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Plant Cell Membranes

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[35] α-Tocopherol and Plastoquinone Synthesis in Chloroplast Membranes

By JÜRGEN SOLL

Plant prenylquinones, α-tocopherol, plastoquinone-9, and phylloquinone, function in chloroplasts as membrane constituents, antioxidants, or electron carriers. For a long time biosynthetic studies were hampered by the fact that no chemical intermediates of the biosynthetic pathway or biochemically active organelles were available. Earlier studies[1,2] proposed pathways with a multitude of chemically possible intermediates. The detailed work[3] on the chemical synthesis of prenylquinones enabled others[4-6] to work out the most probable pathway (Fig. 1) in plastoquinone and tocopherol biosynthesis.

Chemical Synthesis of Prenylquinones

The small-scale synthesis of **IV** (Fig. 2) is described here[4] and can be applied to the synthesis of other prenylquinones (*I*, *II*, *III*, *V*, *VI*) without problems. In many cases methylquinones are not commercially available and have to be prepared in advance. The corresponding phenol (4 mmol) is dissolved in 10 ml methanol and oxidized by 10 mmol of Fremy's salt[7] $\{[(SO_3)_2NO]K_2\}$ in 120 ml water and 4 ml sodium acetate (1 *M*). The reaction is allowed to continue for 30 min and the quinone is then extensively extracted with diethyl ether. The organic solvent is evaporated and the quinone purified by column chromatography (silica gel 60, Merck, FRG) using $CHCl_3$ as developing solvent. Quinone (0.7 mmol) is dissolved in 1.5 ml benzene, 2.5 ml H_2O, and 250 mg $Na_2S_2O_4$ is added. The reduction is completed after 5 min, the quinol is washed with ice water, and dried in a desiccator. Freshly distilled BF_3–etherate (0.3 ml in 1 ml tetrahydrofuran) is added dropwise via a syringe to a solution of 1.1 mmol quinol, 200 mg Al_2O_3 (W-200 basic, Woelm-Pharma, FRG), and 1.1 mmol

[1] W. Janiszowska and J. F. Pennock, *Vitam. Horm. (N.Y.)* **34,** 77 (1976).
[2] D. R. Threlfall and G. R. Whistance, *in* "Aspects of Terpenoid Chemistry and Biochemistry" (T. W. Goodwin, ed.), p. 335. Academic Press, London, 1971.
[3] H. Mayer and O. Isler, this series, Vol. 18C, p. 241.
[4] J. Soll and G. Schultz, *Phytochemistry* **19,** 215 (1980).
[5] J. Soll, M. Kemmerling, and G. Schultz, *Arch. Biochem. Biophys.* **204,** 544 (1980).
[6] S. R. Morris and D. R. Threlfall, *Biochem. Soc. Trans.* **11,** 587 (1983).
[7] H. J. Teuber and W. Rau, *Chem. Ber.* **86,** 1036 (1953).

