Observations on the biosynthesis of α-eleostearic and vernolic acids, and the accumulation of petroselinic acid in somatic carrot embryos

by

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DEDICATION

I would like to dedicate my dissertation

to

my mother

who died of cancer in China while I was studying at Iowa State University.

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ABBREVIATIONS

ABA:	abscisic acid
ACP:	acyl carrier protein
ATP:	adenosine triphosphate
CDP:	cytidine diphosphate
CoA:	coenzyme A
DAF:	days after flowering
DAG:	diacylglycerol
DAGs:	diacylglycerols
DAP:	days after pollination
DTT:	dithiothreitol
FAS:	fatty acid synthase
fr. wt:	fresh weight
LPAAT:	lysophosphatidic acid acyltransferase
NADH:	nicotinamide adenine dinucleotide
NADPH:	nicotinamide adenine dinucleotide phosphate
PC:	phosphatidylcholine
PE:	phosphatidylethanolamine
PI:	phosphatidylinositol
PrOH:	isopropanol
TAG:	triacylglycerol

TAGs:	triacylglycerols
wt:	weight

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CHAPTER 1. GENERAL INTRODUCTION

Dissertation Organization

This dissertation contains a general introduction, including an overview on the chemistry of petroselinic, vernolic and α-eleostearic acids, and an introduction to the biosynthesis of fatty acids in plant seeds. The general introduction is followed by five research papers and a general conclusion.

The Chemistry of Petroselinic, Vernolic and α-Eleostearic Acids

Many important industrial fatty acids and oils exist in limited amounts in nature or come only from certain geographic areas. Substitutes for some fatty acids can be produced by chemical reactions such as epoxidation, but chemical syntheses often produce by-products that cause environmental disposal problems. Because the supplies of these industrial fatty acids and oils are limited, the availability and price of these natural products are unpredictable. Recent developments in biochemistry and biotechnology provide a promising way to produce genetically engineered plants with high content of unusual fatty acids, e. g. rapeseed oil containing lauric acid. This possibility of new sources of useful fatty acids has kindled interest in studying the biosynthesis of unusual fatty acid in plants.

Petroselinic (*cis*-6-octadecenoic), vernolic (12, 13-epoxy-*cis*-9-octadecenoic) and α-eleostearic (*cis, cis, trans*-9, 11, 13-octadecatrienoic) acids are three fatty acids with unusual structures for which there is significant commercial interest. This dissertation reports a

somatic embryo system for petroselinic acid research and explores the biosynthesis of vernolic and α-eleostearic acids in plant seeds.

Petroselinic Acid

Petroselinic (*cis*-6-octadecenoic) acid is an unusual isomer of oleic acid, which is the major fatty acid in most oils and fats and contains the unsaturation in the 9, 10-position. Petroselinic acid was identified as a solid isomer of oleic acid in the fatty oil of parsley seed and named by Vongerichten and Köhler in 1909 (1). In the 1960s, considerable effort was given to searching for commercial sources of petroselinate and to studying its special properties (2-7). Petroselinic acid and its derivatives have proven superior to oleic acid as a detergent and in photogenic emulsions (3). Through oxidative splitting of its Δ^6 double bond, petroselinic acid yields two industrially important products, lauric (12:0) and adipic (6:0 dicarboxylic) acids. Currently, the U. S. spends $ 400 million annually to import lauric acid oils for surfactants and foods (2, 8). Adipic acid is the raw material for nylon 66. Another potential usage of petroselinic acid is in nutritional value-added foods. Petroselinic acid has a melting point of 33°C, compared to 13°C for oleic acid. This property means that petroselinic acid oils are solid or semi-solid at room temperature and would provide a promising way to produce zero-*trans* plastic fats such as margarine and shortening. In addition, pancreatic lipase is less active in hydrolyzing glycerol esters of petroselinic acid than with oleic acid (9, 10). Thus, petroselinate-containing oils may offer a low calorie alternative to conventional vegetable oil.

Although the properties of petroselinic acid have been widely studied and much interest has been shown in using it commercially, there are no commercial sources. The seed oils of the *Apiacea*, formerly *Umbelliferae*, and the closely related *Araliaceae* family are rich in petroselinate. The *Apiacea* includes parsley, celery and carrot. None of them are cultivated for seed and oil production because their agronomic properties are not good for this purpose.

With the development of biotechnology (11-16), it appears promising to produce petroselinic acid economically by transferring the gene(s) responsible for its biosynthesis into oilseed crops such as soybean. An understanding of the biosynthetic pathway is valuable in carrying out such a scheme. Since the plants of *Apiacea* usually are biennial, they are not a convenient source of developing seed, the commonly used tissues for biosynthetic studies of plant fatty acids. Wurtele et al. (17) have shown that it is possible to produce somatic embryos from carrot seedlings. We examined these embryos for production of petroselinate. If petroselinate was produced in the embryos, they would be a more convenient system to study petroselinate biosynthesis. Previous reports indicated that little petroselinate was produced in such somatic embryos (18, 19).

Vernolic Acid

Vernolic (12, 13-epoxy-*cis*-9-octadecenoic) acid is the sole naturally occurring epoxy fatty acid known to exist in seed oils at levels above 15% (20). Currently, the epoxy fatty acids are produced from soybean and linseed oils by treatment with hydrogen peroxide and

acetic acid (25). Epoxy fatty acids are used as plasticizers for polyvinyl chloride (PVC) and raw materials for synthetic fibers (26-31). Another potential commercial usage of epoxy fatty acids is in paint formulation to decrease organic compound emissions during drying. It is believed that at least 160 million pounds of organic compound emissions would be eliminated by adding 1 pint of vernonia oil to each of 325 million gallons of oil-based paints produced annually in the United States (21, 32). Vernonia oils typically contain 79-81% vernolic acid. The oil is pourable below 0°C (21) and is biodegradable.

Gunstone discovered the presence of vernolic acid in *Vernonia anthelmintica* oil in 1954 (48). Since then, 60 species in 12 plant families have been reported to contain vernolic acid, but only four plant families are now known to produce oils with > 15% of epoxy acids and none of them have been successfully domesticated (33). The seed oils of *Euphoria* and *Vernonia* usually contain 58-81 % (22-24). *Vernonia galamensis*, a potential industrial oilseed crop that is native to Africa, is being cultivated in Zimbabwe. However, attempt to domesticate this plant in the United States has revealed the need for a short day length for flowering, which leads to the danger of damaging the plant from frost (21). An alternative to domestication of *Vernonia galamensis* for vernolic acid production is to manipulate the genome of crops such as soybean by introducing the genes for biosynthesis of vernolic acid. The investigation of the biosynthesis of vernolic acid will help provide the information necessary for the gene manipulation.

α-Eleostearic Acid

α-Eleostearic (*cis, cis, trans*-9, 11, 13-octadecatrienoic) acid makes up as much as 85% of the total fatty acids of tung (Chinese wood) oil, and it also is found in the seed oils of species of *Rosaceae, Euphorbiaceae, and Cucurbitaceae* (20). The conjugated trienes can undergo thermal and autoxidative polymerization readily (34-38). This property characterizes tung oil as a drying oil with many important industrial uses in surface coatings, paints and waterproofing agents (39, 40).

Tung trees can grow only in certain geographic areas. Therefore, the availability of tung oil, the sole commercial source of α-eleostearic acid, is limited. The price and availability of tung oil greatly depend on production in China. Tung trees were introduced into the United States from China early of this century and cultivated along the coast of the Gulf of Mexico. There are many difficulties in growing tung trees in the United States, and tung oil production in the U.S. has stopped since a hurricane in 1968 destroyed the tung orchards (Conkertor, E. J., private communication). Recently, the world supply of tung oil has decreased, and the price has become erratic and unpredictable. This has resulted in a new interest in research on the biosynthesis of α-eleostearic acid.

Mormodica charantia, known as bitter gourd, is widely grown throughout Western Africa and Asian countries where its young fruits are used a vegetable and the leaves for medicine (41-43). The seed oil of mature fruits contains about 60-70% α-eleostearate. There is a new interest in growing *M. charantia* for α-eleostearate production (44). *M. charantia* flowers and starts to bear fruits about 30 days after germination under green house

conditions, which makes it a very good candidate for the research on biosynthesis of α-eleostearic acid.

The Biochemistry of Fatty Acids

Introduction

The serious study of the biosynthesis of fatty acids might be regarded as having begun about 1860 when Lawes and Gilbert developed the concept of conversion of carbohydrate to fat (45). In 1890, Emil Fisher (46) proposed that fatty acids could be synthesized from sugar molecules by "hexose condensation." The concept of acetate as an active intermediate in fat synthesis was proposed by Embden and Oppenheimer from their observations in 1912 (47). Later, the role of malonic acid in the synthesis of fatty acids was documented (49). The participation of a biotin-containing enzyme in the biosynthesis of fatty acids was observed by Wakil and his coworkers (50-53). They suggested a reaction for the biosynthesis of palmitic acid in which the first two atoms come from acetyl-CoA and the rest from malonyl-CoA.

Two procedures were used by earlier biochemists for exploring the biogenesis of fatty acid: indirect or compositional analysis, and direct or precursor labeling. Because the levels of complexity and the influence of environmental conditions, compositional studies may lead to erroneous conclusion regarding metabolism. For instance, some early biochemists believed that the saturated and unsaturated fatty acids were synthesized independently based on the observation that all fatty acids increased in ripening seeds

concurrently (54). However, compositional studies account for some successful discoveries, such as the observation of increasing linolenate and linoleate coincident with decreasing stearate and oleate in developing flax seeds (55). Desaturation was suggested as the possible synthetic mechanism for formation of polyunsaturated acids. Generally, the indirect methods can provide data on compositional variation with development, environmental changes or variety. Confirmation must be obtained directly from precursor labeling or direct studies, in which radioactive precursor is fed to the developing tissues, such as seedlings and developing cotyledon slices, and the changes of fatty acid composition in lipids are studied at timed intervals.

There are two kinds of precursors, the general and specific precursors. The general precursor usually is a small molecule that is the starting point or intermediate in the synthetic pathway. The common general precursor is acetate, which is incorporated into fatty acids at the greatest rate among the possible intermediates and precursors, such as propionate, butyrate, pyruvate, fructose or succinate (56). [1-^{14}C]-Acetate was incorporated into fatty acids three times faster than [2-^{14}C]acetate (56). The specific precursor is generally a radioactively labeled fatty acid, which is the immediate precursor of the studied fatty acids, and used to confirm a suspected metabolic reaction. In the history of the study of plant fatty acid biosynthesis, the introduction of a specific precursor into the system for *in vivo* study was the hardest problem because of their property of water insoluble.

Direct studies have been of two kinds: time-course and pulse-chase experiments. Time-course labeling is an experiment during which radioactively labeled precursors are

supplied throughout the experiment, and a portion of samples is taken at the timed intervals. In pulse-chase experiments, tissues are radioactively labeled with a single burst of labeled precursor that is then removed and incubation is continued in a medium free of radioactively labeled precursor to allow for further metabolism. In the first type, radioactivities of all fatty acids increase, and differences in the rate of rise and the order in which fatty acids become radioactively labeled indicate the pathway of biosynthesis (57). The pulse-chase experiment, on the other hand, is more successful in determining precursor-product relationships (58).

Biosynthesis of fatty acids

The biosynthesis of fatty acids in both plants and animals has been demonstrated to start from acetyl-CoA, the immediate precursor in the pathway (59-60). In plants in the presence of ATP and CO_2, acetyl-CoA is carboxylated to form malonyl-CoA in the plastid by the carboxylase, which catalyzes the first committed step in plant fatty acid biosynthesis (61, 62). Malonyl-CoA is converted into its ACP form, which are then used to synthesize palmitoyl-ACP (16:0) by the fatty acid synthases, FAS III and I (63, 64). Stearoyl-ACP (18:0), which is formed by elongation from palmitoyl-ACP by FAS II, is desaturated to form oleoyl-ACP (18:1 n9) by 18:0-ACP desaturase (65). The three fatty acids in the plastid final products, 16:0-ACP, 18:0-ACP and 18:1 n9-ACP, are released by acyl-ACP hydrolase and exported into the cytoplasm (66). During transportation, the free fatty acids are converted to their CoA form by a specific acyl-CoA synthetase located in the outer envelope of plastid

(67). Further modification on the fatty acids occurs in cooperation with the biosynthesis of TAG.

It is believed that in plants TAGs are synthesized according to the Kennedy pathway (68). First, glycerol-3-phosphate is acylated at the sn-1 position by glycerophosphate acyltransferase to form lysophosphatidic acid, which is then acylated to form phosphatidic acid by a second acyltransferase. Phosphatidic acid is the precursor for both DAG and PC. TAG is synthesized by acylation of DAG, which is derived from phosphase hydrolysis of phosphatidic acid. PC is the substrate of membrane-bound enzymes for forming polyunsaturated fatty acids and may also be the substrate for the biosynthesis of other unusual fatty acids such as ricinoleic acids (69-71, 76). The polyunsaturated and other unusual fatty acids can be incorporated into TAG through three pathways. One way is to acylate DAG formed from PC by reverse action of CDP-choline:DAG cholinephospho-transferase. The second is to bring unusual fatty acids into acyl-CoA pool through ester interchange between acyl-CoA and PC catalyzed by lyso-PC acyltransferase. The third is to release unusual fatty acids from PC and then convert the free acid into its CoA form. Linoleic and linolenic acid have been proven to be synthesized and incorporated into TAGs by the first two pathways, and ricinoleic and vernolic acids are thought to be incorporated by the third (69).

Instead of desaturation of 18:0, as in the synthesis of oleate, petroselinate is synthesized by two steps in plastids (72, 73). First 16:0-ACP is converted to 16:1n12-ACP by Δ^4 desaturase. The latter is elongated into 18:1n12-ACP. Petroselinic acid is release by a

specific hydrolase and exported to the cytoplasm, where petroselinate is incorporated into TAGs by acylations. Erucate (22:1n9) is synthesized by elongation of oleoyl-CoA and incorporated specifically sn-1, 3 positions of TAGs because of inability of lysophosphatidic acid acyltransferase (LPAAT) to utilize erucoyl-CoA (76). Ricinoleic acid is synthesized from linoleate on PC and released as the free fatty acid (74). The biosynthesis of vernolic acid has been reported to be synthesized very similarly (33). In the developing seed of *Euphorbia lagascae*, linoleate on PC is the epoxylation precursor and vernolic acid might then be released and incorporated into TAG after conversion to its CoA form. Crombie and Holloway (78, 79) studied the biosynthesis of calendic acid (*cis*, *cis*, *trans*-8, 10, 12-octadecatrienoic acid) in the developing marigold seeds. They found that linoleic acid is the precursor of calendic acid. α-Eleostearic acid was reported to be synthesized from linoleic, 13-hydroxy- and hydroperoxy-octadecadienoic acids (77), but information on the pathway of incorporation into TAG is limited.

References

1. Vongerichten, E., and A. Köhler, *Ber.* 42:1638 (1909).
2. Kleiman, R., and G. F. Spencer, *J. Am. Oil Chem. Soc.* 59:29 (1982).
3. Placek, L. L., *J. Am. Oil Chem. Soc.* 40:319 (1963).
4. Lotti, G. and E. Bogan, *Ric. Sci.* 37:798 (1967).
5. Privett, O. S., J. D. Nadenicek, R. P. Weber, and F. J. Pasch, *J. Am. Oil Chem. Soc.* 40:28 (1963).

6. Chobamov, D., E. Chooparova, A. Popov, and Kamenova, *Fette Scifen Anstrich* 68:447 (1966).

7. Kleiman, R., V. L. Davison, F. R. Earie, and H. J. Dutton, *Lipids* 2:339 (1967).

8. Ohlrogge, J. B., *Plant Physiol.* 104:821 (1994).

9. Heimerman, W. H., R. T. Holman, D. T. Gordon, D. E. Knowalyddhyn, and R. G. Jensen, *Lipids* 8:45 (1973).

10. Weber, N., K. D. Mukherjee, E. Schultz, and K.-D. Richter, *Inform* 5:499 (1994).

11. Cahoon, E. B., J. Shanklin, and J. B. Ohlrogge, *Proc. Natl. Acad. Sci. USA, Plant Biology* 89:11184 (1992).

12. Cahoon, E. B., and J. B. Ohlrogge, *Plant Physiol.* 104:827 (1994).

13. Dormann, P., M. Frentzen, and J. B. Ohlrogge, *Plant Physiol.* 104:839 (1994).

14. Robbelein, G., R. K. Downey and A. Ashri, eds., *Oil Crops of the World*, McGraw-Hill, New York, 1989.

15. Cahoon, E. B., P. Dormann, and J. B. Ohlrogge, *Prog. Lipid Res.* 33:155 (1994).

16. Topfer, R., N. Martin, J. Schell, *Science* 268:681 (1995).

17. Wurtele, E. S., H. Wang, S. Dargerian, B. J. Nikolau, and T. H. Ulrich, *Plant Physiol.* 102:303 (1993).

18. Dutta, P. C., and L. A. Appelqvist, *Plant Sci.* 64:167 (1989).

19. Dutta, P. C., and L. A. Appelqvist, *Plant Sci.* 75:177 (1991).

20. Sonntag, N. O., Structure and Composition of Fats and Oils. in *Bailey's Industrial Oil ant Fat Products*, D. Swarn, 4th ed. (1979) p. 41.

