

METHODS IN ENZYMOLOGY

EDITORS-IN-CHIEF

Sidney P. Colowick Nathan O. Kaplan

Methods in Enzymology

Volume 148

Plant Cell Membranes

EDITED BY

Lester Packer

Roland Douce

DEPARTMENT OF PHYSIOLOGY AND ANATOMY
UNIVERSITY OF CALIFORNIA
BERKELEY, CALIFORNIA

DÉPARTEMENT DE RECHERCHE FONDAMENTALE
CENTRE D'ETUDES NUCLÉAIRES ET UNIVERSITÉ
DE GRENOBLE
GRENOBLE, FRANCE



ACADEMIC PRESS, INC.

Harcourt Brace Jovanovich, Publishers

San Diego New York Berkeley Boston
London Sydney Tokyo Toronto

Table of Contents

CONTRIBUTORS TO VOLUME 148	xi
PREFACE	xvii
VOLUMES IN SERIES	xix

Section I. Cells, Protoplasts, and Liposomes

1. Techniques of Cell Suspension Culture	RICHARD BLIGNY AND JEAN-JACQUES LEGUAY	3
2. Culture and Characteristics of Green Plant Cells	COLIN DALTON AND IAN MACKENZIE	16
3. Preparation of Protoplasts from Plant Tissues for Organelle Isolation	MIKIO NISHIMURA, IKUKO HARA-NISHIMURA, AND TAKASHI AKAZAWA	27
4. Interaction of Plant Protoplast and Liposome	TOSHIYUKI NAGATA	34
5. Liposomes as Carriers for the Transfer and Ex- pression of Nucleic Acids into Higher Plant Pro- toplasts	MICHEL CABOCHE AND PAUL F. LURQUIN	39
6. Interspecific Transfer of Partial Nuclear Genomic Information by Protoplast Fusion	OTTO SCHIEDER	45

Section II. Vacuoles and Tonoplasts

7. Isolation of Mature Vacuoles of Higher Plants: General Principles, Criteria for Purity and In- tegrity	GEORGE J. WAGNER	55
8. Isolation of Vacuoles and Tonoplast from Proto- plasts	ALAIN M. BOUDET AND GILBERT ALIBERT	74
9. Preparation of Tonoplast Vesicles from Isolated Vacuoles	ROBERT T. LEONARD	82
10. Biochemical and Enzymatic Components of a Vacuolar Membrane: Tonoplast of Lutoids from <i>Hevea</i> Latex	J. D'AUZAC, H. CHRESTIN, AND B. MARIN	87

11. Characterization of Tonoplast Enzyme Activities and Transport	BENJAMIN JACOBY	105
12. Preparation of Tonoplast Vesicles: Applications to H ⁺ -Coupled Secondary Transport in Plant Vacuoles	EDUARDO BLUMWALD, PHILIP A. REA, AND RONALD J. POOLE	115
13. Purification and Characterization of the Tonoplast H ⁺ -Translocating ATPase	STEPHEN K. RANDALL AND HEVEN SZE	123
14. High-Performance Liquid Chromatography for Simultaneous Kinetic Measurements of Adenine Nucleotides in Isolated Vacuoles	M. HILL, A. DUPAIX, P. VOLFIN, A. KURKDJIAN, AND B. ARRIO	132