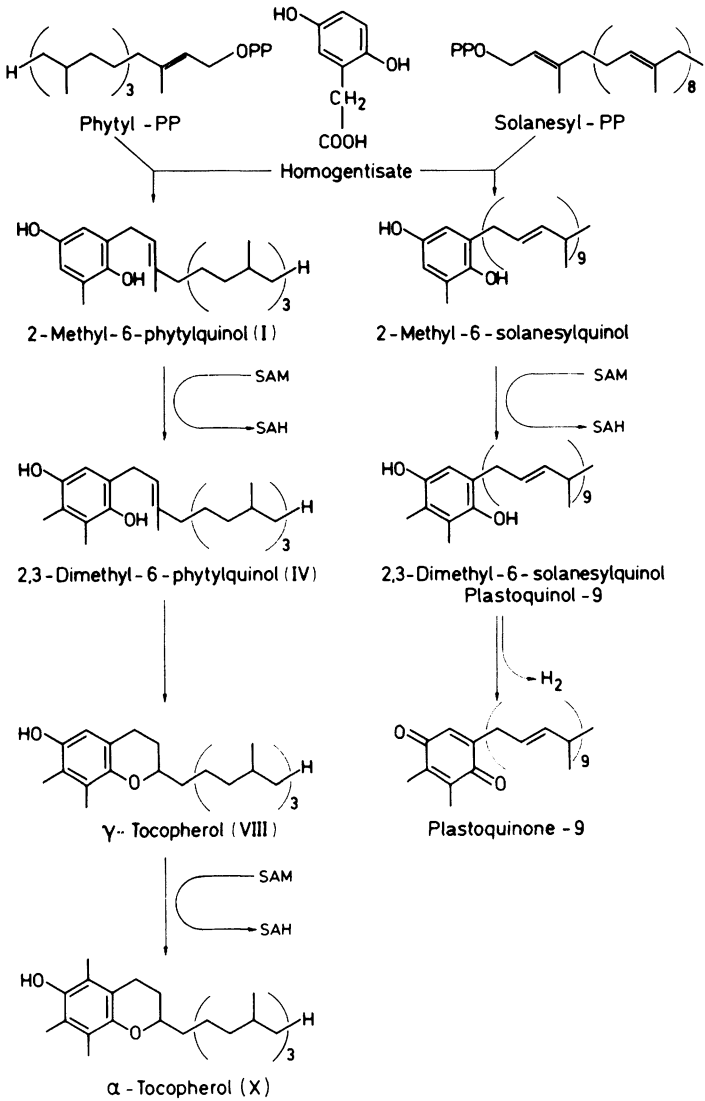
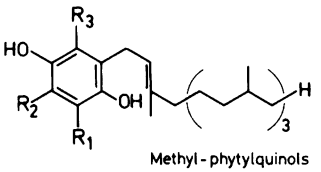
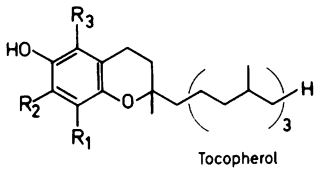


FIG. 1. Proposed pathway of α-tocopherol and plastoquinone-9 synthesis in spinach chloroplasts. From the available data these are the most likely intermediates to be involved in prenylquinone synthesis. A possible bypass in tocopherol synthesis might occur which leads from I via VII to VIII instead of I via IV to VIII (see text). SAM, *S*-Adenosylmethionine; SAH, *S*-adenosylhomocysteine.



Methyl-phytylquinols



Tocopherol

R_1	R_2	R_3		
CH_3	H	H	- 2-Methyl-6-	I
H	CH_3	H	- 2-Methyl-5-	II
H	H	CH_3	- 2-Methyl-3-	III
CH_3	CH_3	H	- 2,3-Dimethyl-5-	IV
CH_3	H	CH_3	- 2,5-Dimethyl-6-	V
CH_3	CH_3	CH_3	- Trimethyl-6-	VI

phytylquinol

R_1	R_2	R_3	
CH_3	H	H	- δTocopherol - VII
CH_3	CH_3	H	- γTocopherol - VIII
CH_3	H	CH_3	- βTocopherol - IX
CH_3	CH_3	CH_3	- αTocopherol - X

FIG. 2. Nomenclature and identification of prenylquinols and tocopherols.

isophytol in 2 ml dry tetrahydrofuran. The mixture is stirred under N_2 in the dark for 35 hr. Residual BF_3 is hydrolyzed on ice, and the prenylated quinols extracted with diethyl ether, the ether solution dried, and the organic solvent evaporated. The resulting quinol (**IV**) is oxidized by 400 mg Ag_2O in dry diethyl ether. Prenylquinones are purified by column chromatography (Silica-gel 60, Merck) developed with petrol (bp 60–80°)–diethyl ether, 15 : 1 (system 1). Purity of the products is verified by thin-layer chromatography (precoated plates on glass, silica gel, G-1500 LS254, Schleicher and Schüll, FRG) in system 1. When the prenylquinones **I**, **II**, and **III** are to be synthesized, care has to be taken to separate the isomers properly. This can be achieved by repeated thin-layer chromatography as above. The succession of prenylquinones in this thin-layer chromatography system is shown in Fig. 3A. Quinones and