21. Anonymous, *Inform* 2:69 (1991).

22. Krewson, C. F., J. Ard and R. W. Reimenschneider, *J. Am. Oil Chem. Soc.* 39:334 (1962)

23. Tallent, W. H., D. G. Cope, J. W. Hagemann, F. R. Earle and I. A. Wolff, *Lipids* 1:335 (1966).

24. Ayorinde, F. O., B. D. Bufler and M. T. Clayton, *J. Am. Oil Chem. Soc.* 67:844 (1990).

25. El-Mallah, M. M. H., H. A. El-Enein, M. Sadek and A. M. Gad, *Seifen-Ole-fette-wachse* 98:75 (1972).

26. Ayorinde, F. O., G. Osman, R. L. Sheparelt and F. T. Powers, *J. Am. Oil Chem. Soc.* 65:1774 (1988).

27. Rakoff, H., *Lipids* 23:4 (1988).

28. Ayorinde, F. O., F. T. Powers, L. D. Streete, R. L. Shepard and D. N. Tabi, *J. Am. Oil Chem. Soc.* 66:690 (1989).

29. Earle, F. R., *Ibid*, 47:510 (1970).

30. Afolabi, A. O., M. E. Aluko, G. C. Wang, W. A. Anderson and F. O. Ayorinde, *Ibid*, 66:983 (1989).

31. Charlson, K. D., V. E. Sohns, R. B. Perkins, Jr. and E. L. Huffman, *Ind. Eng. Chem. Prod. Res. Dev.* 16:95 (1977).

32. Perdue, R. E., Jr., *Agricultural Research* 37:10 (1989).

33. Bafor, M., M. A. Smith, L. Jonsson, K. Stobart and S. Stymne, *Arch. Biochem. Biophys.* 303:145 (1993).

34. Braddey, T. F. and R. W. Tess, *Ind. Eng. Chem.* 41:310 (1949).

35. Cowan, J. C., *Ibid*, 41:1647 (1949).

36. Bragdon, C. R. ed., *Film Forming, Film Properties and Film Deterioration*, Wiley-Interscience, New York, 1958.

37. Chatfield, H. W., ed., *The Science of Surface Coating*, Van Nostrand, Princeton, NJ, 1962.

38. Rheneck, A. E., and R. O. Austin, in *Treatise on Coatings*, Myers, R. R. and J. S. Long, eds., vol. 1, part 2, chapter 4, Marcel Dekker, New York, 1968.

39. Copacz, M., *J. Am. Oil Chem. Soc.* 45:285 (1968).

40. Long, J. S., S. F. Thame, and O. W. Smith, *Ibid*, 45:285 (1968).

41. Yuwai, K., K. S. Rao, C. Kaluwin, P. J. Gwyn and D. E. Rivett, *J. Agric. Food Chem.* 39:1762 (1991).

42. Fatope, M. O., *J. Natural Prod.* 53:1491 (1990).

43. Guevara, A. P., C. Y. Lim-Sylianco, F. M. Dayrit and P. Finch, *Phytochem.* 28:1721 (1989).

44. Chang, M. -K. E. J. Conkerton, D. C. Chapital, P. J. Wan, O. P. Vadhwa and J. M. Spiers, *Inform* 6:462 (1995).

45. Lawes, G. B. and J. H. Gilber, *J. Royal. Agr. Soc. Engl.* 21:433 (1860).

46. Fisher, E., *Ber.* 23:2114 (1890).

47. Embden, G. and M. Oppenheimer, *Biochem. Z.* 45:186 (1912).

48. Gunstone, F. D., *J. Chem. Soc.* 1954:1611 (1954).

49. Smedley-Maclean, I. and D. Hoffert, *Biochem. J.* 20:343 (1926).

50. Wakil, S. J., *J. Am. Chem. Soc.* 80:6465 (1958).

51. Wakil, S. J. and J. Ganguley, *Ibid*, 81:2597 (1959).

52. Wakil, S. J., E. B. Titchener, and D. M. Gibson, *Biochim. Biophys. Acta* 29:225 (1958).

53. Wakil, S. J. and D. M. Gibson, *Ibid*, 41:122 (1960).

54. Gentry, H. S. and R. W. Miller, *Economic. Botany* 19:25 (1965).

55. Theis, E. R. J. S. Long and G. F. Beal, *Ind. Eng. Chem.* 22:768 (1930).

56. Newcomb, E. H. and P. K. Stumpf, *J. Biol. Chem.* 200:233 (1953).

57. Simmons, R. O., and F. W. Quackenbush, *J. Am. Oil Chem. Soc.* 31:601 (1954).

58. Zilversmit D. B., C. Entenmam and M. C. Fisher, *J. Gen. Physiol.* 26:325 (1943).

59. Harwood, J. L., Annu. Rev. *Plant Physiol. Mol. Biol.* 39:101 (1988).

60. Hannapel, D. J. and J. B. Ohlrogge, *Plant Physiol.* 86:1174 (1988).

61. Simcox, P. D., W. Garland, V. Deluca, D. T. Canvin and D. T. Dennis, *Can. J. Bot.* 57:1008 (1979).

62. Post-Beittenmiller, D., G. Roughan, J. B. Ohlrogge, *Proc. Natl. Acad. Sci. Physiol.* 100:923 (1992).

63. Jaworski, J., R. C. Clough and S. R. Barnum, *Plant Physiol.* 90:41 (1989).

64. Shimakata T. and P. K. Stumpf, *Arch. Biochem. Biophys.* 220:39 (1983).

65. Mckeon T. A. and P. K. Stumpf, *J. Biol. Chem.* 257:12141 (1982).

66. Shine, W. E., M. Mancha and P. K. Stumpf, *Arch. Biochem. Biophys.* 172:110 (1976).

67. Joyard, J., R. Douce, Galactolipid Synthesis, in *The Biochemistry of Plants*, P. K. Stumpf, ed., Academic Press, Orlando, FL, vol. 9. p. 215, 1987.

68. Kennedy, E. P., *Fed. Proc. Am. Soc. Exp. Biol.* 20:934 (1961).

69. Bafor, M., M. A. Smith, L. Jonsson, K. Stobart ansd S. Stymne, *Biochem. J.* 280:507 (1991).

70. Galliard, T. and P. K. Stumpf, *J. Biol. Chem.* 241:5806 (1981).

71. Frentzen, M., Acyltransferase and Triacylglycerols, in *Lipid Metabolism in Plants*, J.-C. Kader and P. Mazliac, eds, Kluwer, Dordrecht, Netherlands, 1995, chapter 6, p. 195.

72. Ohlrogge J. B., *Plant Physiol.* 104:821 (1994).

73. Dormann, P., M. Frentzen and J. B. Ohlorgge, *Plant Physiol.* 104:839 (1994).

74. Morris, L. J., *Biochem. Res. Commun.* 29:311 (1967).

75. Banas, A., I. Johansson and S. Stymne, *Plant Sci.* 84:137 (1992).

76. van de Loo, F. J., B. G. Fox and C. Somerville, Unusual Fatty Acids, In *Lipid Metabolism in Plants*, J. -C. Kader and P. Mazliac, eds, Kluwer, Dordrecht, Netherlands, 1995, Chapter 6, p. 91.

77. Noda, M., Biosynthesis of Conjugated Polyenoic Acids in Plant Seeds, in *Structure, Function and Metabolism of Plant Lipids*, P. -A. Siegenthaler and W. Eichenberger, eds., Elsevier Science Publisher B. V., p. 29, 1984.

78 Crombie, L and S. J. Holloway, *J. Chem. Soc. Perkin. Trans.* 2425 (1985).

79. Crombie, L and S. J. Holloway, *J. Chem. Soc., Chem. Commun.* 953 (1984).

CHAPTER 2. PHENYLETHYL ESTERS OF FATTY ACIDS FOR THE ANALYTICAL RESOLUTION OF PETROSELINATE AND OLEATE

A paper published in the Journal of American Oil Chemists' Society [1]

Linsen Liu [2] and E.G. Hammond [2,3]

Abstract

2-Phenylethyl esters of fatty acids were prepared readily by esterification of free fatty acids or transesterification of other lipids. Compared with methyl esters, phenylethyl esters greatly improve the resolution of oleate and petroselinate by both gas and high performance liquid chromatography, and the ultraviolet absorption of the phenylethyl esters facilitates detection of the derivatives by high performance liquid chromatography (HPLC) ultraviolet detectors. The fatty acid compositions of corn and soybean oil obtained by analysis of phenylethyl esters agreed with those obtained with methyl esters. The phenylethyl esters were resolved and eluted on C-18 HPLC columns with much smaller solvent volumes than those reported for other aromatic esters.

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[1]Reprinted with permission from J. Am. Oil. Chem. Soc. 72:749-751 (1995).

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Key words: Fatty acid analysis, gas chromatography, high-performance liquid chromatography, phenylethyl esters, petroselinic acid.

Introduction

Fatty acid compositions are typically analyzed as methyl esters by gas chromatography (GC), but methyl petroselinate and methyl oleate are poorly resolved on most stationary phases (1). In metabolic studies of the fatty acids, high performance liquid chromatography (HPLC) separations also may be necessary, and methyl esters have little absorption in the ultraviolet detectors that are commonly available. Phenyl, naphthacyl and benzyl esters of the fatty acids have been suggested for such HPLC separations (2), but the preparation of these derivatives has required preliminary saponification of the samples, which obviously is inconvenient for routine use. We have developed simple procedures to prepare phenylethyl esters of fatty acids by esterification and transesterification and demonstrate the advantages of this derivative in the chromatographic determination of fatty acid composition.

Materials and Methods

Reagents. Phenylethyl alcohol was purchased from Aldrich Chemical Company (Milwaukee, WI) and used without further purification. HPLC grade hexane and methanol were from Fisher Scientific (Pittsburgh, PA); all other reagents came from Sigma Chemical Company (St. Louis, MO). Phenylethyl alcohol containing 1% sulfuric acid was prepared by

mixing the alcohol and acid in an ice bath. Sodium phenylethoxide (0.5 M) was prepared by reacting freshly cut sodium metal with phenylethyl alcohol.

Acid-Catalyzed Phenylethylation. Approximately 10 mg of lipid was dissolved in 0.5 ml of hexane and combined with 1 ml of 1% sulfuric acid in phenylethyl alcohol, and the mixture was heated in a capped vial for 1 h in a boiling water bath or overnight in a 50°C-oven. Aqueous sodium chloride (1%) was added, and the phenylethyl esters were recovered by extracting twice with 1 ml of hexane. The hexane extract could be used directly for gas chromatographic analysis but it contained some phenylethyl alcohol. The alcohol could be removed by thin-layer chromatography on a silica gel G plate developed with hexane-ether (80:20). For HPLC analysis, the hexane was evaporated with a stream of nitrogen, and 1 ml of methanol was added to dissolve the derivatives.

Base-Catalyzed Phenylethylation. Approximately 10 mg of lipid was dissolved in 0.5 ml of hexane, and 0.5 ml of 0.5 M sodium phenylethoxide in phenylethyl alcohol was added. After 30 min at 50°C or 1 h at room temperature, 1% aqueous sodium chloride was added, and the derivatives were extracted and purified as before.

GC. The phenylethyl and methyl esters of fatty acids were analyzed isothermally at 220°C on an Hewlett Packard (Wilmington, DE) 5890 instrument equipped with an FID

detector and 15 M x 0.244 mm J&W (Deerfield, IL) DB-23 capillary column. Helium was the carrier gas at 1 ml/min.

HPLC. Shimazu (Kyoto, Japan) LC-9A and Beckman (Fullerton, CA) System Gold instruments were used with Supelco (State College, PA) C-18 4 x 250 mm columns and ultraviolet detectors at a wavelength of 210 nm. Acetonitrile or methanol combined with water were the mobile phases.

UV Spectrophotometry. Samples in methanol were scanned between 200-300 nm with a Hitachi (Tokyo, Japan) U-2000 spectrophotometer.

Results and Discussions

Many combinations of acetonitrile or methanol with water can resolve the phenylethyl esters of fatty acids by HPLC on Supelco C-18 columns, but 5% water in methanol gave adequate resolution in the shortest time (Fig. 1). The phenylethyl derivatives of common fatty acids were separated and eluted in about 30 min by 5% water in methanol at 1 ml/min. These elution times are significantly shorter than those reported for other aromatic derivatives of fatty acids (Table 1), thus economizing on time and solvents.

Table 2 shows the relative response factors of several fatty acid phenylethyl esters on a UV detector. The UV factors were determined experimentally with phenylethyl stearate arbitrarily set to 1.0. The factors deviate from 1.0 because of the absorbance of the carboxyl

group and isolated double bonds in the acyl groups at 210 nm (Fig. 2) and the conversion of mole percentages to weight percentages.

The separation of phenylethyl oleate and petroselinate achieved on a 15-M x 0.224 mm J&W DB-23 column is shown in Fig. 3. The hydrogen flame correction factors (Table 2) were calculated according to Ackman and Sipos (5). The fatty acid compositions in Table 3 compare the results obtained from methyl and phenylethyl esters by GC and HPLC. For repeated injections percentages for the various esters by GC are replicated with standard deviations ranging from 0.07 to 0.12. For HPLC, the corresponding standard deviations are 0.03-0.21.

References

1. Wolff, R. L. and F. F. Vandamme, *J. Am. Oil Chem. Soc. 69*:1228 (1992).

2. Beach, R. F., *Anal. Chem. 47*:2437 (1975).

3. Pei, P. T.-S., W. C. Koss and S. Ramachandran, *Lipids 11*:814 (1976).

4. Copper, M. J. and M. W. Anders, *Anal. Chem. 46*:1849 (1974).

5. Ackman, R. G. and J. C. Sipos, *J. Am. Oil Chem. Soc. 41*:377 (1964).

Table 1. The HPLC elution times (min) reported for various aromatic fatty acid derivatives

Fatty Acids	Phenacyl[1]	Bromo-Phenacyl[2]	Naphthacyl[3]	Phenyl ethyl[4]	Phenyl ethyl[5]
18:3	28	13	38		7
18:2	34	18	54	6	14
18:1	50	26	85	8	17
18:0	57	44	-	11	23

[1]90 x 0.64 cm μ-Bondapak C-18, acetonitrile-water mobile phase at 2 mL/min (2).

[2]25 x 0.5 cm Applied Sci. Lab micropat C-18, 1.5 mL/min of 90:10 methanol-water (3).

[3]90 x 0.18 Corosil C-18 Waters Associates, 12 mL/hr of 85:15 methanol-water (4).

[4]25 x 0.4 cm Supelco C-18, 2 mL/min of acetonitrile.

[5]25 x 0.4 cm Supelco C-18, 1 mL/min of 95:5 methanol-water.

Table 2. The relative response factors to give weight percentage of phenylethyl esters of some common fatty acids in an ultraviolet HPLC and hydrogen flame GC detector

Fatty Ester	U.V. Detector [1]	Hydrogen Flame Detector [2]
16:0	0.968	1.012
18:0	1.000	1.000
18:1	0.976	0.995
18:2	0.888	0.990
18:3	0.620	0.984

[1]Empirical values.

[2]Calculated values.

Table 3. The fatty acid compositions of corn and soybean oils as weight percent methyl esters determined as methyl and phenylethyl esters by GC and HPLC

Oils	Derivatives/Methods	16:0	18:0	18:1	18:2	18:3
Soy						
	Methyl/GC	11.4	3.8	25.8	52.1	6.9
	Phenylethyl/HPLC	11.1	4.3	25.3	52.5	6.8
	Phenylethyl/GC	11.8	4.4	26.9	51.2	5.8
Corn						
	Methyl/GC	12.2	1.9	25.4	59.4	1
	Phenylethyl/HPLC	11.8	2.4	25.5	59.0	1
	Phenylethyl/GC	12.0	2.1	26.5	59.0	0.8

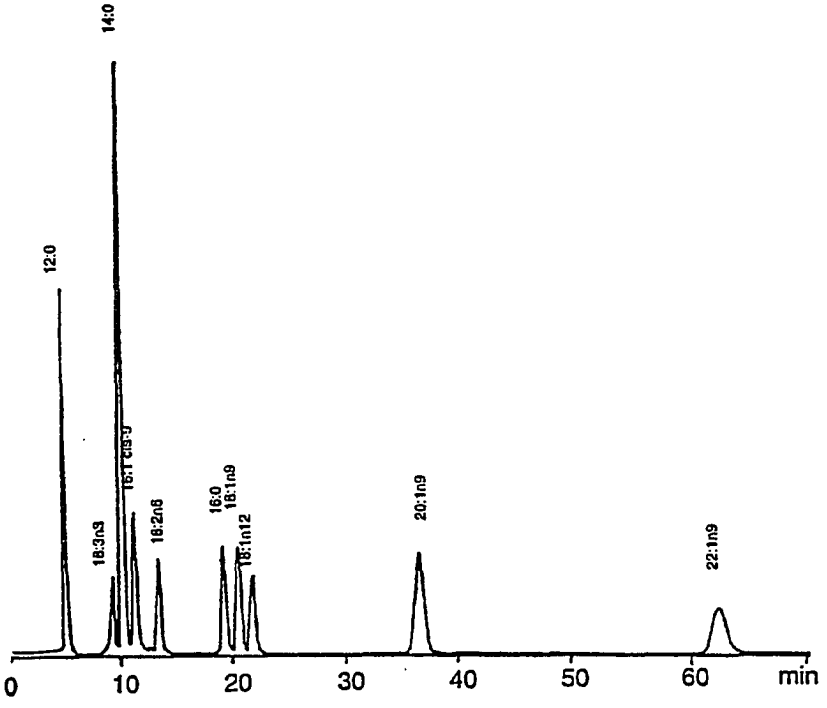

Figure 1. Separation of phenylethyl esters by high-performance liquid chromatography on a C-18 column with methanol/water 95:5 (vol/vol) at 2 mL/min.

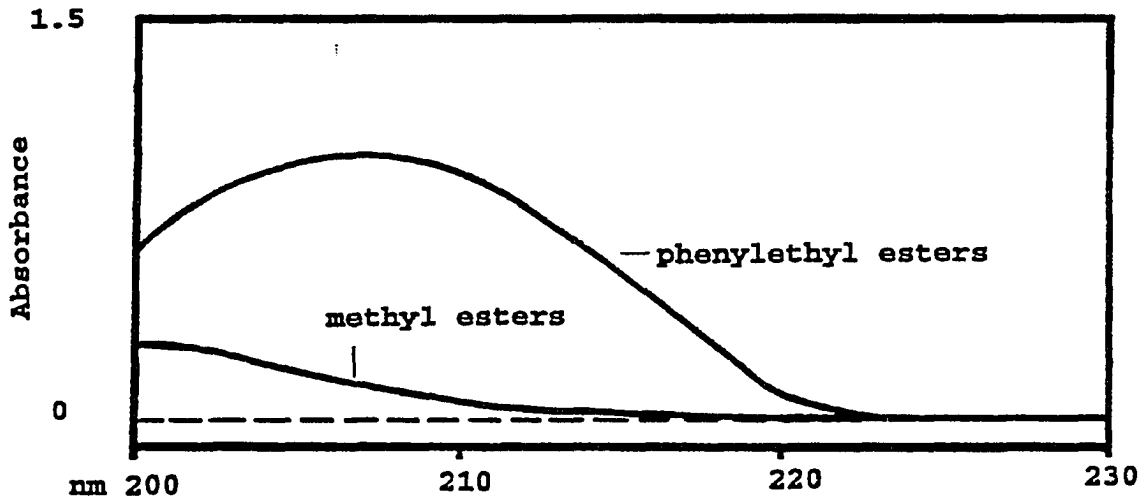

Figure 2. Spectrum of a 5 μM solution of soybean oil esters in methanol illustrating phenylethyl esters and methyl esters.

