in the tocopherol synthesis, the cyclization of 2,3-Me<sub>2</sub>-PQH<sub>2</sub> to  $\gamma$ T was only detected in intact chloroplasts (1).

A very important result is the specificity of the prenyltransferase which uses phytyl-PP as polyprenyl component and not geranylgeranyl-PP. In the methylation of Me-6-PQH<sub>2</sub> and Me-6-GGQH<sub>2</sub> a preference for the GG derivate was found (2, 13). In plants with a tocotrienol pathway (i.e., Hevea, wheat, barley (18)) geranylgeranyl-PP may form the prenyl side chain and not phytyl-PP. The preference for the Me-6-GGQH<sub>2</sub> in comparison to Me-6-PQH<sub>2</sub> for the methylation step in higher plants like spinach might be an "ancient relic" while the really specific point developed during evolution became the enzymatic prenylation.

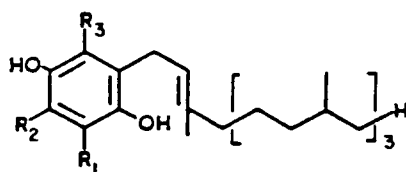
Furthermore, only one isomer among two or three possible ones is formed at each step in the biosynthesis of  $\alpha$ T. As it was shown under Results, only Me-6-PQH<sub>2</sub> and not

Me-5-PQH<sub>2</sub> or Me-3-PQH<sub>2</sub> is formed by the homogentisate decarboxylasepolyprenyltransferase. In the following reaction the methyltransferase is specific to Me-6-PQH<sub>2</sub> (1). The resulting biosynthetic sequence and its compartmentation for  $\alpha$ T and PQ-9 is shown in Fig. 6.

Plastoquinone-9 synthesis takes place at both the thylakoid and the envelope membrane. Stroma protein had no stimulating effect. The sequence of PQ-9 synthesis is similar to that of  $\alpha$ T. First, homogentisate is prenylated with a polyprenyl-PP, in this case solanesyl-PP. Me-6-SQH<sub>2</sub> is formed as only position isomer. It is methylated with SAM as cofactor to yield PQH<sub>2</sub>-9. A low enzymatic activity was found at the thylakoid membranes. Because of the high thylakoid to envelope protein ratio in the chloroplast (about 50:1 (11)), the rate of PQ-9 synthesis by the thylakoids in total is not as different from the envelope ones as it is calculated on the basis of specific activity per milligram of protein. As confirmed under Results, a contamination of thylakoids with envelope membranes could be excluded.

The chloroplast envelope contains all the enzymes involved in the prenylquinone synthesis. This is important during the thylakoid biogenesis, because then massive biosynthesis of the two prenylquinones should occur at the envelope.  $\alpha$ T is needed as membrane constituent of the thylakoid, PQ-9 as electron carrier between the two photosystems in the thylakoids. Transport of PQ-9 and  $\alpha$ T from the envelope to thylakoids may happen as membrane flow as proposed in Refs. (4, 19).

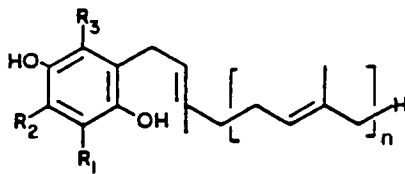
APPENDIX



Phytylquinols

R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>		
CH <sub>3</sub>	H	H	Me-6-PQH <sub>2</sub>	2-Methyl-6-
H	CH <sub>3</sub>	H	Me-5-PQH <sub>2</sub>	2-Methyl-5-
H	H	CH <sub>3</sub>	Me-3-PQH <sub>2</sub>	2-Methyl-3-
CH <sub>3</sub>	CH <sub>3</sub>	H	2,3-Me <sub>2</sub> -PQH <sub>2</sub>	2,3-Dimethyl-5-

phytylquinol



$n = 3$  Geranylgeranylquinols

$R_1$   $R_2$   $R_3$  as above

$n = 8$  Solanesylquinols, Plastoquinols

$R_1$   $R_2$   $R_3$

$CH_3$  H H Me-6-SQH<sub>2</sub> 2-Methyl-6-solanesylquinol

$CH_3$   $CH_3$  H PQH<sub>2</sub>-9 Plastoquinol-9

The corresponding quinones are signified in the text with Q instead of QH<sub>2</sub>

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