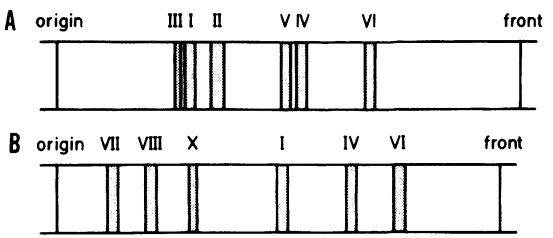


FIG. 3. Separation of prenylquinones and tocopherols by thin-layer chromatography. (A) The separation of mono-, di-, and trimethylphytylquinones on precoated thin-layer plates, silica gel G-1500, with diethyl ether : petrol (1 : 15, v : v) as developing solvent. The purification of prenylquinones and tocopherols using the same thin-layer plates but diethyl ether : petrol, (1 : 10, v : v) as solvent system. R_f values and separation are variable with silica gel plates obtained from different manufacturers. Precoated plates on glass give better resolution than those on plastic or aluminum foil (see also text).

quinols have a tendency to oxidize and polymerize during chromatography. We have obtained the best results using the systems described; other systems caused more oxidation, decomposition, and poor separation of quinones or prenylquinone isomers. All products should be stored and purified in the quinone form and not in the quinol form which is more susceptible to uncontrolled breakdown. Quinones and prenylquinones are detected on thin-layer plates with fluorescence indicator at 254 nm, while quinoles and prenylquinoles are visualized with $FeCl_3$/2,2′-dipyridyl 0.1%:0.25% (w/w) in ethanol.

Chemicals Needed for the Enzymatic Assays

As can be seen from Fig. 1, homogentisate, polyprenyl diphosphate, and *S*-adenosylmethionine are necessary for the incubation. Unlabeled and labeled *S*-adenosylmethionine and homogentisate are commercially available, while phytyl diphosphate and solanesyl diphosphate have to be prepared.[8-10] Dry trichloroacetonitrile (15 mmol), 5 mmol ditriethylammonium phosphate,[10] and 30 ml dry acetonitrile are mixed in a round-bottom flask. Two millimoles of phytol in 15 ml acetonitrile is added dropwise over a 3-hr period. The mixture is stirred for another 12 hr, then 50 ml of acetone is added and concentrated ammonia is dropped into the solution until no further precipitation occurs. Precipitation occurs for 2 hr at 0°. The solid is repeatedly washed with 0.28 *M* ammonia in methanol to eliminate prenyl monophosphates. The resulting prenyl diphosphate is dried and used in the enzyme assay. Product analysis showed that this preparation is still heavily contaminated by inorganic phosphates which, however, do not interfere with the enzyme assays. If further purification is desired this can be achieved by recrystallization in $CHCl_3$–methanol.[8] [^{3}H]Homogentisate, labeled by tritium exchange service, has to be purified prior to use in the following system: silica gel precoated thin-layer plates on glass and toluene/methanol/acetic acid (80/20/4 v/v/v) as developing solvent.

Preparation of Chloroplasts and Chloroplast Components

Chloroplasts are isolated from spinach leaves by standard procedures[11] and further purified on silica sol gradients.[12] Chloroplast compo-

[8] C. N. Joo, C. E. Park, J. K. G. Kramer, and M. Kates, *Can. J. Biochem.* **51,** 1527 (1973).

[9] R. Widmaier, J. Howe, and P. Heinstein, *Arch. Biochem. Biophys.* **200,** 609 (1980).

[10] G. Popjak, J. W. Cornfarth, R. H. Cornfarth, R. Ryhage, and S. de Witt Goodman, *J. Biol. Chem.* **237,** 56 (1962).