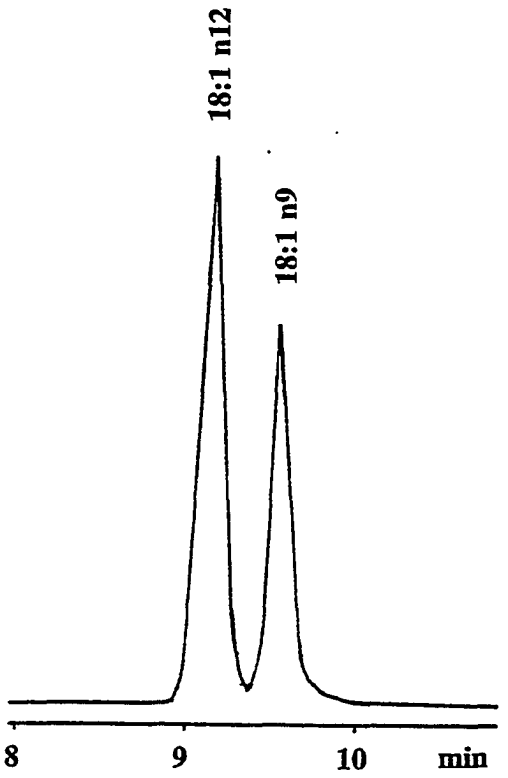

Figure 3. Separation of phenylethyl petroselinate and oleate on a DB-23 15-M capillary column (J&W, Deerfield, IL) operated isothermally at 220°C with helium as carrier gas at 1 mL/min

CHAPTER 3. ACCUMULATION OF PETROSELINIC ACID IN DEVELOPING SOMATIC CARROT EMBRYOS

A paper published in Phytochemistry [1]

Linsen Liu [2], Earl G. Hammond [2] and Eve Syrkin Wurtele [3]

Abstract

Developing somatic embryos of carrots accumulated up to 23 mg g^{-1} dry wt of petroselinate, most of which was incorporated into triacylglycerols. Both triacylglycerol and petroselinate accumulation in the embryos were increased by addition of abscisic acid to the culture medium.

Introduction

Carrots are domestic biennial plants cultivated for their roots. The seeds are rich in petroselinate, 6-*cis*-octadecenoate (18:1 n12), which can be oxidatively cleaved into the commercially important lauric and adipic acids. A survey of the 418 lines in the United States carrot collection showed that the petroselinate percentage in seed ranges from 55

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[1] Reprinted with permission from Phytochemistry 1993, 37, pp 749-753.

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(PI285861) to 88% (PI324241) of the total fatty acids (Liu and Hammond, unpublished results). Carrots may, therefore, provide a useful system for studying the factors that regulate petroselinic acid biosynthesis. It is impractical to use embryos dissected from maturing seeds of carrots to study petroselinic acid biosynthesis, but carrots are one of the few species in which cultured somatic cells can be induced to undergo embryogenesis at a high rate and thus provide large quantities of embryos at specific stages of development [1, 2]. Because the embryos are grown in culture, they are readily accessible to experimental manipulation [2]. However, success in inducing petroselinic acid synthesis during somatic embryogenesis has been very limited to date. Dutta and Appelqvist [3] reported the production of a maximum of 1.4% petroselinate in somatic embryos, but in a later study [4], their embryos produced no petroselinate.

This study describes the accumulation of petroselinate (up to 24% of the triacylglycerol fatty acids) in developing somatic embryos of carrot. The changes in fatty acid composition, triacylglycerol and phospholipid content during development and in response to abscisic acid (ABA) are reported.

Results and Discussion

Although somatic embryos mimic zygotic embryos in many aspects of their development; many proteins and other biomolecules used by the embryo as storage products may accumulate to lower levels [1-5]. To examine whether they accumulate petroselinate and, if so, begin to establish conditions that might maximize this accumulation, cultured

carrot cells were induced to form embryos by transfer to medium lacking the synthetic auxin, 2,4-D. The resulting somatic embryos accumulated petroselinate. In a preliminary experiment, petroselinate in the triacylglycerol fraction increased from 2% of the total fatty acids at 14 days after induction, to 16% at 18 days and then decreased to 12% at 23 days. The total triacylglycerol content in these embryos was 0.4-0.7% on a dry wt basis.

ABA is a plant growth regulator that can repress somatic embryo germination and promote maturation so that somatic embryos more closely mimic the maturation of zygotic embryos in seeds [5]. To investigate the influence of ABA on petroselinate accumulation in somatic embryos, carrot cells (cell line 49A) were induced to form embryos in media supplemented with ABA. Initial experiments were conducted to determine the range of ABA that should be added. In these experiments, the yield of embryos in medium containing 500 μM ABA was low (2.0 g fr.wt 500 ml^{-1} of medium, compared with 11.2 g fr. wt for the control after 14 days of culture), and the embryos were brown and abnormal in appearance; therefore, ABA levels of 100 μM or less were used in subsequent experiments to test the effects of ABA.

Cells were induced to form embryos by transfer to media lacking 2,4-D but containing 0, 10 or 100 μM ABA and analyzed at various times after induction. In medium containing no added ABA, embryos developed to the heart and torpedo stages by days 14 and 18 after induction; they had turned green at the shoot end and were beginning to germinate by day 23 after induction. In contrast, in the medium containing 10 μM ABA, most embryos were arrested in the heart to torpedo stages, and no embryos turned green.

Embryos in medium containing 100 μM ABA were generally similar in development and appearance to those growing in medium containing 10 μM ABA. Table 1A shows the yield (fresh and dry wt) of embryos per flask when cell lines 49A and GH1 were cultured in media containing 0, 10 or 100 μM ABA. The yield of embryos, by dry or fr. wt, decreased in response to 10 and 100 μM ABA. In contrast, the lipid yields g^{-1} fr. wt or dry wt (Table 1B) were highest in embryos growing in media containing ABA. Embryos growing in ABA contained 80-204 mg of triacylglycerol g^{-1} dry wt at 18 days after induction, an amount approaching that of the embryo within a seed. For the two cell lines, this value was 36 and seven times the level of triacylglycerol that had accumulated in embryos growing in medium lacking ABA 18 days after induction.

Table 1B shows the phospholipid content in the somatic embryos, which consisted mainly of phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol. The total phospholipid content ranged from about 5 to 11 mg g^{-1} dry wt in the embryos growing in medium without added ABA, but increased by about two-five fold in embryos growing in the presence of ABA. Even so, this overall increase was much less than that observed for the triacylglycerol fraction.

The fatty acid composition of the lipid fraction isolated from carrot embryos growing in 0, 10 or 100 μM ABA, respectively, was investigated at different times after induction of embryogenesis (Table 2 and 3). This changed both with development and with ABA levels in the media. The most abundant fatty acid ester in the triacylglycerols was linolenate (Table 2A and 2B), whose content ranged from 37.7 to 69% of the total fatty acids. Petroselinate

Table 1A. Yields of somatic carrot embryos grown in media containing various ABA concentrations for various time

Yields (g 500 ml^{-1})	Ages (days)*	Carrot cell line					
		49 A			GH1		
		0	10	100	0	10	100
Fr. wt	0	0.25	NA	NA	0.3	NA	NA
	14	11.2	5	2.8	5.5	3.5	2.8
	18	14.5	6	2.9	9.4	5.6	4.1
	23	26.4	11	5.2	14	11.1	11
Dry wt	0	0.01	NA	NA	0	NA	NA
	14	1	0	0.3	0.5	0.4	0.3
	18	1.4	1	0.3	0.9	0.5	0.4
	23	2.6	1	0.5	1.4	1.1	1

*Days after induction of embryogenesis

NA = not applicable

Table 1B. Yields and amounts of triacylglycerol (TAG) and phospholipids (PL) in somatic embryos of carrot grown in media containing various ABA concentrations for various time

Yields (mg g^{-1} dry wt)	Ages (days)*	Carrot cell line					
		49 A			GH 1		
		0	10	100	0	10	100
TAG	0	1	NA	NA	1	NA	NA
	14	3	10	24.6	89	106	54.9
	18	2.2	18	80	30	204	157
	23	16	38	59.3	51	75	68.4
PL	0	1	NA	NA	3	NA	NA
	14	5.2	8	8.7	29	11.8	25.9
	18	6.7	10	25.3	25	83	121
	23	10.9	15	25.2	29	32.3	26.7

*Days after induction of embryogenesis

NA = not applicable

Table 2A. Fatty acid compositions (wt %) of triacylglycerol in 49A line of somatic carrot embryos of carrot grown in media containing various ABA concentrations for various time

ABA (μM)	Age (day)	16:0	18:0	18:1n12	18:1n9	18:2	18:3	Others
0	0	31	-†	-	-	69	-	-
	14	14.6	-	8.4	8.5	59.1	5.3	tr*
	18	20.4	5.3	20.5	5.1	45.2	3.4	0.1
	23	14.8	5.4	4.5	21	47	4.8	2.5
10	14	17.7	6.1	14.7	7.1	53.8	tr	tr
	18	20.7	6	19	8.1	44.8	1.5	tr
	23	16	5.4	14.4	14.7	45.2	2.9	1.6
100	14	17.6	5.6	12.5	15.2	49	tr	tr
	18	22.2	5.9	14.2	19.5	37.8	0.5	tr
	23	19.6	5.9	24.1	10.4	37.7	0.9	1.3

* < 0.1

† Not detected

Table 2B. Fatty acid compositions (wt %) of triacylglycerol in GH1 line of somatic carrot embryos of carrot grown in media containing various ABA concentrations for various time

ABA (μM)	Age (day)	16:0	18:0	18:1n12	18:1n9	18:2	18:3	Others
0	0	16.5	2.3	1.9	6.6	63.6	3.7	5.3
	14	19.8	6.1	9.6	6.9	63.5	2.2	1.9
	18	19.9	6.8	10.2	6.8	52.9	1.2	2.1
	23	18.1	5	5	9.7	57.7	2.1	2.4
10	14	17.6	5.8	13.8	10.9	48.2	1.3	2.4
	18	16.5	2.7	11.4	9.3	57	1.7	1.3
	23	17.5	5.1	11.4	10.4	51.5	1.3	2.8
100	14	13.6	5	12.2	11.3	53.6	1.6	2.8
	18	12.3	3	8.7	12	60	1.9	2
	23	15.2	4.5	9.2	14.6	52.8	1.1	2.7

was also incorporated into triacylglycerols in all media and at all stages of development, and ranged from 1.9 to 24.1% of the total fatty acids. In contrast, it was incorporated into the phospholipids at very low levels (Table 3A and 3B), with small amounts found in both phosphatidylcholine and phosphatidylethanolamine. The highest petroselinate yield for line 49A was 14.3 mg g^{-1} dry wt at 23 days after induction in embryos growing in medium containing 100 μM ABA; for line GH1 the corresponding value was 23 mg g^{-1} dry wt at 18 days and 10 μM ABA.

Our results are the first report of petroselinic acid accumulation as a significant component of triacylglycerols in carrot somatic embryos. ABA has been shown to increase the levels of storage proteins in cultured zygotic embryos and in somatic embryos [5], and several reports indicate that triacylglycerol contents may also be increased [3,6,7]. Our data indicate that the levels of both total triacylglycerol and petroselinate increased in somatic embryos of carrot in response to ABA addition to the growth medium. The yield of petroselinate dry wt^{-1} of embryo was related to the amount of triacylglycerols in the embryos, but the proportion of petroselinate in the fatty acids varied independently from the amount of triacylglycerols present. Only in the more mature embryos (day 23) did addition of ABA to the medium cause a substantial increase in the percentage of petroselinate in the triacylglycerols. Whether this difference in fatty acid composition was caused by increased synthesis or decreased turnover of petroselinate relative to other fatty acids is not known. It is possible that the effect of ABA on petroselinate and triacylglycerol accumulation is in part an indirect consequence of ABA arresting embryo development in the heart to young torpedo

Table 3A. Fatty acid compositions (wt %) of phosphatilipids in 49A line of somatic carrot embryos of carrot grown in media containing various ABA concentrations for various time

ABA (μM)	Age (day)	16:0	18:0	18:1n12	18:1n9	18:2	18:3	Others
0	0	29.1	-†	-	-	69.5	-	1.4
	14	25	4.2	1.8	4.1	56.3	5.9	2.5
	18	27.3	3.7	0.6	2.9	57.3	6.6	0.7
	23	23.8	3.3	1.1	3.5	59.2	8.3	0.7
10	14	31	4.2	1.1	4.3	53.9	4.4	1.1
	18	31.4	2.2	tr	4	51.3	4.8	tr*
	23	26.5	2.6	1.7	4.4	59.2	4.6	0.9
100	14	27.2	3	1.1	4.4	60.1	4	tr
	18	29.1	3.3	2	9.5	52.8	2.4	0.9
	23	27	2.7	2.7	6.4	56.7	3.4	1

* < 0.1

† Not detected

Table 3B. Fatty acid compositions (wt %) of phosphatilipids in GH1 line of somatic carrot embryos of carrot grown in media containing various ABA concentrations for various time

ABA (μM)	Age (day)	16:0	18:0	18:1n12	18:1n9	18:2	18:3	Others
0	0	27.2	1.2	-†	2.2	61.8	-	7.6
	14	27.8	3.5	0.5	2.9	59.9	3.5	1.8
	18	24.9	1.9	0.5	5.3	62.5	3.6	1
	23	25.1	2.9	0.6	4.5	61.3	3.4	2.1
10	14	26.8	3	1.5	5.5	57.5	3.5	2.1
	18	23.2	1.3	0.3	3	67.9	3.5	0.8
	23	24.3	2.3	0.9	4.8	62	3.5	2.2
100	14	27.8	2.9	1	4.9	58.2	2.8	2.3
	18	22.4	1.6	0.1	3.4	67.5	4	1.1
	23	20.6	2.1	0.7	9.7	62	3	1.8

† Not detected

stages, since the latter state is when oil accumulation begins. The greatest accumulation of triacylglycerol and petroselinate in somatic embryos approached that found in zygotic embryos of mature carrot seeds. The failure of Dutta and Appelqvist [3,4] to produce significant amounts os petroselinate in somatic embryos of carrots is attributed to minor changes in culture conditions. For example, in our hands, the size of cell clusters, cell density and the past history of the cells used as an inoculum all have a substantial effect on the ability of the embryos to accumulate lipids. Somatic embryogenesis of carrot can, therefore, provide a useful system for the study of the regulation of petroselinate biosynthesis.

Experimental

Cell culture. Cell cultures were initiated from roots of wild carrot (*Daucus carota* L.) seedlings and maintained in liquid culture in MS medium [8] containing 0.5 mg l^{-1} of the exogenous auxin, 2,4-D [9]. Two cell lines, 49A and GH1, each from a different genotype, were established, Induction medium was similar to maintenance medium, but without the presence of 2,4-D. The induction medium was autoclaved, and ABA (mixed isomers from Sigma) was added aseptically at 0, 10 or 100 μM.

To induce embryo formation, suspension cells were transferred at a density of 0.5 mg fr. wt of cell ml^{-1} induction medium [10]. The somatic embryos were harvested at 14, 18 and 23 days after induction. Embryos from individual culture flasks were harvested and weighed for use in lipid determinations. Dry wts were obtained by determining the residual wt of a

known fr. wt of embryos after heating the embryos in a vacuum oven (20 mm Hg) at 70°C for 5 hr.

Lipid extraction and analyses. Methods recommended by Christie [11] were modified as follow: 1-10 g fr. wt somatic embryos or cells were soaked in boiling i-PrOH (1:3, w/v) for 5 min, crushed in a mortar, centrifuged (5125 g for 2 min), with the supernatant retained. The solid residues were re-suspended in $CHCl_3$-MeOH (1:3, w/v), mixed with a vortex mixer and centrifuged as before. The solids were extracted twice more with $CHCl_3$-MeOH (2:1). The supernatants were combined and evaporated to near dryness in vacuo, with the residual lipids dissolved in 80 ml $CHCl_3$-MeOH (2:1), and 20 ml 1% NaCl (w/v) in H_2O added. The upper phase was removed, and the $CHCl_3$ layer was washed once with a quarter of its vol. of MeOH-H_2O (1:1). The $CHCl_3$ layer was concentrated to 100-300 µl and applied to a 20 x 20 x 0.05 cm silica G TLC plate that had been activated at 120°C for 2 hr. The TLC plate was developed in hexane-Et_2O-HOAc (70:30:1). The phospholipid classes that were present were separated by TLC in $CHCl_3$-MeOH-HOAc-H_2O (25:15:4:2). Triacylglycerol and phospholipid bands were visualized by comparison with standards after spraying with 0.1% (w/v) 2',7'-dichlorofluorescein in MeOH. The lipids on the silica gel were directly transesterified to 2-phenylethyl esters by heating the silica gel in 2-6 ml of 1.5% sulfuric acid in 2-phenylethyl alcohol overnight at 45-50°C. Next, 0.5-1 ml H_2O was added, and 2-4 ml hexane was used to extract the esters. Heptadecanoic acid was used as an internal standard. PL contents were calculated from their fatty acid content as

phosphatidylcholine. Phenylethyl esters were separated and identified by GC equipped with FID and a J & W DB-23 fused-silica WCOT capillary column (15 m x 0.244 mm). The GC was operated in the split mode (split ratio 1:10) isothermally at 220°C. Helium was used as the carrier gas at a flow rate of 7 ml min^{-1}.