Section III. Plastids

15. Isolation of Intact Chloroplasts: General Principles and Criteria of Integrity	DAVID A. WALKER, ZORAN G. CEROVIC, AND SIMON P. ROBINSON	145
16. Isolation of Plastids in Density Gradients of Percoll and Other Silica Sols	CARL A. PRICE, JOHN C. CUSHMAN, LETICIA R. MENDIOLA-MORGENTHALER, AND ELLEN M. REARDON	157
17. Purification of Chloroplasts Using Silica Sols	K. W. JOY AND W. R. MILLS	179
18. Separation of Chloroplasts and Cytosol from Protoplasts	SIMON P. ROBINSON	188
19. Use of Thermolysin to Probe the Cytosolic Surface of the Outer Envelope Membrane from Plastids	JACQUES JOYARD, ALBERT-JEAN DORNE, AND ROLAND DOUCE	195
20. Characterization of Plastid Polypeptides from the Outer and Inner Envelope Membranes	JACQUES JOYARD, MARYSE A. BLOCK, JACQUES COVÈS, CLAUDE ALBAN, AND ROLAND DOUCE	206
21. Isolation of Amyloplasts from Suspension Cultures of Soybean	TOM AP REES AND FRASER D. MACDONALD	218
22. Isolation of Amyloplasts from Developing Endosperm of Maize (<i>Zea mays</i> L.)	JACK C. SHANNON, EDGARDO ECHEVERRIA, AND CHARLES BOYER	226
23. Isolation of Plastids from Buds of Cauliflower (<i>Brassica oleracea</i> L.)	ETIENNE-PASCAL JOURNET	234
24. Isolation of Membranous Chromoplasts from Daffodil Flowers	BODO LIEDVOGEL	241

25. Structure and Function of the Inner Membrane Systems in Etioplasts	SATORU MURAKAMI	246
26. Isolation Procedures for Inside-Out Thylakoid Vesicles	HANS-ERIK ÅKERLUND AND BERTIL ANDERSSON	252
27. Characterization of Chloroplast Cytochromes	DEREK S. BENDALL AND STEPHEN A. ROLFE	259
28. Cell-Free Reconstitution of Protein Transport into Chloroplasts	MICHAEL L. MISHKIND, KAREN L. GREER, AND GREGORY W. SCHMIDT	274
29. Polar Lipids of Chloroplast Membranes	DAVID J. CHAPMAN AND JAMES BARBER	294
30. Isolation and Reconstitution of Thylakoid Lipids	SALLIE G. SPRAGUE AND L. ANDREW STAEHELIN	319
31. Long-Chain Fatty Acid Synthesis and Utilization by Isolated Chloroplasts	GRATTAN ROUGHAN	327
32. Phosphatidylglycerol Synthesis in Chloroplast Membranes	J. BRIAN MUDD, JAEN E. ANDREWS, AND S. A. SPARACE	338
33. Galactolipid Biosynthesis in Chloroplast Membranes	NORA W. LEM AND JOHN P. WILLIAMS	346
34. Chlorophylls and Carotenoids: Pigments of Photosynthetic Biomembranes	HARTMUT K. LICHTENTHALER	350
35. α -Tocopherol and Plastoquinone Synthesis in Chloroplast Membranes	JÜRGEN SOLL	383
36. Solubilization and Reconstitution of Carotenogenic Enzymes from Daffodil Chromoplast Membranes Using 3-[(3-Cholamidopropyl)dimethylammonio]-1-propane Sulfonate	P. BEYER	392

Section IV. Mitochondria

37. Isolation of Plant Mitochondria: General Principles and Criteria of Integrity	ROLAND DOUCE, JACQUES BOURGUIGNON, RENAUD BROUQUISSE, AND MICHEL NEUBURGER	403
38. Purification of Plant Mitochondria on Silica Sol Gradients	ANTHONY L. MOORE AND MICHAEL O. PROUDLOVE	415
39. Isolation of Mitochondria from Leaves of C_3 , C_4 , and Crassulacean Acid Metabolism Plants	GERALD E. EDWARDS AND PER GARDESTRÖM	421

40. Separation of Spinach Leaf Mitochondria according to Surface Properties: Partition in Aqueous Polymer Two-Phase Systems	PER GARDESTRÖM AND INGEMAR ERICSON	434
41. Isolation of Submitochondrial Particles with Different Polarities	IAN M. MØLLER, ANNIKA C. LIDÉN, INGEMAR ERICSON, AND PER GARDESTRÖM	442
42. Isolation of the Outer Membrane of Plant Mitochondria	CARMEN A. MANNELLA	453
43. Characterization of Channels Isolated from Plant Mitochondria	MARCO COLOMBINI	465
44. Phosphoglycerides of Mitochondrial Membranes	JOHN L. HARWOOD	475
45. Ubiquinone Biosynthesis in Plant Mitochondria	FRIEDHELM LÜTKE-BRINKHAUS AND HANS KLEINIG	486
46. Purification of Complexes II and IV from Plant Mitochondria	MASAYOSHI MAESHIMA, TSUKAHO HATTORI, AND TADASHI ASAHI	491