[11] H. Nakatani and J. Barber, *Biochim. Biophys. Acta* **461,** 510 (1977).

nents, e.g., envelope and thylakoid membranes and soluble chloroplast protein, are prepared as described.[13]

Enzyme Assay for the Synthesis of Tocopherol and Its Intermediates in Chloroplasts

As outlined in Fig. 1, the synthesis of α-tocopherol comprises a number of reaction steps, catalyzed by the following enzymes: homogentisate decarboxylase-phytyltransferase; *S*-adenosylmethionine : methyl-6-phytylquinol methyltransferase; 2,3-dimethylphytylquinolcyclase; *S*-adenosylmethionine : γ-tocopherol methyltransferase (no EC numbers available). Of the precursors and cosubstrates used only homogentisate, polyprenyl diphosphate, and *S*-adenosylmethionine are water soluble. Prenylquinones, prenylquinols, and tocopherols are not water soluble and it is difficult to determine their real concentration in the test. They are either added in ethanol (no more than 1% ethanol final concentration in the enzyme assay) or in diethyl ether, which is evaporated to dryness prior to the assay. Introduction of the methyl groups into the aromatic moiety is only possible at the quinol stage and not in the quinone form.[4] The same is valid for the formation of the chromanol stage (**IV** → **VIII**). A photometrically adjusted amount of quinone (UV maxima, see Refs. 14 and 15; extinction coefficients, see Ref. 3) was dissolved in 1 ml methanol, reduced with a little solid $NaBH_4$ for 2 min, transferred to diethyl ether, and washed with H_2O. The diethyl ether is evaporated to dryness under N_2 in the reaction vials which were used later in the enzyme assay. Substrate concentrations described[4,14-16] are 50–100 μM prenyl diphosphate, 100 μM *S*-adenosylmethionine, 100–200 μM prenylquinol or tocopherol at pH 7.6–8.2 with $MgCl_2$ as cofactor (1–10 mM) and chloroplasts equivalent to 0.5–1 mg of chlorophyll. Other cofactors like cysteine, dithiothreitol (DTT), light, and Mn^{2+} do not seem to be necessary.[4,15,16] Increased solubilization of quinols and tocopherols can be achieved by detergents (Tween 80) which do not seem to inhibit the *S*-adenosylmethionine : γ-tocopherol methyltransferase.[17]

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[12] G. Mourioux and R. Douce, *Plant Physiol.* **67,** 470 (1981).

[13] R. Douce and J. Joyard, *Adv. Bot. Res.* **7,** 1 (1979).

[14] J. Soll, G. Schultz, J. Joyard, R. Douce, and M. A. Block, *Arch. Biochem. Biophys.* **238,** 290 (1985).

[15] P. S. Marshall, S. R. Morris, and D. R. Threlfall, *Phytochemistry* **24,** 1705 (1985).

[16] B. Camara, F. Bardat, A. Seye, A. d'Harlingue, and R. Moneger, *Plant Physiol.* **70,** 1562 (1982).

[17] B. Camara and A. d'Harlingue, *Plant Cell Rep.* **4,** 31 (1985).

Identification and Purification of Labeled Products

The intermediates obtained in tocopherol synthesis are generally purified by repeated thin-layer chromatography[4,18,19] or HPLC.[15,20] The incubation mixture is extracted with $CHCl_3$: MeOH (1 : 2, v : v).[21] The chloroform phase contains prenylquinols, prenylquinones, tocopherols, other lipids, and pigments. Initial results have shown that prenylquinols are the products formed in this and the following reactions, if the products are analyzed under nonoxidizing conditions.[4,22] In general quinols are oxidized by air prior to thin-layer chromatography (for reasons, see above). A 25-μg aliquot of standard substances corresponding to the possible reaction products is added to the chloroform phase.