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References

1. Halperin, W. and Wetherell, D. F. (1964) Am. J. Botany 51, 274.
2. Wurtele, E. S., Wang, H., Durgerian, S., Nikolau, B. J. and Ulrich, T. H. (1993) Plant Physiol. 102, 303.
3. Dutta, P. C. and Appelquist, L. -A. (1989) Plant Sci. 64, 167.
4. Dutta, P. C. and Appelquist, L. -A. (1989) Plant Sci 75, 177.
5. Finkelstein, R. R. and Crouch, M. L.(1986) Plant Physiol. 81, 907.
6. Pence, V. C.(1991) Plant Physiol. 98, 1391.
7. Attree, S. M, Pomeroy, M. K. and Fowke L. C. (1992) Planta 187, 395.
8. Murashige, T. and Skoog, F. (1962) Plant Physiol. 15, 135.

9. Wurtele, E. S., Keller, G., Nikolau, B. J. and Ulrich, T. U. (1988) J. Plant Physio. 132, 683.

10. Keller, G., Nikolau, B. J., Ulrich, T. U. and Wurtele, E. S. (1988) Plant Physiol. 86, 451.

11. Christie, W. W. (1982), Lipid Analysis, 2nd ed, pp. 22-23. Pergamon Press, Oxford.

CHAPTER 4. THE BIOSYNTHESIS OF VERNOLIC ACID IN THE SEED OF *VERNONIA GALAMENSIS*

A paper to be submitted to the journal of Lipids

Linsen Liu [1], Earl G. Hammond [1] and Basil J. Nikolau [2]

Abstract

The biosynthesis of vernolic (12, 13-epoxy-*cis*-9-octadecenoic) acid in *Vernonia galamensis* was investigated by incubating developing seed with [1- ^{14}C] acetate in 0.1 M phosphate buffer, pH 7.2. Time course and pulse chase experiments were conducted. Vernolate was synthesized and incorporated into TAGs within 30 min., but ^{14}C-labeled vernolate accounted for only about 1% of the radioactivity in PC and was undetectable in DAG. The results suggested that linoleate is the apparent precursor of vernolate and linoleoyl PC might be the substrate for epoxidation.

Introduction

Epoxidized fatty acids are widely used as plasticizers (Gunstone, 1979; Smith, 1979; Ayorinde et al., 1990) and are produced by the treatment of unsaturated esters with peracetic acid. This is a relatively expensive process, and it has been suggested that plant seed oils

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that contain vernolic (12,13-epoxy-cis-9-octadecenoic) acid could be substituted for chemical epoxidation. Only a few plants contain large percentages of vernolate in their seed TAGs, and none of them are cultivated presently in the United States. *Vernonia galamensis*, whose seed contains 40% oil of which 80% is vernolate, has been successfully cultivated in Africa (Anonymous, 1991), but little has been done to improve the plant's agronomic properties. An alternative to growing *V. galamensis* as a cultivated plant is to introduce genes for producing vernolate into an oilseed crop such as soybean. A prerequisite to such a transfer is a better understanding of the biosynthetic pathway for producing vernolate.

Epoxidation related to the biosynthesis of cutin polymers has been studied extensively in plants, and free fatty acids have been identified as substrates (Blee and Schuber, 1990, Bafor et al., 1993). Little is known about epoxydation in oilseeds. Bafor et al. (1993) studied the formation of vernolate in the microsomes of *Euphorbia lagasca* and postulated that linoleoyl PC was the substrate. The resulting vernoloyl PC was hydrolyzed to give free vernolic acid, which was incorporated into TAGs after activation as a CoA ester. In this report, we present an exploration of the biosynthesis of vernolate in the seeds of *V. galamensis* using [1-^{14}C]acetate.

Materials and Methods

Materials

V. galamensis was grown from seeds in a green house under 14-h artificial illumination for 90 d followed by 10-h illumination for 30 d to promote flowering. Seeds

were collected 21 days after flowering (DAF), were expelled from their seed coats and kept in 0.1 M pH 7.2 phosphate buffer at 0°C until used.

Sodium [1-^{14}C]acetate (55 Ci/mol) solution was purchased from ICN (Irvine, CA). Chemicals and biochemicals were obtained from Sigma (St. Louis, MO) and Fisher Chemicals (Pittsburgh, PA).

In vivo radioisotopic labeling

In the time-course experiments, ~ 6 g of decoated seeds were incubated at room temperature (~ 25°C) while shaking at 100 rpm in 2 mL of 0.1 M phosphate buffer (pH 7.2) containing 10 μCi of sodium [1-^{14}C]acetate. At 0.25, 0.5, 1, 2 and 4 h time intervals after the star of the incubation, ~ 1 g of seeds was removed and washed three times with 3 mL of ice-cold water and heated for 15 min with 1 mL of water in a boiling water bath (Christie, 1982). The inactivated seeds were homogenized to extract lipid immediately or otherwise stored at -30°C until lipid extraction.

For the pulse-chase experiments, ~ 7 g of decoated seeds were incubated as above in the presence of 20 μCi of [1-^{14}C] acetate for 1 h. Following this pulse of the radioactive label, the buffer containing the radioisotope was removed, and ~1 g of seeds was washed and heated in water as described. The remaining seeds were washed three times with 5 mL of 10 mM sodium acetate in phosphate buffer, and incubated as before in 2 mL of fresh phosphate buffer in the absence of the radioisotope. At various times after the start of the chase incubation, about one-gram sample of seeds was removed, processed and stored as described.

Lipid extractions

The seeds were homogenized with 1 mL of methanol in a 7-mL Broeck Tissue Grinder (Fisher Scientific, Pittsburgh, PA). The homogenates were placed in 2-dram vials and centrifuged. The supernatant was removed, and the residual solids were extracted twice with 2 mL of chloroform-methanol (2:1, v/v) and once with 2 mL of chloroform. The extracts were combined with 0.9 mL of water, and the chloroform layer, which contained the lipids, was recovered and concentrated by evaporation under reduced pressure at ambient temperature. The recovered lipids were dissolved in 1 mL of chloroform-methanol (2:1) and stored under nitrogen at -30°C.

Lipid separations and analyses

Lipid classes were fractionated by TLC on silica gel H plates (200 x 20o x 0.5 mm) impregnated with 0.8 mM sodium carbonate (5). Polar lipids were fractionated with the solvent chloroform-methanol-acetic acid-water (25:15:8:4, v/v/v/v). Neutral lipids were fractionated with hexane-diethyl ether-acetic acid (60:40:1, v/v/v). These fractionations were undertaken either on separate plates, or by double development. In the latter, the polar lipids were first fractionated by developing the plate half way, the solvent was evaporated under carbon dioxide, and the neutral lipids, which were in the solvent front, were separated by developing the plate all the way with the second solvent. Following chromatography, the solvents were removed under carbon dioxide, and the lipid bands were located by blowing the vertical edges of the plates with iodine vapor.

For fatty acid analysis, each lipid band was scraped from the TLC plates and derivatized to methyl esters without removal of the lipids from the silica gel (4). The derivatization reactions were carried out by mixing the collected silica with 1 mL of diethyl ether and 0.5 mL of 1 M sodium methoxide in methanol and shaking for 1 h at ambient temperature. The reactions were terminated by adding 20 μL of glacial acetic acid and 1 mL of water, and the esters were recovered by extraction with diethyl ether. The ether was evaporated under carbon dioxide, and the residue was dissolved in 0.1-0.3 mL of methanol. The methyl esters were analyzed immediately after derivatization by HPLC on a Beckman (Fullerton, CA) System Gold fitted with a 4 mm x 25 cm Supelco C-18 column (State College, PA) using 5% water in methanol as the solvent. The eluate was monitored with on-line Beckman 166 UV and Beckman 171 radioactivity detectors equipped with a 1-mL cell using Ready Flow III (Beckman Instruments) as the on line scintillation cocktail. The elution of the methyl esters was detected by the absorbance at 202 nm. The methyl ester peaks were identified by comparing their retention volume with those of standards.

The fatty acid compositions of lipids from developing and mature seeds were analyzed as methyl esters, which were prepared by reacting the lipids with 1.0 M sodium methoxide in methanol (5). The esters were analyzed by gas chromatography on a Hewlett Packard (Wilmington, DE) 5890 instrument fitted with a flame ionization detector and a 15-m X 0.244 mm J & W (Deerfield, IL) DB-23 capillary column operated 200°C with a helium flow of 1 mL/min. Peaks were identified by comparing retention times with those of standards.

Determination of radioactivity

An aliquot of lipid sample was mixed with 4 mL of ScintiVerse BD (Fisher Scientific, Pittsburgh, PA) in 4-mL polypropylene vials and radioactivity detected on a Packard 2200CA Scintillation Analyzer (Downers Grove, IL). Lipid bands on TLC plates were revealed with iodine vapor, the lipid-containing silica was scraped into vials and radioactivity was determined as above.

Data treatment

Each sample was analyzed twice and each experiment was replicated. The mean of the four data was reported.

Results and Discussion

Lipid accumulation during seed development

In the green house conditions used in these experiments *V. galamensis* seeds matured in 35 days after flowering (DAF). During this period the lipid content of the seeds increased from 0 to 34% (Fig. 1). Furthermore, most of the lipid accumulated in the period between 21 and 28 DAF. Thus, in the experiments described below, seeds of 21 DAF were used, this being the period when lipids started to rapidly accumulate. As shown in Table 1, vernolic acid occurs mainly in the neutral lipids, TAG and DAG, where it accounts for 61.6% and 59.3% of the total fatty acids, respectively. The only other lipid in which

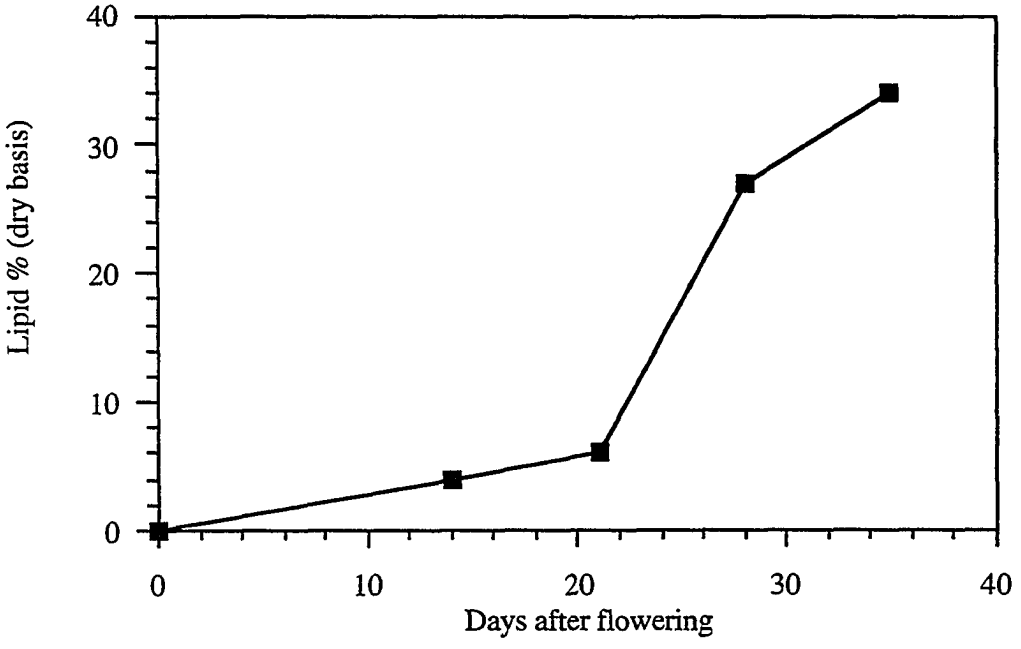

Figure 1. Lipid accumulation during growth in the seed of *Vernonia galamensis*

Table 1. The fatty acid compositions (wt%) of the various lipid fractions in developing seeds 21 days after flowering

Lipids	16:0	18:0	18:1	18:2	Vernolic
TAG	6.4	3.0	5.3	23.4	61.9
DAG	9.3	3.9	10.5	21.6	54.6
PC	15.5	5.9	13.7	59.4	5.3

vernolic acid was detected was PC, but only in small quantities (1.4%), in which the major fatty acid was linoleate.

In vivo labeling of seed lipids with [1-^{14}C]acetate

The results from *in vivo* labeling of lipids of developing seeds are presented in Fig. 2. [1-^{14}C]Acetate was rapidly incorporated into lipids in the first four hours of incubation (Fig. 2A), whereas in the subsequent 4 h incubation there was little increase of radioactivity either in total or individual lipids. PC was the most heavily radioactively labeled lipid throughout the incubation time. Initially DAG was more heavily radioactively labeled than TAG, however after 2-h radioactivity in DAG increased so that by 4-h TAG was the second most heavily radioactively labeled lipid. The only other lipids that incorporated radioactivity to any significant extent were PE and PI, which accounted for 3 to 6% of the radioactivity each and are not shown on the figure.

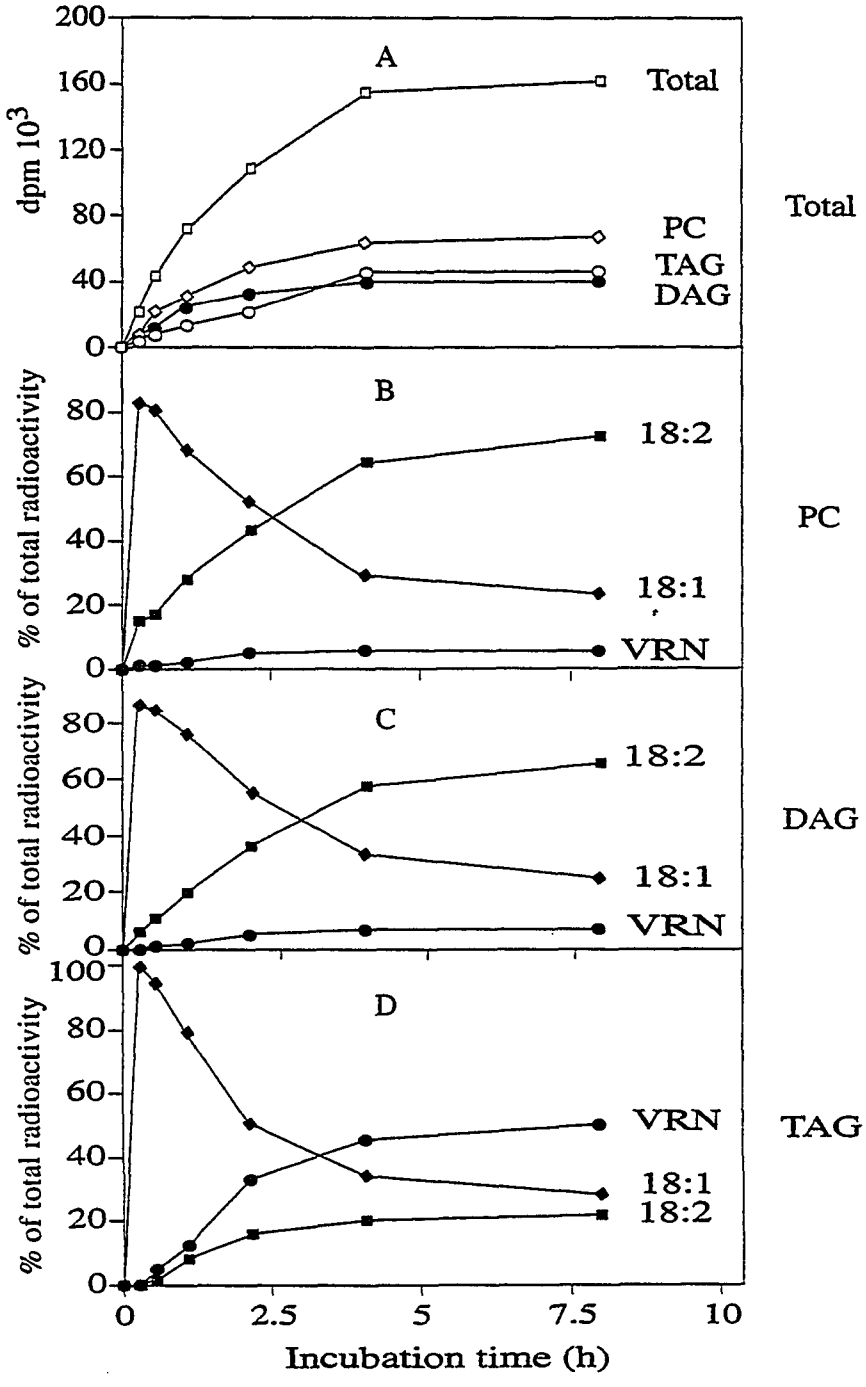

Figure 2. The incorporation of [1-^{14}C]acetate into the individual lipids and their fatty acids during the time course incubation. VRN: vernolate; 18:1: oleate; 18:2: linoleate.

The distributions of radioactivity in each acyl group of the various lipids were determined by transesterification of each isolated lipid to form the methyl esters, which were analyzed by radio-HPLC. The distributions of the radioactivity among the fatty acids of PE and PI are not shown because these lipids contained only a small proportion of the radioisotope and no vernolate was detected among them.

Oleic acid on PC (Fig. 2B) was the most heavily labeled fatty acid in the initial stages of the incubation. With increasing time of incubation, radioisotopes in the oleoyl moiety of PC declined and radioisotope was incorporated into first linoleoyl and then into vernoloyl moieties of PC. Indeed, vernolic acid first became radioactively labeled on PC (Table 2 and Fig. 2). This observation suggested that vernolate was synthesized in PC. Because linoleate was radioactively labeled earlier than vernolate, linoleate may be the precursor of vernolate. With longer time of incubation radioactive label was incorporated sequentially into DAG and TAG (1 h and longer), and the proportion of labeled linoleic and vernolic acids in these lipids increased (Fig. 2 C and D). In all these lipids, saturated fatty acids (16:0 and 18:0, not shown in the figure) were the least labeled (~ 1%) and did not change with the incubation times.

The fatty acid profile in DAG was similar to that of PC, which may indicate that the DAG pool involved in vernolic acid biosynthesis was derived from PC. In TAG, radioactively labeled vernolic acid increased steadily and finally became the major labeled fatty acid (45-50%) after 4-h incubation.

The results indicated that vernolate may be synthesized from linoleate which was

Table 2. The amount of radioactive vernolic acid (% of total radioactive fatty acids) among the lipids at different times of incubation of seeds with [1-^{14}C]acetate.