Section V. Peroxisomes and Glyoxysomes

47. Isolation of Glyoxysomes and Purification of Glyoxysomal Membranes	EUGENE L. VIGIL, TUNG K. FANG, AND ROBERT P. DONALDSON	505
48. Peroxisomes and Fatty Acid Degradation	BERNT GERHARDT	516
49. Proteins and Phospholipids of Glyoxysomal Membranes from Castor Bean	HARRY BEEVERS AND ELMA GONZÁLEZ	526

Section VI. Nuclei, Endoplasmic Reticulum, and Plasma Membrane

50. Isolation of Nuclei from Soybean Suspension Cultures	PAUL KEIM	535
51. Isolation of the Plasma Membrane: Membrane Markers and General Principles	DONALD P. BRISKIN, ROBERT T. LEONARD, AND THOMAS K. HODGES	542
52. Preparation of High-Purity Plasma Membranes	CHRISTER LARSSON, SUSANNE WIDELL, AND PER KJELLBOM	558

53. Possible Approaches to Surface Labeling of the Plasma Membrane	J. L. HALL	568
54. Isolation of Endoplasmic Reticulum: General Principles, Enzymatic Markers, and Endoplasmic Reticulum-Bound Polysomes	J. MICHAEL LORD	576
55. Phosphoglyceride Synthesis in Endoplasmic Reticulum	THOMAS S. MOORE, JR.	585

Section VII. General Physical and Biochemical Methods

56. Electron Microscopy of Plant Cell Membranes	JEAN-PIERRE CARDE	599
57. Two-Dimensional Electrophoresis in the Analysis and Preparation of Cell Organelle Polypeptides	R. REMY AND F. AMBARD-BRETTEVILLE	623
58. Plant Membrane Sterols: Isolation, Identification, and Biosynthesis	MARIE-ANDRÉE HARTMANN AND PIERRE BENVENISTE	632
59. Separation of Molecular Species of Plant Glycolipids and Phospholipids by High-Performance Liquid Chromatography	J. KESSELMEIER AND E. HEINZ	650
60. Movement of Phospholipids between Membranes: Purification of a Phospholipid Transfer Protein from Spinach Leaf	JEAN-CLAUDE KADER, CHANTAL VERGNOLLE, AND PAUL MAZLIAK	661
61. Pesticides and Lipid Synthesis in Plant Membranes	C. ANDING	667
62. Rapid Filtration Technique for Metabolite Fluxes across Cell Organelles	YVES DUPONT AND MARIE-JOSÉ MOUTIN	675
63. Application of Nuclear Magnetic Resonance Methods to Plant Tissues	R. G. RATCLIFFE	683
AUTHOR INDEX		701
SUBJECT INDEX		729

[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 work³ on the chemical synthesis of prenylquinones enabled others⁴⁻⁶ 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 here⁴ 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 salt⁷ $\{[(\text{SO}_3)_2\text{NO}]\text{K}_2\}$ 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 $\text{Na}_2\text{S}_2\text{O}_4$ 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).