The first reaction in tocopherol and plastoquinone synthesis involves the prenylation of homogenisate with simultaneous decarboxylation (Fig. 1). The decarboxylation proceeds with stereochemical retention during the biosynthetic process.[23] While the chemical synthesis of monomethylprenylquinols using methylquinol, phytol, and BF_3 as described earlier yields a mixture of **I**, **II**, and **III**, the enzymatic prenylation of homogenisate yields only one product (**I**).[5,15] This is probably due to the directing influence of intermediates occurring during decarboxylation. Analysis of this reaction was done using spinach chloroplasts, [^{3}H]homogentisate, and phytyl diphosphate. The products were purified by thin-layer chromatography using two different systems in succession [first run, silica gel, petrol (bp 60–80°) : diethyl ether, 10 : 1 (system II); rechromatography, cellulose plates impregnated with 7% paraffin, acetone : H_2O, 85 : 15 (system III), or by HPLC (Lichrosorb Si 60, 5 μm, Merck, 0.06% dioxane in isooctane[15]]. Substances are recovered from thin-layer plates by elution of the zones in question twice with 1 ml of methanol. The methanol is evaporated under N_2 and the residual dissolved in acetone. Only **I** was found to be labeled, this very specific initial reaction excludes already many further intermediates.[5,15] This initial step of prenylquinone formation is followed by methylation of the aromatic moiety with *S*-adenosylmethionine as methyl group donor (see Fig. 4). Since the prenylation reaction yields only **I** the following methylation can occur only from two

[18] J. Soll and G. Schultz, *Biochem. Biophys. Res. Commun.* **91,** 715 (1979).
[19] H. K. Lichtenthaler, *in* "Lipids and Lipid Polymers in Higher Plants" (M. Tevini and H. K. Lichtenthaler, eds.), p. 231. Springer-Verlag, Berlin and New York, 1977.
[20] H. K. Lichtenthaler and U. Prenzel, *J. Chromatogr.* **135,** 493 (1977).
[21] E. G. Bligh and W. J. Dyer, *Can. J. Biochem. Physiol.* **37,** 911 (1959).
[22] K. G. Hutson and D. R. Threlfall, *Biochim. Biophys. Acta* **632,** 630 (1980).
[23] R. Krügel, K. H. Grumbach, H. K. Lichtenthaler, and J. Rètey, *Bioorg. Chem.* **13,** 187 (1985).

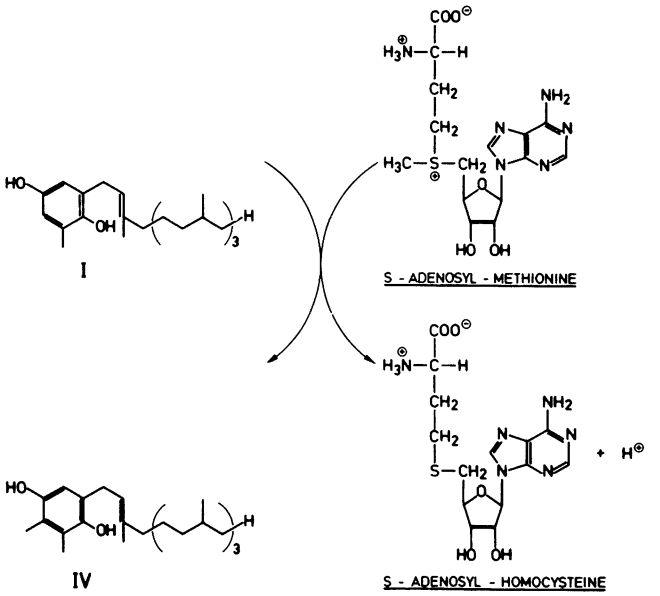

FIG. 4. Methylation of 2-methyl-6-phytylquinol by *S*-adenosylmethionine. Reaction products are 2,3-dimethyl-6-phytylquinol and *S*-adenosylhomocysteine.

different intermediates: (1) **I**, (2) after cyclization of **I** from **VII**. *In vitro* studies using isolated chloroplasts[4] demonstrated that methylation of **I** was about three times higher than from **VII**. This specificity is underlined also by the finding that **II** and **III** are methylated at 10 and 5% of the rate of **I**, respectively, with experimental conditions as described above.

The methylation product of **I** is **IV**, which is processed in a homologous sequence of events to form **X**. **X** formation takes place via **VIII**, which is methylated to form **X**. Products are purified on systems II and III (see Fig. 3). This sequence was supported from the data that **IV** is not methylated to **VI**.[4,15] In all reports known so far the cyclization of the prenylquinol to form the chromanol ring was not studied directly and it seems to be the slowest step in the α-tocopherol formation.