Lipids	Incubation time (h)					
	0.25	0.5	1	2	4	8
PC	0.7	0.6	1.8	4.5	5.4	3.1
DAG	-	1.1	1.9	5.5	7.2	7.2
TAG	-	3.5	11.4	32.3	45.3	50.0

derived from oleate on PC. Because no radioactive free fatty acids were detected and the fatty acid profiles of DAG and PC were quite similar, the vernolic acid was incorporated into TAG by DAG derived from PC by hydrolysis and/or also possibly through vernoloyl-CoA formed by ester interchange between acyl-CoA and PC.

In vivo pulse-chase experiments

To further investigate the biosynthesis of vernolic acid, decoated *V. galamensis* seeds were exposed to [1-^{14}C]acetate, and, after 1 h, the radioactive acetate was replaced with nonradioactive acetate and the incubation continued for an additional 8 h. The results from this experiment are shown in Fig. 3. Fig. 3A shows that the total amount of radioactive label recovered in lipids was relatively constant during the chase phase of the experiment.

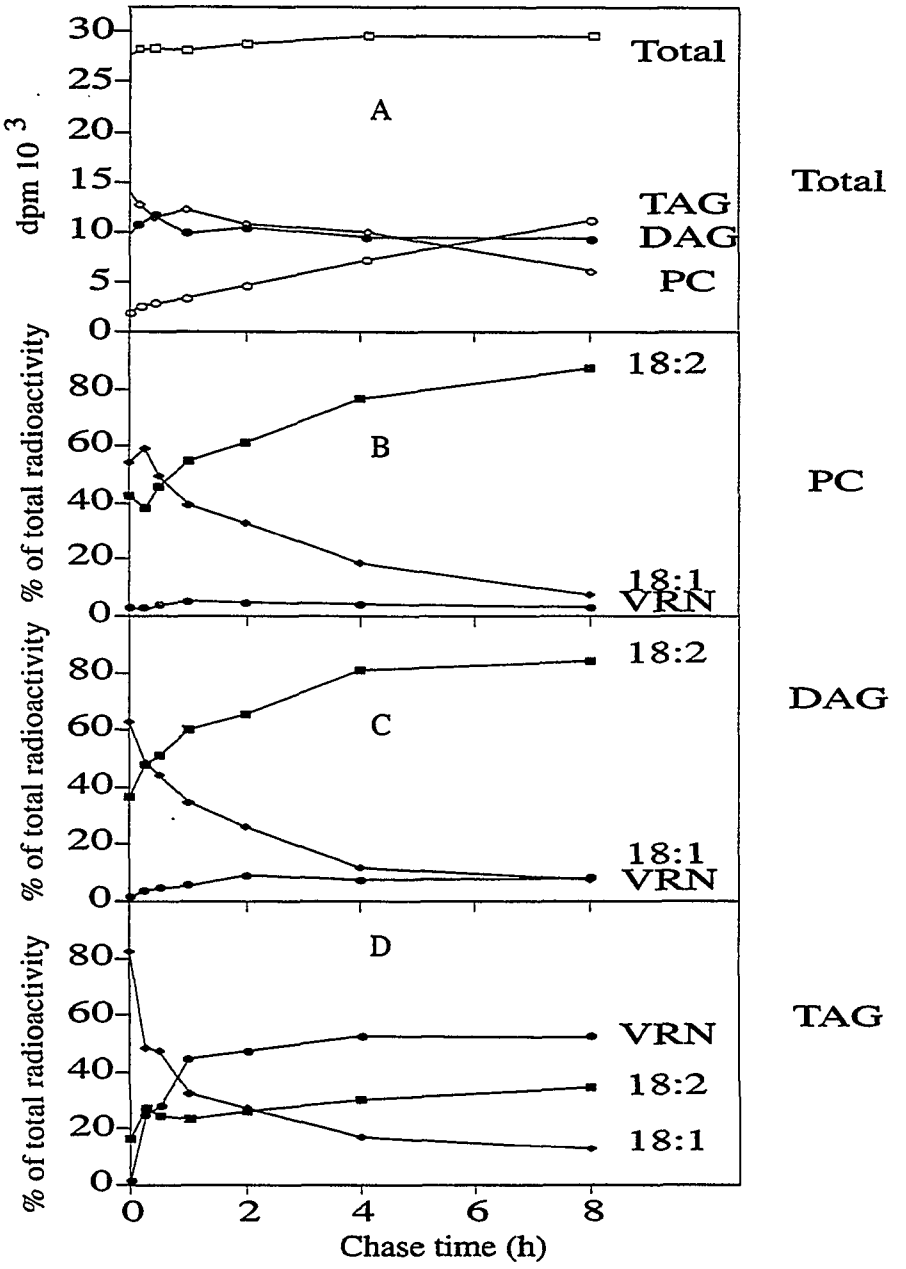

Figure 3. The incorporation of [1-^{14}C]acetate into the individual lipids and their fatty acids during the chase time. VRN: vernolate; 18:1: oleate; 18:2: linoleate.

A little increase during the early incubation might be the continuous incorporation of short chain intermediates formed during radioactive label incubation. PC was the most heavily labeled lipid at the beginning of the chase. The amount of radioactive label in PC declined steadily with time disappearing from the PC appeared in the TAG, which finally became the most heavily labeled lipid. Radioactivity in DAG was constant over time.

The distribution of radioactivity in each acyl group of the various lipid classes was consistent with the results of the experiment shown in Fig. 2. Oleic acid on PC (Fig. 3B) was initially the most heavily labeled fatty acid. With an increase of chase time, the oleoyl moiety lost radioactivity and the linoleoyl moiety in PC continuously gained radioactivity. Radioactive vernolate on PC increased at the early stages of the chase and then slightly declined at the later stages; the amount of radioactive vernolic acid in PC was always low (less than 5%). Phosphatidyl -ethanolamine (PE) and -inositol (PI) were found to have few radioactive labels and the amount did not change much with the chase (data not shown).

As in the experiment shown in Fig. 2, the radioactive fatty acid profile of DAGs (Fig. 3C) also had a similar pattern to that of PC, which suggested that DAG was derived from PC by hydrolysis. In TAGs (Fig. 3D), the radioactivity of vernolate increased and became the most heavily labeled after about 1 h of chase. No free fatty acids were detected; therefore, vernolic acid was incorporated into TAG likely through the DAG and acyl-CoA pools. Saturated acids had few radioactive labels, quite similar to that in the incubation experiment (data not shown). The major radioactively labeled fatty acid in PE and PI was linoleic acid, with minor amounts of palmitic acid (data not shown).

Both experiments showed that the order in which lipids became radioactively labeled was from PC to TAG through DAG as the intermediate, and oleate was radioactively labeled first. Then linoleate and vernolate became radioactively labeled. Vernolate was first detected in PC. These results indicated that vernolate was formed on PC and accumulated in TAG through DAG as the intermediate. Linoleic acid derived from oleic acid seems to be the substrate for epoxidation. The results also show that the seed of *V. galamensis* has a well-developed system to keep vernolic acid from being incorporated into PC even though it might be formed on this lipid. Vernolate was preferentially accumulated into TAGs.

Conclusions

Radioactively labeled vernolic acid was first found in PC, which also was the chief labeled glycerol lipid. Oleate was initially the most heavily labeled fatty ester, but with time the radioactive label moved into linoleate and vernolate. The results suggested that the linoleoyl PC derived from oleoyl PC is the precursor of vernoloyl PC.

References

1. Anonymous (1991) U.S. Efforts focus on variety of new crops. *Inform* 2, 685-692.
2. Ayorinde, F. O., Butler, B. D., Clayton, M. T. (1990) Vernonia galamensis: A rich source of epoxy acid. *J. Am. Oil. Chem. Soc.* 67, 844-845.
3. Bafor M, Smith, M.A., Jonson, L., Stobart, K., Stymne, S. (1993) Biosynthesis of

vernolate (cis-12-epoxyoctadeca-cis-9-enoate) in microsomal preparations from developing endosperm of *Euphorbia lagacase. Arch. Biochem. Biophys.* 303, 145-151.

4. Blee, E., Schuber, F. (1990) Efficient epoxidation of unsaturated fatty Acids by a hydroperoxide-dependent oxygenase. *J. Biol. Chem.* 265, 12887-12894.

5. Christie, W.W. (1982) *Lipid Analysis*, 2nd ed., Pergamon Press Inc, New York, pp 17-23.

6. Gunstone, F. D. (1979) The formation and reactions of long-chain epoxy acids. in *Fatty Acids*, E. H. Pryde, ed., American Oil Chemists' Society, Champaign, IL, pp 379-390.

7. Krewson, C. F., Ard, J. S., Riemenschneider, R. W. (1962) *Vernonia anthelmintica (L)* Willd. Trivernolin, 1, 3-divernolin and vernolic (epoxyoleic) acid from the seed oil. *J. Am. Oil. Chem. Soc.* 39, 334-340.

8. Ohlrogge, J. B., Browns, J., Somerville, C. (1991) The genetics of plant lipids. *Biochem. Biophys. Acta* 1082, 1-26.

9. Skipski, V. P., Peterson, R. F., Barclay, M. (1964) Quantitative analysis of phospholipids by thin-layer chromatography. *Biochem. J.* 90, 362-378.

10. Smith, C. R. (1979) Unusual seed oils and their fatty acids. in *Fatty Acids*, E. H. Pryde, ed., American Oil Chemists' Society, Champaign, IL, pp 29-47.

CHAPTER 5. THE BIOSYNTHESIS OF α-ELEOSTEARIC ACID IN THE SEED OF *MORMODICA CHARANTIA* L.

A paper to be submitted to the journal of Plant Physiology

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Abstract

Time-course and pulse-chase radioactive labeling experiments using sodium [1-^{14}C]-acetate, ammonium [1-^{14}C]oleate and ammonium [1-^{14}C]linoleate were conducted to reveal the biosynthetic pathway for the formation of α-eleostearic acid in the seed of *M. charantia*.

In [1-^{14}C]acetate labeling, radioactively labeled α-eleostearate was first found in PC, which also was the chief radioactively labeled glycerolipid. Oleate was initially the most heavily labeled fatty acid, but with time the radioactive label moved into linoleate and α-eleostearate. The results indicated that linoleoyl PC is the precursor of α-eleostearoyl PC. Results with [1-^{14}C]oleic and [1-^{14}C] linoleic acids were consistent with this conclusion.

Introduction

The most common trienoic fatty acid that occurs in plants is linolenic acid (*cis-9, cis-12, cis*-15-octadecatrienoic acid). This compound is the most prevalent fatty acid of the

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photosynthetic membranes of chloroplast, and occurs at various levels in the seed oils of many plants. In particular, linolenate is the major fatty ester in the seeds of linseed, from whence it derives its name. In a limited number of plant families, an isomer of linolenic acid, α-eleostearic acid (*cis*-9, *trans*-11, *trans*-13-octadecatrienoic), accumulates to high levels in the seed oil. Typically 77-86% of the fatty acids of the seed oil from the tung tree is α-eleostearic acid. The three conjugated double bonds make α-eleostearic acid readily susceptible to oxidation and polymerization. Thus, oils containing α-eleostearic acids, such as tung oil, are prized as a drying oil and can be used as constituents of specialty surface coatings (1).

Crombie (2, 3) studied the biosynthesis of calendic (*cis, cis, trans*-8, 10, 12-octadecatrienoic) acid in marigold, and Noda (4) briefly investigated the precursor of eleostearic acid. But, the information on biogenesis of these two conjugated fatty acids is still limited. This manuscript describes *in vivo* radiotracer studies of the biosynthesis of α-eleostearic acid in the developing seeds of the bitter gourd, *M. charantia* (6).

Materials and Methods

Materials

Plant. *M. charantia* was grown from seed in a green house under 14-h artificial illumination at 22-25°C. Under these conditions, plants flowered 4 weeks after planting and the flowers were pollinated manually. The fruits matured 30-35 days after pollination (DAP), and seeds from 16-day-old fruits were used in radioactive labeling experiments.

Seeds were dissected from the fruit, the seed coat was removed, and the developing seeds were kept on ice in 0.1 M phosphate buffers (pH 6.65) until use.

Radioisotopes. Sodium [1-^{14}C]acetate (55 Ci/mol) in an ethanol solution was purchased from ICN (Irvine, CA). Chemicals and biochemicals were obtained from Sigma (St. Louis, MO) and Fisher Chemicals (Pittsburgh, PA). [1-^{14}C]Oleic and linoleic acids in toluene (50 Ci/mol) were from Amersham Life Science (Arlington Heights, IL). The ammonium salts of the fatty acid were prepared by evaporating the toluene from 0.5 mL of radioactive fatty acid solution and heating the residual with 0.2 mL of 2 M NH_4OH at 60°C for 10 min. Nitrogen was bubbled through the resulting solution to remove residual ammonia.

***In vivo* radioisotopic labeling**

Thirty-six developing seeds (approximately 6 g) were incubated at room temperature (~ 25°C) in 2 mL of 0.1 M phosphate buffers (pH 6.65) containing 10 μCi of the radio labels: sodium [1-^{14}C]acetate, ammonium [1-^{14}C]oleate or ammonium [1-^{14}C]linoleate. At various times after the start of the incubation, six seeds were washed three times with 1 mL of ice-cold water and heated in 1 mL of water for 15 min in a boiling bath of water to inactivate phospholipases. The heated seeds were stored at -30°C until the lipid was extracted.

For pulse-chase experiments, 42 seeds (~ 7 g) were incubated as above in the presence of 20 μCi of radio labels: sodium [1-^{14}C]acetate or ammonium [1-^{14}C]linoleate for 1 h.

Following this pulse of radioactive label, the buffer containing the radioisotope was removed, and a six-seed sample was washed and heated in water as described. The remaining seeds were washed three times with 5 mL of 10 mM sodium acetate in phosphate buffer and incubated as before in 2 mL of fresh buffer without the radioisotope. At various times after the start of the chase incubation, ~ 1 g samples of seeds were removed, processed and stored as described.

Lipid extractions (7)

The seed was homogenized with 1 mL of methanol in a 7-mL Broeck Tissue Grinder (Fisher Scientific, Pittsburgh, PA). The homogenate was centrifuged, the supernatant was removed and the residual solid was extracted twice with 2 mL of chloroform-methanol (2:1, v/v) and once with 2 mL of chloroform. The extracts were pooled and combined with 0.9 mL of water. The chloroform layer containing the lipids was recovered and concentrated by evaporation under reduced pressure at ambient temperature. The recovered lipid was dissolved in 1 mL of chloroform-methanol (2:1) and stored under nitrogen at -30°C.

Lipid separations and analyses (7)

Lipid classes were fractionated by TLC on silica gel H plates (200 x 200 x 0.5 mm) impregnated with 0.8 mM sodium carbonate (5). Polar lipids were fractionated with the solvent chloroform-methanol-acetic acid-water (25:15:8:4, v/v/v/v). Neutral lipids were fractionated with hexane-diethyl ether-acetic acid (60:40:1, v/v/v). These fractionations

were undertaken either on separate plates, or by double development. In the latter, the polar lipids were first fractionated by developing the plate half way to the top, the solvent was evaporated under carbon dioxide, and the neutral lipids, which were in the solvent front, were separated by developing the plate all the way with the second solvent. This double development method was not suitable for analysis of fatty acid composition because of the ease with which α-eleostearate oxidized. Following chromatography, the developing solvents were removed under carbon dioxide, and the neutral lipid bands were located by spraying the vertical edges of the plates with 0.1 % (w/v) 2',7'-dichlorofloresein in methanol; similarly the polar lipids were detected with 0.01 % (w/v) aqueous Rhodamine 6G.

For fatty acid analysis, the silica gel containing each lipid was scraped from the TLC plates, and the fatty acids were directly converted to methyl esters without removal of the lipids from the silica gel (4). For the polar lipid, only the portions of the band in the unsprayed areas of the plates were taken for derivatization, to avoid the water in the Rhodamine 6G solution. The derivatization reactions were carried out by mixing the collected silica with 0.5 mL of 1 M sodium methoxide and incubating the mixture with shaking at 30-40°C for 1 h. The reactions were terminated by adding 1 mL of water, and the methyl esters were recovered by extraction with hexane. The hexane was evaporated under carbon dioxide, and the residue was dissolved in 0.1-0.3 mL of methanol. The methyl esters were analyzed immediately after derivatization by HPLC on a Beckman System Gold (Fullerton, CA) fitted with a 4-mm x 25 cm Supelco C-18 column (State College, PA) with 5% water in methanol or acetonitrile as the mobile phase solvents. The eluate was monitored

at 202 nm with an on-line Beckman Model 166 UV detector and for radioactivity with a Beckman 171 detector equipped with a 1-mL cell using Beckman Ready Flow III as the on line scintillation cocktail. The methyl ester peaks were identified by comparing their retention volume with those of standards.

Because of the extreme susceptibility of lipids containing α-eleostearic acid to oxidation, the TLC fractionations of lipids and subsequent fatty acid analyses were undertaken without delay and with the minimal exposure of the samples to air.

The fatty acid compositions of lipids from developing and mature seeds were analyzed as methyl esters by gas chromatography on a Hewlett Packard 5890 (Wilmington, DE) fitted with a flame ionization detector and a 15-m x 0.244 mm J & W DB-23 (Deerfield, IL) capillary column operated 200°C with a helium flow of 1 mL/min. Peaks were identified by comparing retention times with those of standards.

Determination of radioactivity

A liquor of lipid sample was mixed with 4 mL of ScintiVerse BD (Fisher Scientific, Pittsburgh, PA) in 4-mL polypropylene vials and radioactivity detected on a Packard 2200CA Scintillation Analyzer (Downers Grove, IL). Lipid bands on TLC plates were revealed with iodine vapor, and the lipid-containing silica was counted.

Data treatment

Unless indicated, each sample was analyzed twice and each experiment was replicated. The mean of these four data was reported.

Results and Discussion

Lipid accumulation during seed development

In the green house conditions used in these experiments *M. charantia* fruit matured in 30-35 days after pollination. During this period, seeds grew from small flat ellipsoids (5 mm x 2 mm x 0.5 mm) at 14-d after pollination to full size (15 mm x 7 mm x 2 mm) by 28-d after pollination. Subsequently, the seeds were desiccated. During this period, the fresh weight of the seeds increased from 60 mg to 182 mg per seed. Table 1 illustrates that lipid constitutes nearly one third of the dry weight of the mature seed (33 days after planting). Furthermore, these data reveal that lipid accumulation was about 80% completed by 18 days after pollination. Thus, in the experiments described here, seeds between 14 and 18 days after pollination were used, this being the period when lipid was rapidly accumulating.