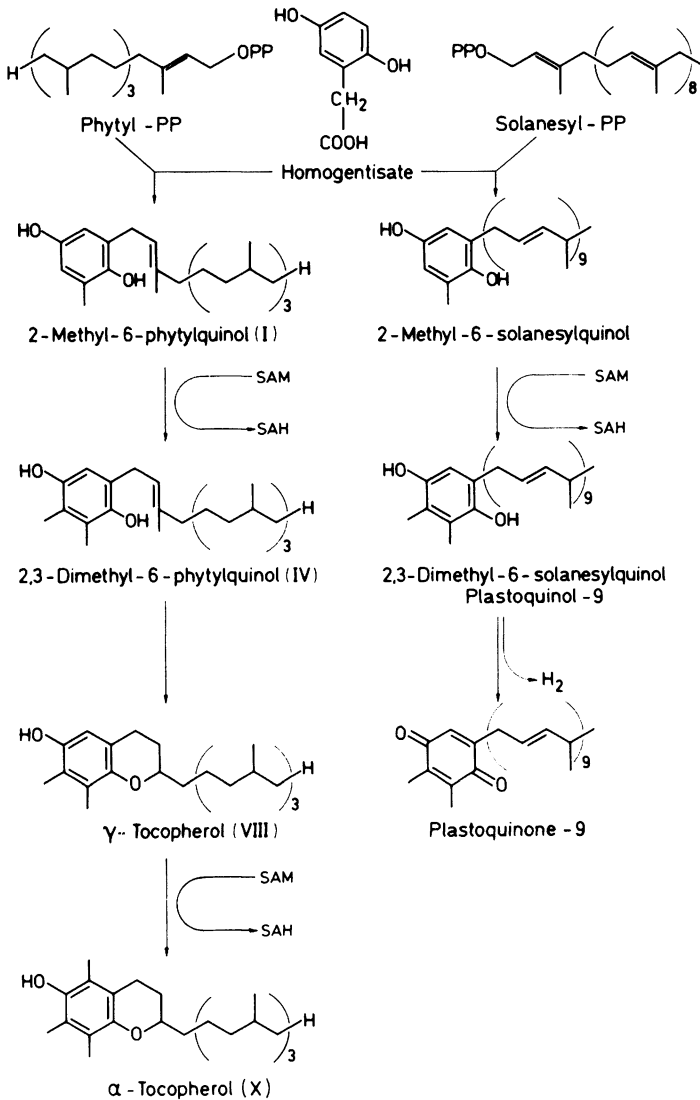


FIG. 1. Proposed pathway of α -tocopherol and plastoquinone-9 synthesis in spinach chloroplasts. From the available data these are the most likely intermediates to be involved in prenylquinone synthesis. A possible bypass in tocopherol synthesis might occur which leads from I via VII to VIII instead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

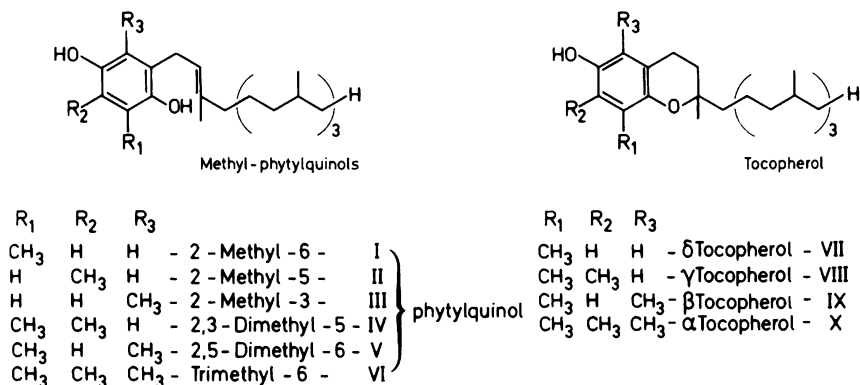


FIG. 2. Nomenclature and identification of prenylquinols and tocopherols.

isophytol in 2 ml dry tetrahydrofuran. The mixture is stirred under N₂ in the dark for 35 hr. Residual BF₃ 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 Ag₂O 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