The reactions mentioned above described only one or two steps at a time of a series leading to α-tocopherol. If it is necessary to look at the whole sequence, all cosubstrates have to be included (homogentisate, phytyl diphosphate, *S*-adenosylmethionine). The incorporation rates vary from pmol to nmol/hr · mg chlorophyll for single reaction steps, which makes it obvious that only highly active chloroplast preparations can be used for these approaches. When these multiple step analyses are done

the products formed are **I**, **IV**, **VII**, **VIII** and **X**,[4,15,24] confirming the results described earlier. It should be stressed again that it is difficult to separate the different possible isomers in tocopherol synthesis. Monomethylphytylquinols can only be separated by repeated thin-layer chromatography on precoated plates on glass or HPLC[4,15,19,20] ($\lambda_{max\ I}$ 254, $\lambda_{max\ II}$ 253, $\lambda_{max\ III}$ 249 nm). Compounds **IV** and **V** are separated by simple chromatography on precoated thin-layer plates on glass using system II. **VIII** and **IX** should be purified as nitroso derivatives[25] or by HPLC.[15,20] It is obvious from the literature and our own experience that successful separation of isomers depends strongly on the brand of chromatography plates used.

Plastoquinone Synthesis

Plastoquinone synthesis (Fig. 1) occurs essentially via reactions similar for tocopherol; homogentisate (20 μM) and solanesyl diphosphate (80 μM) are condensed to form the equivalent to **I**[5] (2-methyl-6-solanesylquinol), which is then methylated by *S*-adenosylmethionine (70 μM) to yield plastoquinol-9[5] (2,3-dimethylsolanesylquinol) (Fig. 1). The rates of synthesis are again in the picomolar range per hr · mg chlorophyll. Purification of the incubation products is done by two successive thin-layer chromatography systems (first system; system I; rechromatography, cellulose plates impregnated with 7% paraffin, acetone : H_2O, 90 : 10, system IV).

Localization of Prenylquinone Synthesis in Chloroplasts

Recently developed methods for the fractionation and purification of chloroplast components[13] enabled us[5,14,24,26] to localize all but one enzyme in tocopherol and plastoquinone synthesis at spinach chloroplast envelopes. Thylakoids or soluble chloroplast protein had no enzymatic activity. Recombination of membranes with soluble chloroplast extract did not increase prenylquinone synthesis. These observations are now extended to envelope membranes from pea chloroplasts (J. Soll, unpublished). All test and purification conditions were essentially as described for chloroplasts. The amount of membranes used was between 50 and 100 μg protein per assay. The available data do not demonstrate the enzyme responsible for the cyclization of **IV** to yield **VIII**. Though some enzymatic activity in plastoquinone synthesis is found associated with the thylakoid

[24] G. Schultz, J. Soll, E. Fiedler, and D. Schulze-Siebert, *Physiol. Plant.* **64,** 123 (1985).
[25] S. Marcinkiewicz and J. Green, *Analyst* **84,** 304 (1959).
[26] J. Soll, R. Douce, and G. Schultz, *FEBS Lett.* **112,** 243 (1980).

membrane it is probably due to contamination of this membrane fraction by envelopes.

The envelope forms a two-membrane barrier, which surrounds the chloroplast and is present at all stages of chloroplast development.[27] It is now possible to separate the two membranes into outer envelope and inner envelope membrane.[27,28] Applying these methods it is possible to align tocopherol and plastoquinone synthesis with the inner envelope membrane.[14] Again, all enzymes but the cyclization enzyme (**IV** → **VIII**) were demonstrated.