The fatty acid composition of the lipids isolated from mature seeds of *M. charantia* is very similar to the fatty acid composition of the lipids of seeds 16-d after pollination (Table 2). α-Eleostearate accounts for nearly two-thirds of the fatty acids of these seeds, and stearic acid is the other major fatty acid. Table 2 also presents the fatty acid composition of the various lipid fractions in developing seeds, 16 d after pollination. α-Eleostearate occurs mainly in the neutral lipids, TAG and DAG, where it accounts for 62.2% and 29.6% of the

Table 1. Growth and lipid accumulation in developing *M. charantia* seeds

Days after flowering	Seed weight (mg)		Lipid content (%)
	Dry	Fresh	(dry basis)
14	5.9	60.0	15
18	15.8	165.2	26
28	74.8	181.6	31
38	86.0	161.0	33

Table 2. The fatty acid compositions (wt %) of lipids from the seed of *M. charantia* 16 days after pollination

Lipid	16:0	18:0	18:1	18:2	α-Eleostearate
TAG	1.5	31.8	2.3	2.2	62.2
DAG	7.7	31.4	7.7	27.6	29.6
PC	11.4	5.0	13.3	68.6	1.7
PE	17.3	14.3	13.0	55.4	-
PI	12.1	26.1	10.8	50.9	-

fatty acids, respectively. The only other lipid in which α-eleostearate was detected was PC, but only in small quantities (1.7%). The other major fatty acid in TAGs and DAGs was stearate.

The three phospholipids found in these seeds, PE, PI and PC, had fatty acid intermediate between the fatty acid composition of TAGs and phospholipids. Indeed, these compositions that were distinct from the neutral lipids. Linoleate was the most abundant fatty acid in the phospholipids, constituting about one half to two-thirds of the fatty acids in these lipids. The PC was slightly more unsaturated than PI and PE, with a higher linoleate and lower stearate content in the former lipid. The fatty acid composition of the DAG was data are consistent with there being two metabolic pools of DAG, one which acts as an immediate precursor for TAG synthesis, and a second that is in equilibrium with the phospholipids. The later pool of DAG would be characterized by its content of linoleate, which is not incorporated into TAG, whereas the former DAG pool would contain α-eleostearate.

***In vivo* labeling of seed lipids with [1-^{14}C]acetate**

The *in vivo* radioactive labeling of lipids of developing seeds was undertaken in three separate experiments, with very similar results. The data from one such experiment are presented here (Fig. 1). As shown in Fig. 1A, [1-^{14}C]acetate was rapidly incorporated into lipids in the first hour of incubation, whereas in the subsequent 7-h incubation the rate of incorporation decreased by a factor of 20-fold. PC was the most heavily labeled lipid in the

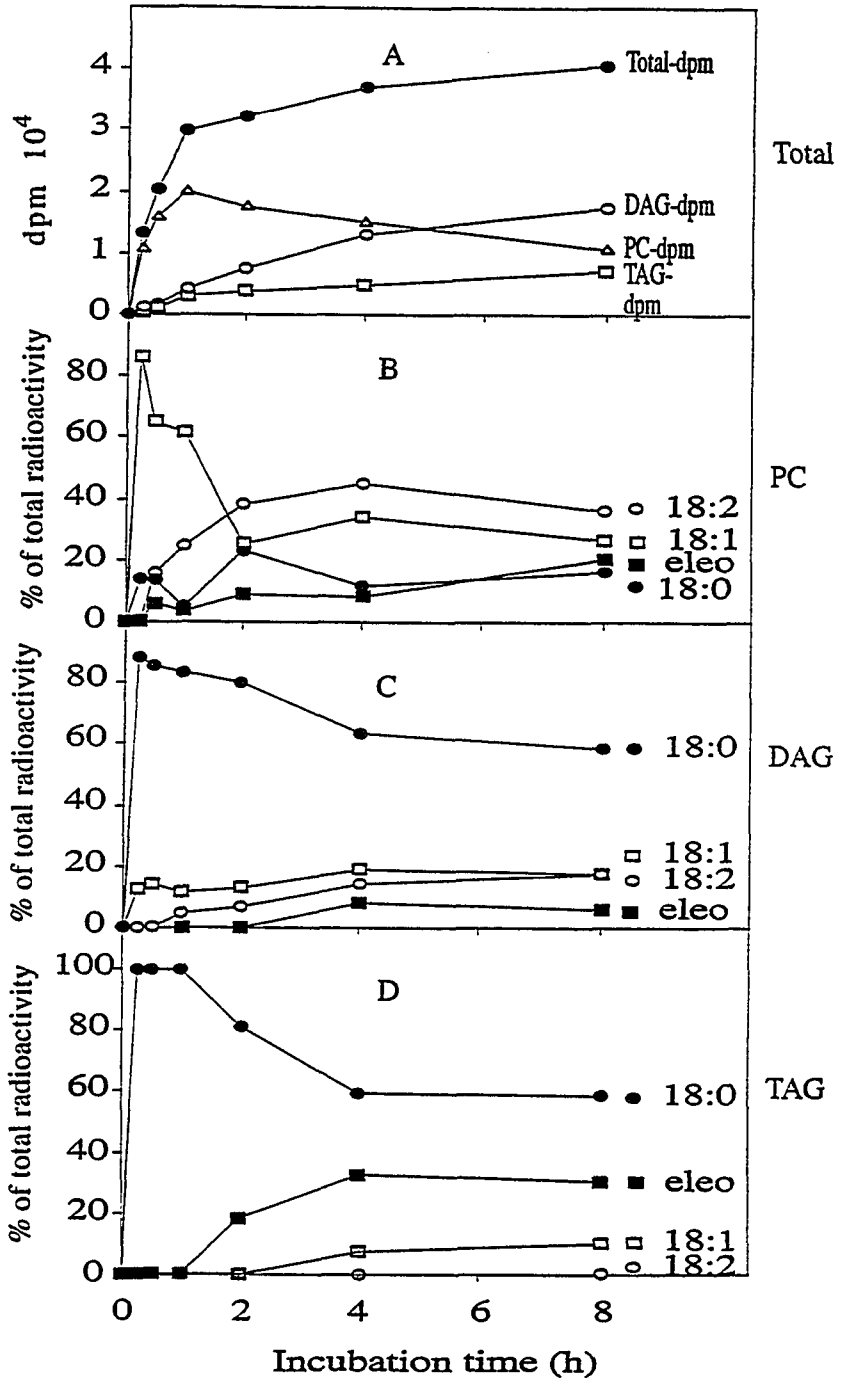

Figure 1: The incorporation of [1-^{14}C]acetate into the individual lipids and their fatty acids during the time course incubation. eleo: α-eleostearate; 18:0: stearate; 18:1: oleate; 18:2: linoleate.

initial time points of the incubation. Indeed, at the first time point (15 min), radioactivity in PC accounted for nearly 80% of the radioactive label incorporated into lipids. Subsequently, the proportion of radioactivity in PC steadily declined to 26% by 8 h. As radioactivity in PC declined, the proportion of radioactive label in DAGs and TAGs increased; but, DAGs were always more heavily labeled than TAGs (ratio of radioactivity in DAG:TAG ranged between 1.5 to 2.5). The only other lipids that incorporated radioactivity to any significant extent were PE and PI, which accounted for 3 to 6% of the radioactivity each (data not shown). PE and PI are not involved in this pathway.

The distribution of radioactivity in each acyl group of the various lipid classes was determined by transesterification of each isolated lipid class to methyl esters, which were analyzed by radio-HPLC. The distributions of the radioactivity among the fatty acids of PC, DAG, and TAG are shown in Fig. 1B, 1C and 1D. Those of the PE and PI are not shown because these lipids contained a small proportion of the radioisotopes, and no eleostearic acid was detected in them.

Oleate on PC was the most heavily labeled fatty acid in the initial stages of the incubation. With increasing time of incubation, radioisotopes in the oleoyl moiety of PC declined and radioisotopes were incorporated into first linoleoyl and then to eleostearoyl moieties of PC. Indeed, **α**-eleostearate first became radioactively labeled on PC. At longer incubation times (1 h and longer), the radioactive label was incorporated sequentially into DAG and TAG, and the proportion of radioactively labeled linoleate and **α**-eleostearate in these lipids increased. Stearate was the most heavily labeled fatty ester in the neutral lipids,

TAGs and DAGs, but did not contribute more than 25% of the acyl moiety of PC throughout the time points of the incubation. These results suggested that stearate was incorporated directly into TAGs through the Kennedy pathway without involving PC. On longer incubation, the proportion of radioactivity in stearate declined because radioactivity in other fatty esters increased. In DAG, oleate and linoleate became radioactively labeled prior to the incorporation of radioactive label in α-eleostearate. In TAG, α-eleostearate became radioactively labeled prior to oleate.

In vivo pulse-chase experiments with [1-^{14}C]acetate

To further investigate the biosynthesis of eleostearic acid, decoated *M. charantia* seeds were exposed to [1-^{14}C]acetate, and after 1 h, the radioactive acetate was replaced with nonradioactive acetate and the incubation continued for an additional 8 h. The results are shown in Fig. 2. Fig. 2A shows the incorporation of total radioactivity into the lipids of the seeds at various times after the radioactive label was removed, and the relative proportions in the various lipid classes. The total amount of radioactive label recovered in lipids was relatively constant during the chase phase of the experiment. PC was the most heavily labeled lipid at the beginning of the chase time. The amount of radioactive label in PC increased during the first hour of the chase and subsequently declined steadily for the remainder of the experiment. The radioactive label disappearing from the PC appeared in the DAG and TAG, with the former always containing a considerably greater proportion of radioactive label.

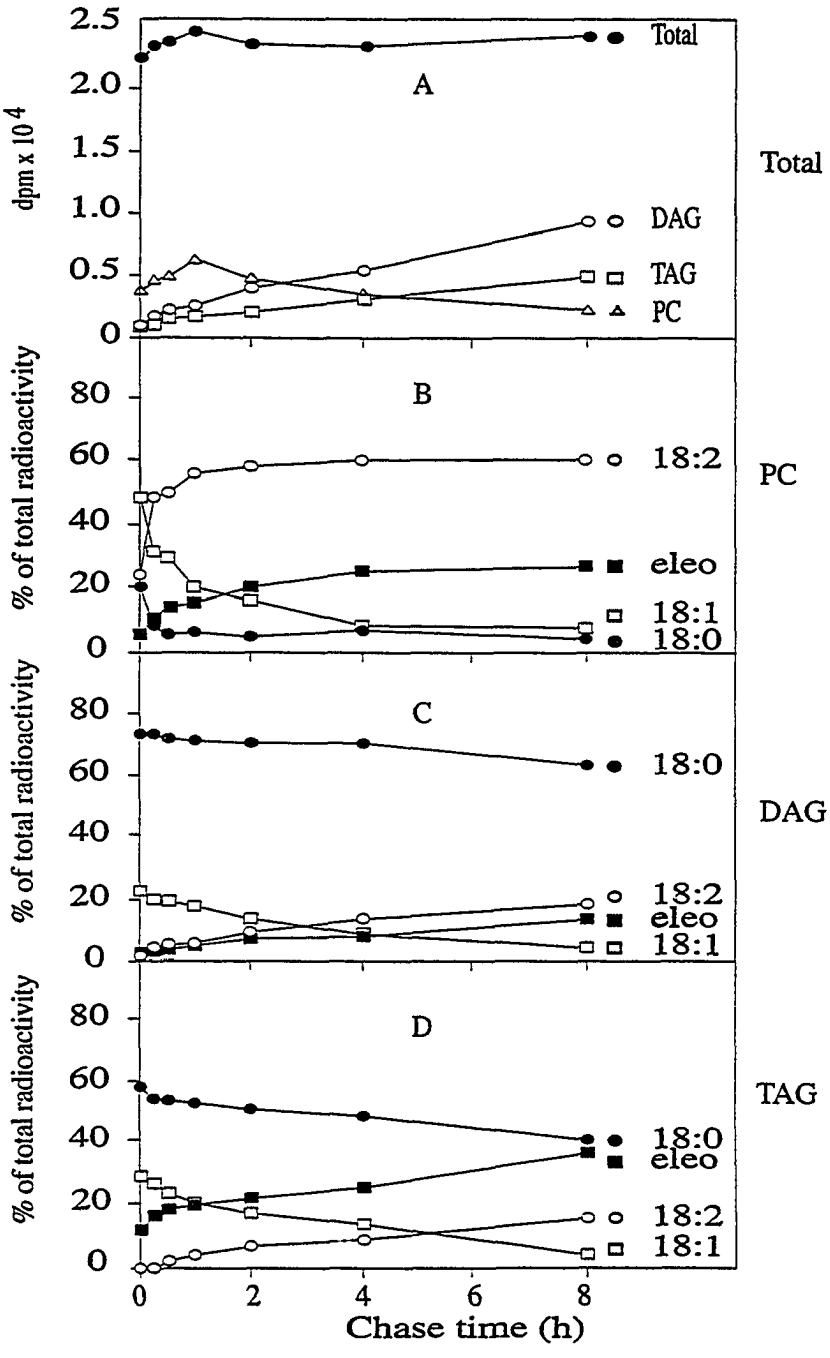

Figure 2: The incorporation of [1-^{14}C]acetate into the individual lipids and their fatty acids during the chase time. eleo: α-eleostearate; 18:0: stearate; 18:1: oleate; 18:2: linoleate.

The distribution of radioactivity in each acyl group of the various lipid classes was consistent with the results of the above incubation experiment. Oleic acid on PC (Fig. 2B) was initially the most heavily labeled fatty acid. With increasing chase time, the oleoyl moiety lost its radioactivity and linoleoyl and eleostearoyl moieties in PC gained in radioactivity. The radioactivity of the stearoyl moiety declined in the first 0.5-h incubation and then stayed constant. In TAGs and DAGs, the radioactivities of stearate and oleate declined with the chase time, whereas those of linoleate and α-eleostearate increased. These trends are similar to those in Fig. 1 C and D except that in Fig. 1 the oleate increased with time.

***In vivo* labeling of seed lipids with ammonium [1-^{14}C]- oleate and linoleate**

[1-^{14}C]Oleate. To confirm the results from [1-^{14}C]acetate labeling, seeds were exposed to [1-^{14}C]oleate in a time course protocol. Fig. 3 shows the results from oleate radioactive labeling. The radioactive label was continuously incorporated into the lipids with incubation time. PC was the most heavily labeled lipid at all times, and TAG was always more heavily labeled than DAG.

Oleate on PC (Fig. 3A) was the most heavily labeled fatty ester at first and then declined with its metabolism into linoleate and α-eleostearate. Following the synthesis of linoleate, α-eleostearate was synthesized after 2 h. No radioactive saturated fatty acids were found in the lipids, which indicated that oleic acid was metabolized as an entire molecule instead of first being fragmented and reassembled into fatty acids. α-Eleostearate was

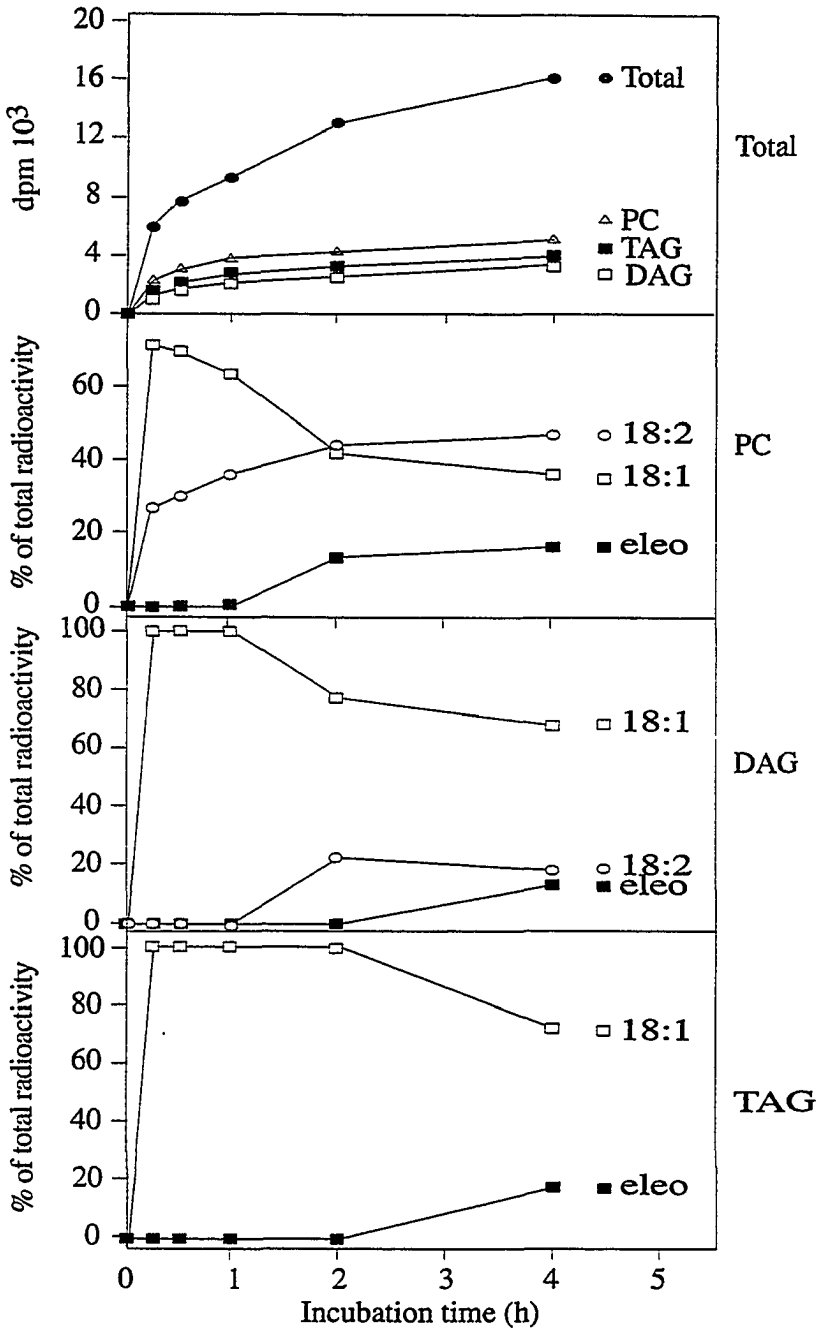

Figure 3: The incorporation of [1-^{14}C]oleate into the individual lipids and their fatty acids during the time course incubation. eleo: α-eleostearate; 18:1: oleate; 18:2: linoleate.

incorporated much later into the neutral lipids, DAG and TAG, than in PC. No detectable amount of linoleate was incorporated into TAG. Obviously, linoleoyl-PC is the substrate for eleostearoyl-PC. PE and PI contained little radioisotope and no eleostearic acid (data not shown).