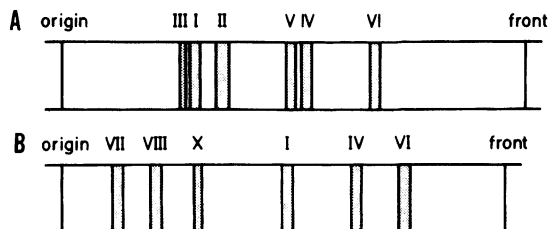


FIG. 3. Separation of prenylquinones and tocopherols by thin-layer chromatography. (A) The separation of mono-, di-, and trimethylphytylquinones on precoated thin-layer plates, silica gel G-1500, with diethyl ether : petrol (1 : 15, v : v) as developing solvent. The purification of prenylquinones and 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 $\text{FeCl}_3/2,2'$ -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 phytol 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 [³H]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 procedures¹¹ 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. Heinstejn, *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 \rightarrow 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 NaBH₄ for 2 min, transferred to diethyl ether, and washed with H₂O. The diethyl ether is evaporated to dryness under N₂ in the reaction vials which were used later in the enzyme assay. Substrate concentrations described^{4,14-16} are 50–100 μ M prenyl diphosphate, 100 μ M *S*-adenosylmethionine, 100–200 μ M prenylquinol or tocopherol at pH 7.6–8.2 with MgCl₂ as cofactor (1–10 mM) and chloroplasts equivalent to 0.5–1 mg of chlorophyll. Other cofactors like cysteine, dithiothreitol (DTT), light, and Mn²⁺ 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

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, [^3H]homogenisate, 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 isoctane¹⁵). 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. Prenzler, *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).

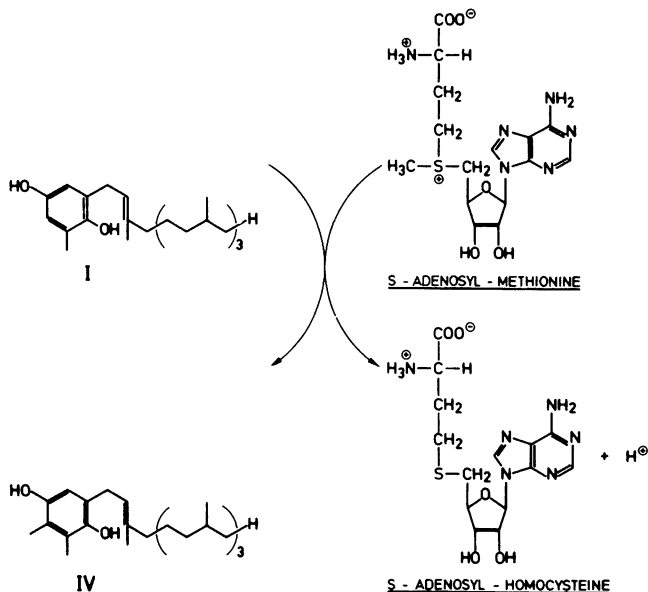


FIG. 4. Methylation of 2-methyl-6-phytylquinol by *S*-adenosylmethionine. Reaction products are 2,3-dimethyl-6-phytylquinol and *S*-adenosylhomocysteine.

different intermediates: (1) **I**, (2) after cyclization of **I** from **VII**. *In vitro* studies using isolated chloroplasts⁴ 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} ($\lambda_{\max \text{ I}}$ 254, $\lambda_{\max \text{ II}}$ 253, $\lambda_{\max \text{ III}}$ 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 derivatives²⁵ 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-9⁵ (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:H₂O, 90:10, system IV).

Localization of Prenylquinone Synthesis in Chloroplasts

Recently developed methods for the fractionation and purification of chloroplast components¹³ 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*) chloroplasts was shown to catalyze α -tocopherol synthesis via the same intermediates as described for chloroplasts¹⁶ (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 mM DTT, and Tween 80 (1 mg/ml), followed by $(\text{NH}_4)_2\text{SO}_4$ precipitation (20–60%). The protein is purified by column chromatography²⁹: (1) blue Sepharose CL-6B, 50 mM KH_2PO_4 , 1 mM DTT, 1 mM EDTA, pH 6.2; (2) blue Sepharose CL-6B, 50 mM Tris-HCl, 1 mM DTT, 1 mM EDTA, pH 8; (3) DEAE-Sephacel, 50 mM Tris-HCl, pH 7.6, 1 mM 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 Da²⁹ (pH optimum, 8.2; K_m S-adenosylmethionine, 2.5 μM ; K_m γ -tocopherol, 13.7 μM).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 $\text{CHCl}_3/\text{MeOH}$ (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 $\mu\text{g}/\text{mg}$ 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).