Purification of Enzymes Involved in Tocopherol Synthesis

A membrane fraction obtained from pepper (*Capsicum annuum*) chromoplasts was shown to catalyze α-tocopherol synthesis via the same intermediates as described for chloroplasts[16] (Fig. 1). The methods used were essentially as above. This membrane fraction was then used as a source for the enzyme purification. An acetone powder is obtained from the membranes at −20° which is then solubilized in 0.1 *M* phosphate buffer (pH 7.0), 5 m*M* DTT, and Tween 80 (1 mg/ml), followed by $(NH_4)_2SO_4$ precipitation (20–60%). The protein is purified by column chromatography[29]: (1) blue Sepharose CL-6B, 50 m*M* KH_2PO_4, 1 m*M* DTT, 1 m*M* EDTA, pH 6.2; (2) blue Sepharose CL-6B, 50 m*M* Tris–HCl, 1 m*M* DTT, 1 m*M* EDTA, pH 8; (3) DEAE–Sephacel, 50 m*M* Tris-HCl, pH 7.6, 1 m*M* DTT eluted with a gradient of 0–0.4 *M* KCl. Analysis of the active fractions obtained, by SDS–polyacrylamide gel electrophoresis showed only one band at about 33,000 Da[29] (pH optimum, 8.2; K_m *S*-adenosylmethionine, 2.5 μ*M*; K_m γ-tocopherol, 13.7 μ*M*).[29]

Analysis of Envelope Membranes for Tocopherol and Plastoquinone

Determination should be done from fresh or deep-frozen material. Freeze-dried membranes contain less quinols and tocopherols than fresh membranes and more quinone and tocoquinone instead.[5,14,30,31] (see Table I). Membranes are extracted either by $CHCl_3$/MeOH (see earlier) or by

[27] R. Douce, M. A. Block, A. J. Dorne, and J. Joyard, *Subcell. Biochem.* **10,** 1 (1984).
[28] K. Cline, J. Andrews, J. Mersey, E. H. Newcomb, and K. Keegstra, *Proc. Natl. Acad. Sci. U.S.A.* **78,** 3595 (1981).
[29] A. d'Harlingue, F. Villat, and b. Camara, *C. R. Seances Acad. Sci., Ser. 3* **6,** 233 (1985).
[30] H. K. Lichtenthaler, U. Prenzel, R. Douce, and J. Joyard, *Biochim. Biophys. Acta* **641,** 99 (1981).
[31] G. Schultz, H. Bickel, B. Buchholz, and J. Soll, *in* "Chloroplast Development" (G. Akoyunoglou, ed.), p. 311. Balaban Int. Sci. Serv, Philadelphia, Pennsylvania, 1981.

TABLE I
PRENYL LIPID CONCENTRATION IN CHLOROPLAST MEMBRANES FROM SPINACH LEAVES[a]

Prenylquinone	Thylakoid	Envelope mixture	Inner envelope	Outer envelope	Retention time (min)
α-Tocopherol	1.1	2.8	6.7	9.8	5.6
α-Tocoquinone	0.24	0.2	—	—	3.8
Phylloquinone K_1	0.34	0.1	0.07	0.05	7.3
Plastoquinone-9	3.9[b]	1.2[b]	1.63	1.1	25.5
Plastoquinol-9	—		1.54	1.1	11.3
Total prenylquinones	5.5	4.3	10.0	12.1	—

[a] Adapted from Refs. 14, 30, and 31; values are expressed in μg/mg protein.
[b] Represents the sum of plastoquinone and plastoquinol.[30]

hexane/acetone (10:4, v:v).[30] The lipid extract is further analyzed by HPLC (RP8, 7-μm mesh, Merck) using methanol:water (95.7:4.3, v:v) as developing solvent (1.5 ml flow rate)[14,32] and two UV detectors set at 250 nm (to detect quinones) and 292 nm (to detect tocopherol and quinol), respectively.[14,33]

Acknowledgments

I would like to thank Prof. G. Schultz for his continuous support and encouragement during the progress of this work.

[32] H. K. Lichtenthaler, *in* "Handbook of Chromatography" (H. K. Mangold, ed.), p. 115. CRC Press, Boca Raton, Florida, 1984.
[33] D. R. Threlfall and T. W. Goodwin, *Biochem. J.* **103,** 573 (1967).