[1-^{14}C]Linoleate. One-time course incubation and one-pulse chase experiment were conducted using [1-^{14}C]linoleate as the label. These results were shown in Fig. 4 and 5, respectively. In the time course experiment (Fig. 4), linoleic acid was incorporated continuously with time. PC and TAG were the most and least heavily labeled, respectively, and the radioactive label in both lipids increased continuously with time. DAG was the second most heavily labeled lipid and reached a constant value at 1 h. α-Eleostearate was found in PC, DAG and TAG (Fig. 4 B, C and D) after 0.5 h incubation. The amount of α-eleostearic acid in PC was the highest and in TAG was the second. In all lipids, the amount of α-eleostearic acid increased with metabolism of linoleate. No other radioactively labeled fatty esters were detected, which suggested that linoleic acid was incorporated and metabolized as an entire molecule without any degradation.

In the pulse chase experiment as shown in Fig. 5, linoleic acid was incorporated continuously during the first 2-h incubation and then reached a equilibrium value after 2 h. PC, initially the most heavily labeled lipid, increased its radioactive for 2 h and then declined slightly throughout the rest incubation. The amount of radioactive label in DAG was low and did not change with time. During the chase time, TAG became the most heavily labeled lipid class. Among all three lipids, PC, DAG and TAG, α-eleostearate

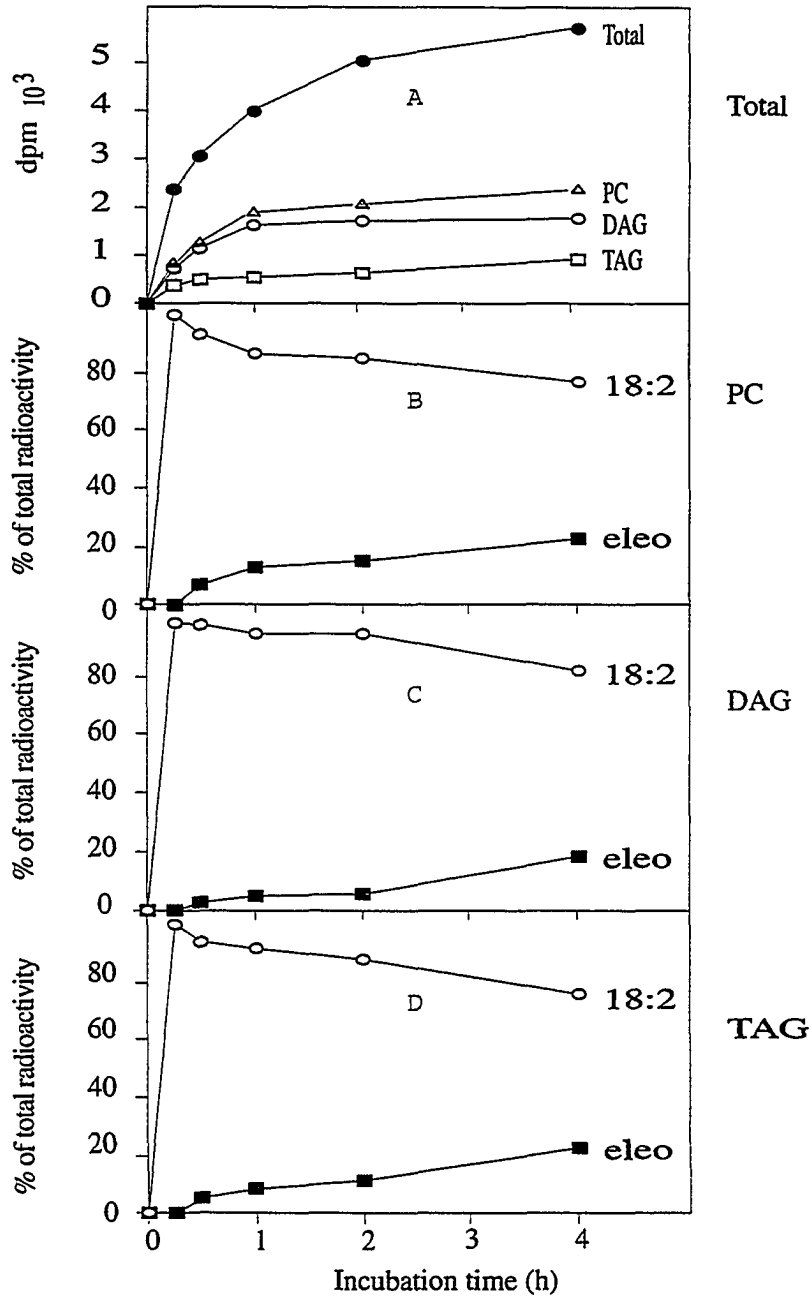

Figure 4: The incorporation of [1-^{14}C]linoleate into the individual lipids and their fatty acids during the time course incubation. eleo: α-eleostearate; 18:2: linoleate.

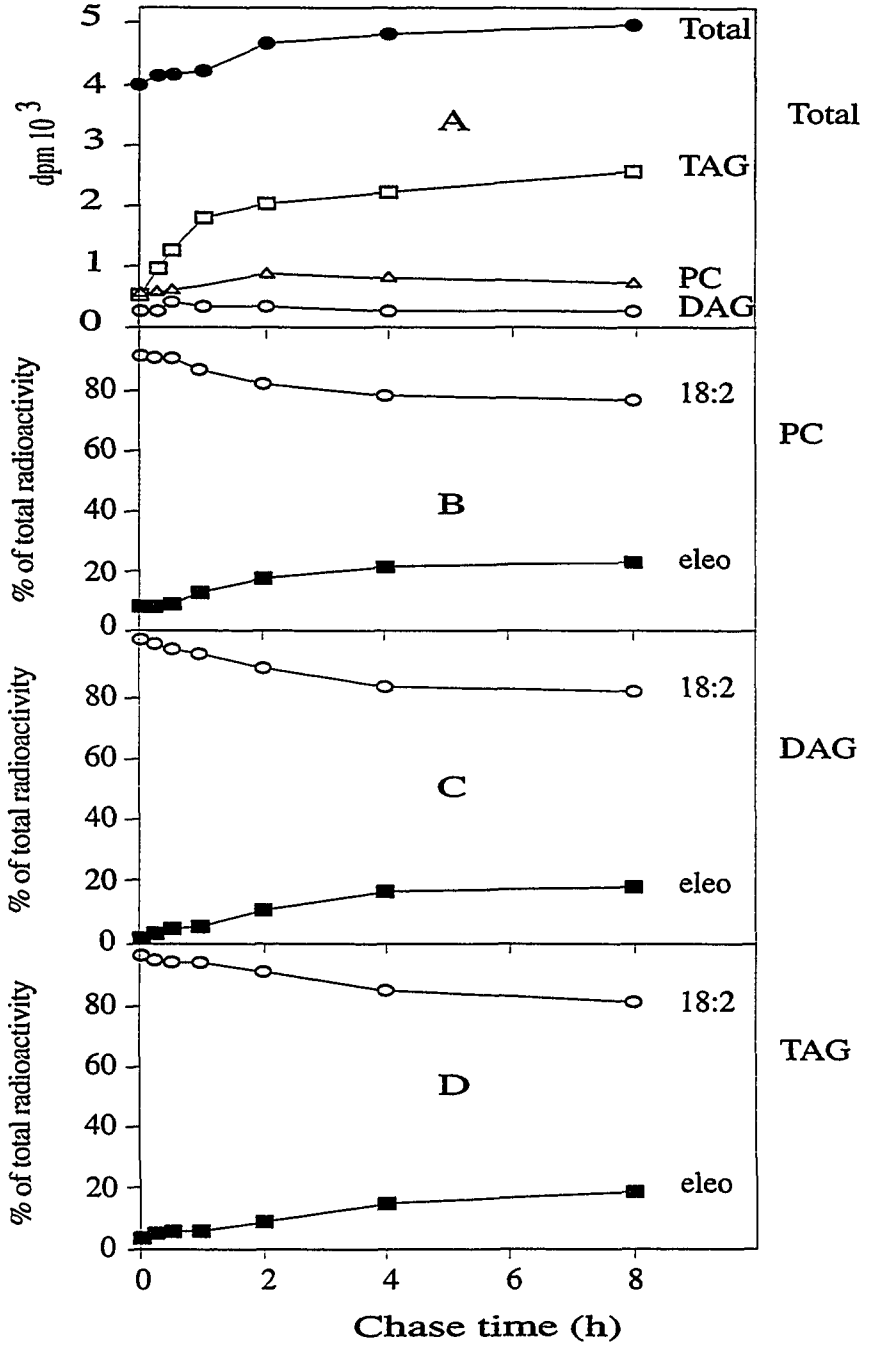

Figure 5: The incorporation of [1-^{14}C]linoleate into the individual lipids and their fatty acids during the chase time. eleo: α-eleostearate; 18:2: linoleate.

increased with the decrease of linoleate. PC contained more α-eleostearate than did the neutral lipids.

These results demonstrate a precursor-product relationship between linoleate and α-eleostearate. The substrate for biosynthesis of α-eleostearate was PC. PE and PI were not involved in the pathway. The α-eleostearate was incorporated into TAG from PC, probably through DAG as the intermediate.

Conclusion

The results indicated that the linoleoyl PC derived from oleoyl PC is the precursor of α-eleostearoyl PC.

References

1. Hammond, E. G. (1991) The raw materials of the fats and oils industry, In *Fats and Oils Processing*, P Wan, ed., AOCS, Champaign, IL, pp 1-15
2. Crombie, L and S. J. Holloway, *J. Chem. Soc. Perkin Trans* 2425 (1985).
3. Crombie, L and S. J. Holloway, *J. Chem. Soc., Chem. Commun.* 953 (1984).
4. Noda, M., Biosynthesis of Conjugated Polyenoic Acids in Plant Seeds, In *Structure, Function and Metabolism of Plant Lipids*, P. -A. Siegenthaler and W. Eichenberger, eds., Elsevier Science Publisher B. V., p. 29, 1984.
5. Skipski, V. P., R. F. Peterson , M. Barclay, *Biochem. J.* 90:362-378 (1964).

6. Hopkins, C.Y. (1990) Fatty acids of *Cucurbitaceae* seed oils in relation to taxonomy., In *Biology and Utilization of the Cucurbitaceae*, D. M.Bates, R. W. Robinson, C. Jeffrey, eds., Cornell U. Press, Ithaca, NY.

7. Christie, W.W., *Lipid Analysis*, 2nd ed., Pergamon Press Inc, New York, pp. 17-23, 1982.

CHAPTER 6. THE BIOSYNTHESIS OF α-ELEOSTEARIC ACID *IN VITRO*

A paper to be submitted to the journal of Plant Physiology

Linsen Liu [1], Earl G. Hammond [1] and Basil J. Nikolau [2]

Abstract

The biosynthesis of α-eleostearic acid from [1-^{14}C]linoleic acid *in vitro* was stimulated by the cofactors, NADH, ATP and CoA, at optimum concentrations of 5 mM, 6 mM and 5 mM, respectively, and inhibited by DTT. The optimum pH was 5.5. The *in vivo* incubation indicated that α-eleostearate was synthesized from linoleate on PC. No radioactive α-eleostearate was detected in the acyl-CoA class.

Introduction

In a previous paper in this dissertation (pp. 62-83), we showed that α-eleostearic acid was synthesized by developing seed of *M. charantia in vivo* from linoleate on PC. To characterize and purify the enzymes involved in the biosynthesis, an *in vitro* assay system is necessary. This paper presents the results of an investigation on the biosynthesis of α-eleostearic acid *in vitro*.

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Methods and Materials

Plant and chemicals

M. charantia were grown in the green house under 14-h lumination. Seeds developing for 16-d after pollination were used to make cell-free preparations. TLC plates of silica gel G and all the organic solvents were from Fisher Scientific (Pittsburgh, PA). [1-^{14}C]linoleic acid (50 Ci/mol) were from Amersham Life Science (Arlington Heights, IL). All other chemicals were from Sigma Chemical (St. Louis, MO).

Buffers and stock solutions

Hepes-KOH buffer. A 3X Hepes buffer containing 0.24 M Hepes, 0.9 M sucrose and 45 mM $MgCl_2$ was prepared and titrated to pH 7.2 using 1 M of KOH. The stock buffer was stored at 0°C and diluted three fold to make a 80 mM Hepes buffer just before use.

Ammonium [1-^{14}C]linoleate (3) The ammonium salts of the fatty acid were prepared by evaporating the toluene from 0.5 mL of radioactive fatty acid solution and heating the residual with 0.2 mL of 2 M NH_4OH at 60°C for 10 min. Nitrogen was bubbled through the resulting solution to remove residual ammonia.

Cofactors stock solutions. The following cofactor stock solutions (100X) were prepared: 0.5 M coenzyme A, 0.05 M NADPH, 0.2 M NADH, 0.1 M ATP and 0.2 M DTT.

In vitro incubation

A cell-free homogenate. A cell-free homogenate (1) was prepared by grinding 15

decoated developing seeds of 16-d after pollination in 2 mL of 80 mM Hepes buffer with a chilled pestle and mortar. The homogenate was filtered through a double layer of cheese cloth to give the cell-free preparation.

Cofactor determinations. Two experiments were conducted to identify the critical cofactors for the biosynthesis of α-eleostearic acid using ammonium [1-^{14}C]linoleate. In the first experiment, the cell free homogenate containing 5 mM ATP was used as the blank and the cofactors tested (DTT, CoA, NADH and NADPH) were added singly to the incubation mixtures. In a 0.5 mL (pH 7.2) of total assay volume, the incubation mixture contained the following components: 1 μCi ammonium [1-^{14}C]linoleate, 80 mM Hepes-KOH, 25 mM ATP, 250 μL of cell free homogenate, and 5 μL of the tested cofactor stock solution (2 mM DTT, 5 mM CoA, 0.5 mM NADPH or 2 mM NADH). In the second experiment, the cofactors, ATP, CoA, NADH and NADPH, were omitted one at a time from a 0.5 mL (pH 7.2) of complete incubation mixture which contained 1 μCi ammonium [1-^{14}C]linoleate, 80 mM Hepes-KOH, 5 mM ATP, 2 mM NADH and 0.5 mM NADPH, 5 mM CoA and 250 μL of cell free homogenate. The reactions were stopped by heating the incubation mixture to 100°C for 3 min.

Optimum cofactor concentrations. The NADH was tested at 0, 0.2, 0.5, 1, 2, 5, 10 mM with 5 mM CoA and 5 mM ATP at pH 7.2. The CoA was tested at 0, 0.5, 1, 2, 5, 10, 15 with 5 mM ATP, 5 mM NADH. The concentration of ATP was tested at 0, 1, 2, 4, 6, 10 mM with 5 mM NADH, 5 mM CoA at pH 7.2.

Optimum pH. The optimal pH was studied at different pH with 1 μCi ammonium [1-

^{14}C]linoleate, 5 mM NADH, 5 mM CoA, 6 mM ATP, 170 μL of 0.5 M pH buffer solution and 250 μL cell free homogenate. Two experiments were conducted at pH ranging from 3.0 to 6.0 and 5.5 to 9.0, respectively. Citric acid-sodium citrate (0.5 M) was used as the buffer for pH from 3.0 to 6.0. For buffers of pH 5.5 to pH 9.0 in the second experiment, 0.5 M MES was used for pH 5.5, 6.0 and 6.5; 0.5 M Hepes used for pH 7.0, 7.5 and 8.0; AMPSO for pH 8.5 and 9.0.

In vitro incubation. A cell free homogenate was incubated with 20 μCi [1-^{14}C]linoleate under the optimized cofactor concentrations, 5 mM NADH, 5 mM CoA and 6 mM ATP, at pH 5.5. A total volume of 2.4 mL of incubation mixture was incubated at 37°C and 0.5 mL was removed and heated at different times.

Lipid extraction and analysis

Two mL of chloroform-methanol (2:1, v/v) was added to the 0.5 mL of incubation mixture (2). The extracts were stired and centrifuged for 1 min. The bottom chloroform layer containing glycerolipids and the upper methanol-water layer containing acyl-CoA were recovered separately (3). After evaporating the chloroform, benzene was usually added and evaporated to remove residual moisture, and the residual lipids were dissolved in 0.4 mL of chloroform-methanol (2:1). The methanol-water extracts were frozen with liquid nitrogen and evaporated in a SpeedVac Concentrator (Savant Instruments, Inc., Farmingdale, NJ), the residual material was redissolved in 0.2 mL of water.

TAG, free fatty acids and DAG were separated from other lipids on TLC silica gel G

plates developed with hexane-diethyl ether-acetic acid (70:30:2, v/v/v). PC, PE and PI were resolved by development with chloroform-methanol-acetic-water (170:30:25:5, v/v/v/v) (3). Acyl-CoA was purified on TLC silica gel G plates developed with *n*-butanol-acetic acid--water (5:2:3, v/v/v) (4).

Methyl esters were prepared by reacting up to 20 mg of lipids or bands scraped from TLC plates with 0.5 mL of 1 M sodium methoxide in methanol for 1-h at room temperature with occasionally shaking. After addition of 1 mL of hexane, transesterification was terminated by adding 20 μL of glacial acetic acid and 0.5 mL of water. The hexane layers were recovered and evaporated to almost dryness. The methyl esters were dissolved in 50 μL of acetonitrile and separated on a Supelco (Bellefonte, PA) C-18 4 x 250 mm silica column and detected by radio-HPLC (System Gold, Beckman Instruments, Fullerton, CA) with acetonitrile as the mobile phase (2 mL/min).

Data treatment

Each sample was analyzed twice and each experiment was replicated. The mean of the four data was reported.

Results and Discussion

Cofactors

The results from the first experiment (Table 1) on cofactors indicated that the biosynthesis of α-eleostearic acid was inhibited by DTT and enhanced by NADPH, NADH

and CoA. The effect of cofactors was ordered as following: NADH > CoA > NADPH. The second experiment results (Table 2) were consistent with those of first experiment and indicated that the effect on the biosynthesis of α-eleostearic acid of ATP was between NADH and CoA. The order of contribution to the biosynthesis of α-eleostearic acid was NADH > ATP > CoA > NADPH. NADPH did not show a significant contribution to the biosynthesis, and only CoA, NADH and ATP were added as the cofactors in the following experiments.

Table 1. Cofactor requirement for the conversion of [1-^{14}C]linoleate to [^{14}C]α-eleostearate*

Cofactors	Blank	+ DTT (2 mM)	+ NADPH (0.5 mM)	+ CoA 5 mM	+ NADH (2 mM)
α-Eleostearate % of total radioactivity	0.4	0	0.6	0.8	1.4

* A *M. charantia* seed extract was incubated with [1-^{14}C]linoleate with the addition of the indicated cofactors.

Table 2. Cofactor requirement for the conversion of [1-^{14}C]linoleate to [^{14}C]α-eleostearate*

Cofactors	Blank§	Complete†	- NADPH	- CoA	- ATP	- NADH
α-Eleostearate % of total radioactivity	0.4	2.4	2.3	2.2	1.7	0.7

* A *M. charantia* seed extract was incubated with [1-^{14}C]linoleate in the presence or absence of the following cofactors (pH 7.2): 5 mM CoA, 0.5 mM NADPH, 2 mM NADH, 5 mM ATP and 15 mM $MgCl_2$.

§ Contained no indicated cofactors.

† Contained all the indicated cofactors.

Optimal incubation conditions

Four experiments were used to determine the optimal concentrations of the three major as well as pH. In each of these experiments, the concentration of individual cofactors was changed over a range while the others remained constant.

Fig. 1 and 2 indicated that the optimal concentrations of both NADH and CoA were 5 mM. The yield of α-eleostearate increased linearly with ATP concentration up to 6 mM

(Fig. 3). The effect of pH from both experiments (Fig. 4) indicated that the yield of α-eleostearic acid was highest at pH 5.5.

***In vitro* time course incubation**

Fig. 5 shows the radioactivity was incorporated into different lipid classes with incubation time. PC was the most heavily labeled lipid at all times that were tested and reached its maximal radioactive label at 8 min. The radioactivity in PC then declined for the rest of the incubation. The radioactive label in DAG reached its peak earlier at 4 min, declined to its lowest point at 15 min, and then slowly increased. The radioactive label in TAG increased steadily with time. [1-^{14}C]Linoleate was rapidly incorporated into acyl-CoA in 1 min, in which the radioactivity decreased steadily during the incubation. In the Kennedy pathway, fatty acids are esterified with glycerol-3-phosphate to form phosphatidic acid, which is then hydrolyzed to form DAG. This DAG is then converted into PC. The desaturation and conjugation of α-eleostearic acid occurs on PC, which is hydrolyzed to form DAG, which is acylated to form TAG. PE and PI contained less than 5 % of total radioactivity and contained no eleostearic acid (data not shown).

The analysis of fatty acid composition indicated that α-eleostearate and linoleate were the only radioactive fatty esters. The percentage of α-eleostearic acid in various lipids during the time course of the experiment is shown in Fig. 6. α-Eleostearic acid was first found in PC, and the amount of radioactive label peaked at 8 min and declined. DAG contained labeled α-eleostearate earlier than TAG and the labeled α-eleostearate increased steadily for

30 min. TAG contained labeled α-eleostearic acid after 15 min of incubation. These results indicated that [14 C]-linoleate was first incorporated into PC for biosynthesis to α-eleostearate. Then, the synthesized α-eleostearate was transferred into TAG through DAG as the intermediate. The slow rise of radioactive label in DAG, after a minimum at 15 min, coincided with the incorporation

of α-eleostearate into TAG. Analysis of acyl-CoA has not revealed any amount of α-eleostearoyl-CoA but linoleoyl-CoA.

Conclusions

The results show that α-eleostearic acid can be synthesized *in vitro*. The biosynthesis was enhanced by the cofactors NADH, ATP and CoA and inhibited by DTT. The optimal concentrations for the biosynthesis was 5 mM NADH, 6 mM ATP and 5 mM CoA at pH 5.5. Linoleoyl PC was indicated as the substrate for the biosynthesis of eleostearoyl PC.

References

1. Pollard, M. R., T. McKeon, L. A. Gupta, and P. K. Stumpf, Lipids 14:651 (1979).
2. Christie, W. W., Lipid Analysis, 2nd ed., Pergamon Press, Oxford, 1982.
3. Stobart, A. K. and S. Stymne, Triacylglycerol Biosynthesis, *in* Methods in Plant Biochemistry, Vol. 4, p. 39, Academic Press Limited, 1990.
4. Juguelin, H. and C. Cassagne, Anal. Biochem. 142: 329 (1984)

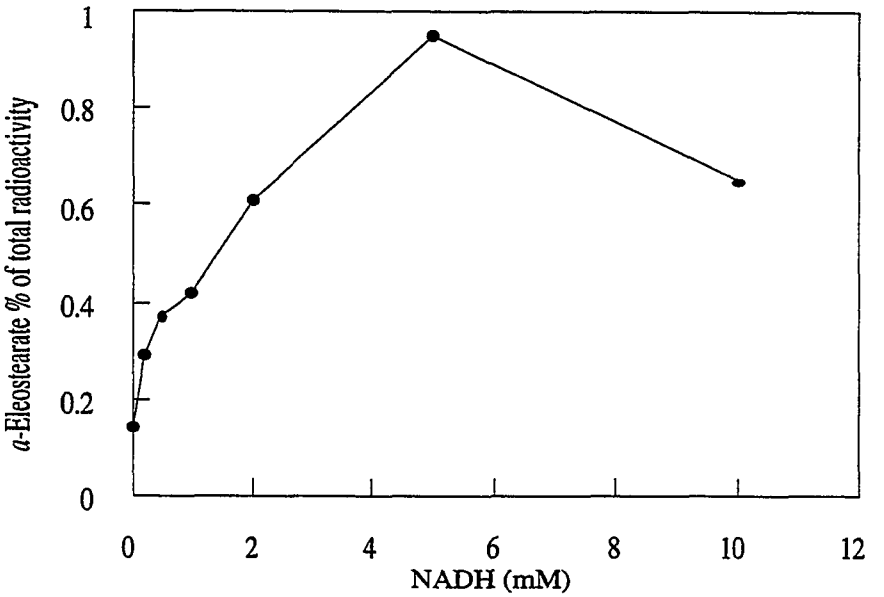

Figure 1. The effect on the yield of *a*-eleostearate of varying the NADH concentration in the *M. charantia* seed homogenate incubation mixture in the presence of the following cofactors: 5 mM CoA, 5 mM ATP and 15 mM $MgCl_2$, at pH 7.2.

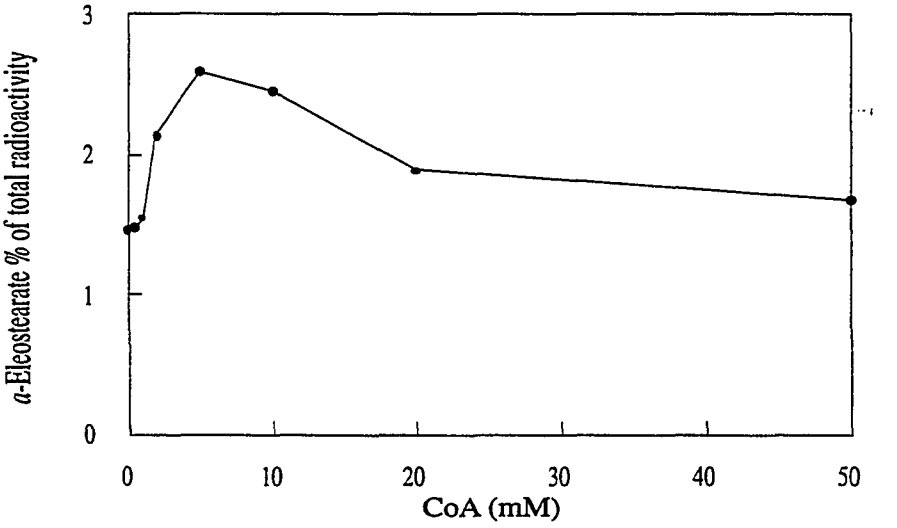

Figure 2. The effect on the yield of *a*-eleostearate of varying the CoA concentration in the M. charantia seed homogenate incubation mixture in the presence of the following cofactors: 5 mM ATP, 5 mM NADH and 15 mM $MgCl_2$, at pH 7.2.

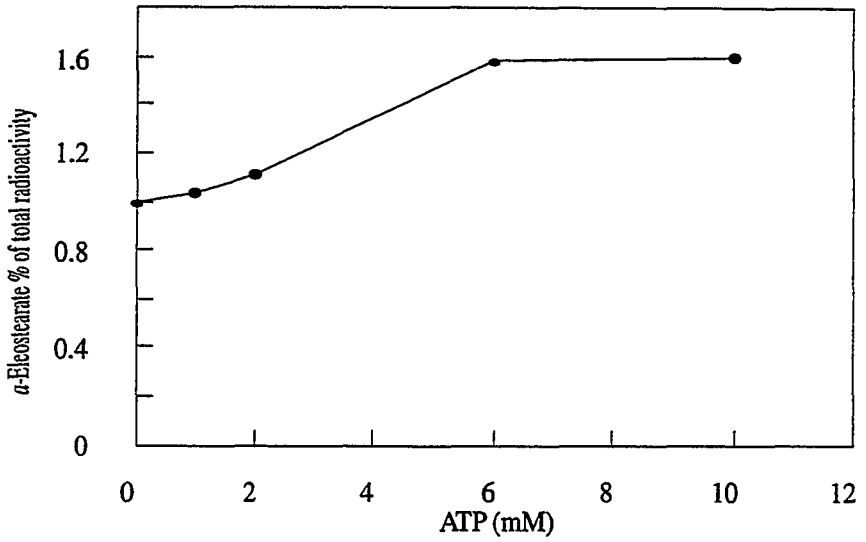

Figure 3. The effect on the yield of *a*-eleostearate of varing the ATP concentration in the *M. charantia* seed homogenate incubation mixture in the presence of the following cofactors: 5 mM NADH, 5 mM CoA and 15 mM $MgCl_2$, at pH 7.2.

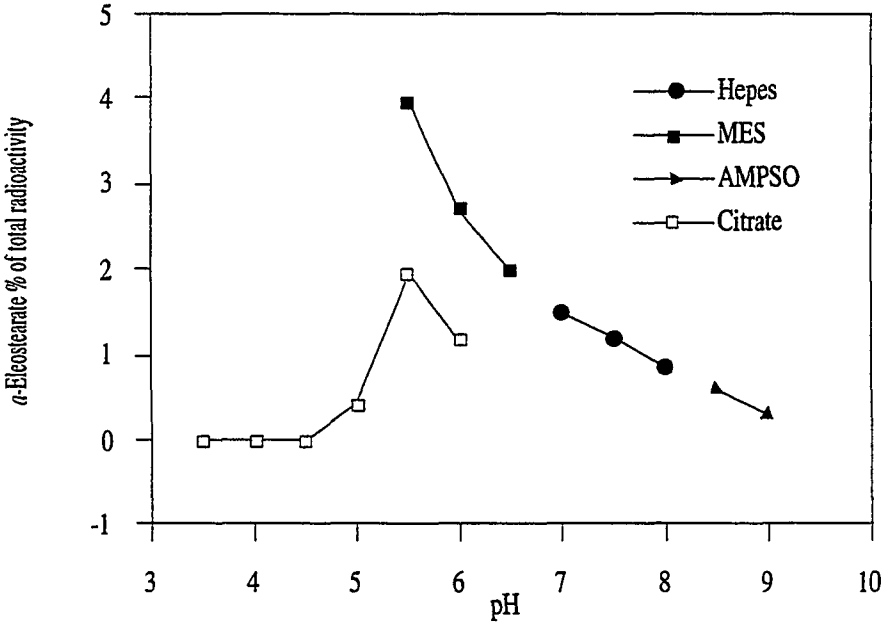

Figure 4. The effect on the yield of *a*-eleostearate of varing the pH of the *M. charantia* seed homogenate incubation mixture in the presence of the following cofactors: 5 mM NADH, 5 mM CoA, 6 mM ATP and 15 mM $MgCl_2$.

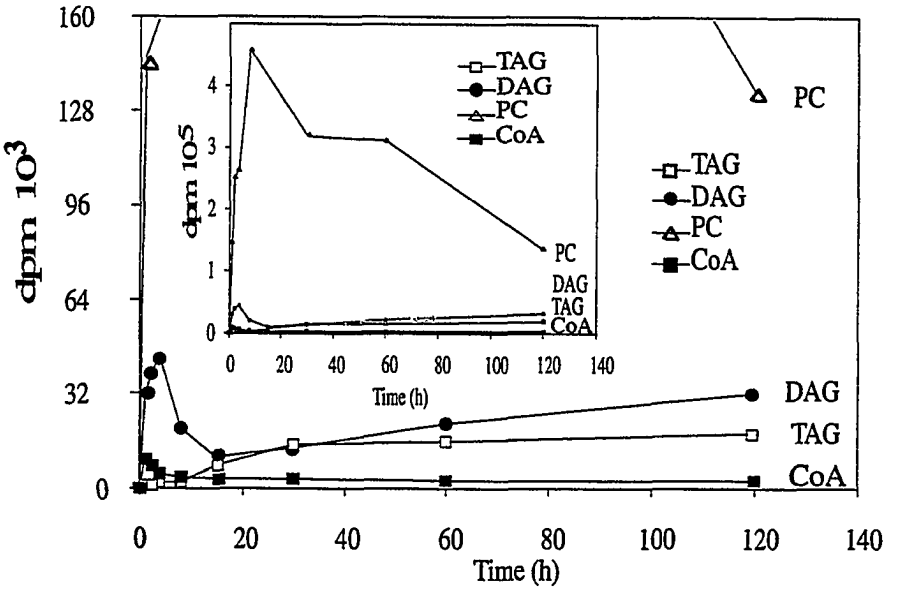

Figure 5. The incorporation of [1-14C]linoleate into various lipid classes when incubated with *M. charantia* seed homohenates, 5 mM NADH, 5 mM CoA, 6 mM ATP and 15 mM $MgCl_2$, at pH 5.5.

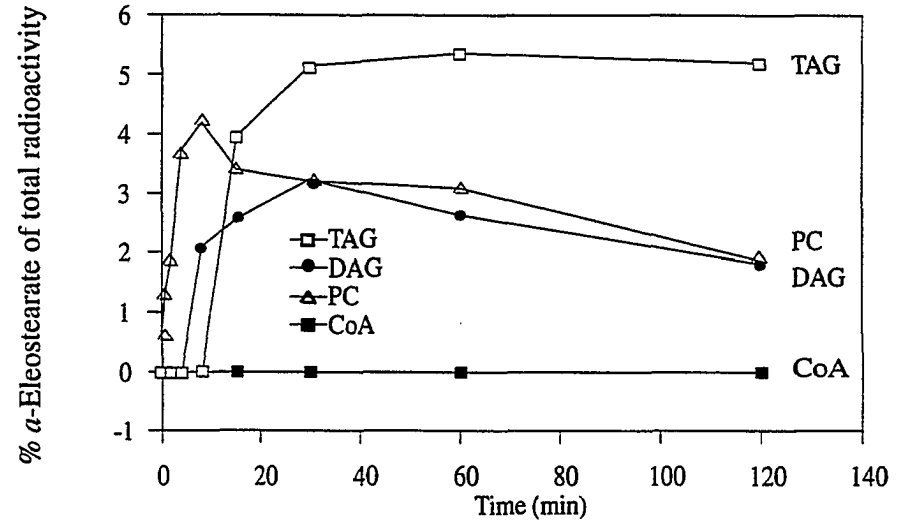

Figure 6. The incorporation of [1-14C]linoleate into *a*-eleostearate in various lipid classes when incubated with *M. charantia* seed homohenates, 5 mM NADH, 5 mM CoA, 6 mM ATP and 15 mM $MgCl_2$, at pH 5.5.

CHAPTER 7. GENERAL CONCLUSIONS

The biosynthesis of three unusual fatty acids, petroselinate, vernolate and α-eleostearate was explored in this dissertation. These acids have potential industrial and food uses, and the transfer of the genes responsible for their biosynthesis to high-yielding oil seed crops is contemplated.

Two lines of somatic carrot embryos were successfully induced, in which up to 20% of petroselinate occurred in their TAG. These somatic embryos can provide a convenient source of tissue for studying the biosynthesis of petroselinic acid. An analytical method also was developed that can separate petroselinate from oleate . This method was based on the novel phenylethyl esters of the fatty acids and gave good separations both by gas chromatography and high performance liquid chromatography. This derivative offers the fastest and most efficient method for petroselinate analysis among the published methods. Together petroselinate-producing somatic carrot embryos and phenylethyl esters derivatives provide a very efficient system for the research on biosynthesis and analysis of petroselinate.

Vernolate was shown to be synthesized from linoleate on PC in developing seeds of *Vernonia galamensis* by an *in vivo* labeling experiment. This result provides a basic understanding of the biosynthesis of vernolic acid in *Vernonia galamensis*.

In *Momordica charantia* seed, α-eleostearate was shown to be synthesized from linoleate on PC by both *in vivo* and *in vitro* incubations. The biosynthesis of α-eleostearic acid *in vitro* was enhanced by ATP, NADH and CoA, and inhibited by DTT. The optimum pH for

the biosynthesis of α-eleostearic acid *in vitro* was 5.5. α-Eleostearic acid was incorporated into TAG through DAG.