Formation of γ-Linolenic Acid in the Higher Plant Evening Primrose (*Oenothera biennis* L.)

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Seed lipids of evening primrose (*Oenothera biennis* L.), in contrast to those of most higher plants, contain predominantly γ-linolenic acid rather than α-linolenic acids as the constituent octadecatrienoic acid. Compositional changes in the lipids of developing evening primrose seeds indicate that, at the early stages of maturation, both α-linolenic acid and γ-linolenic acid are synthesized by Δ^{15} and Δ^{6} desaturation of linoleic acid, respectively, but at later stages the Δ^{6} desaturation predominates. The α-linolenic acid formed is channeled almost exclusively to the phospholipids and glycolipids that are constituents of the cell membranes, whereas γ-linolenic acid is esterified mainly to the storage lipids, i.e. triacylglycerols. Dilinoleoyl-γ-linolenoylglycerols are the major molecular species of triacylglycerols formed that contain γ-linolenoyl moieties. Changes in the composition of acyl moieties of phospholipids and glycolipids are consistent with the possible involvement of phosphatidylcholines and/or monogalactosyldiacylglycerols as substrates for the desaturation of the linoleoyl moieties. In contrast to the seeds, the leaves of evening primrose have α-linolenic acid as the major constituent of lipids and they do not contain γ-linolenic acid at all.

γ-Linolenic acid, *all-cis*-6,9,12-octadecatrienoic acid, is a ubiquitous constituent of lipids of animals (Kuksis, 1978), protozoa (Erwin and Bloch, 1963), zooflagellates (Korn et al., 1965; Meyer and Holz, 1966), fungi (Shaw, 1965), phytoflagellates (Erwin and Bloch, 1964; Haines et al., 1962), alga (Nichols and Wood, 1968), and mosses (Schlenk and Gellerman, 1965). In animal organisms, γ-linolenic acid is formed by Δ^{6} desaturation of linoleic acid, *all-cis*-9,12-octadecadienoic acid (Jeffcoat and James, 1984). A similar pathway of biosynthesis of γ-linolenic acid has been shown to occur in alga (Nichols and Wood, 1968), and it may also be operative in other organisms.

Lipids containing γ-linolenic acid are unusual constituents of tissues of higher plants, which are generally abundant in α-linolenic acid, *all-cis*-9,12,15-octadecatrienoic acid. Seeds of several plants belonging to the families, such as Onagraceae, Boraginaceae, and Saxifragaceae, have been found to contain substantial proportions of γ-linolenic acid as a constituent of their lipids (Hudson, 1984; Traitler et al., 1984; Wolf et al., 1983).

It is now well established that in higher plants linoleic acid is formed by desaturation of oleoyl moieties of phosphatidylcholines (Murphy et al., 1984; Roughan and Slack, 1982; Slack et al., 1979; Stymne and Appelqvist, 1978), but the pathways of biosynthesis of α- and γ-linolenic acids are not yet clearly understood. Seeds of evening primrose, *Oenothera biennis* L., have been reported to contain γ-linolenic acid, rather than α-linolenic acid, as the major constituent octadecatrienoic acid (Hudson, 1984; Wolf et al., 1983). In the present study we have investigated the formation of lipids containing γ-linolenic acid vs those containing α-linolenic acid in developing seeds of evening primrose at various stages of development. These investigations should form the basis of radiolabeling studies aimed at elucidation of the pathways of formation of γ-linolenic acid in higher plants.

EXPERIMENTAL SECTION

Materials. Seeds of evening primrose (*O. biennis* L.) were provided by Efamol Ltd., Woodbridge Meadows, Surrey, England. The evening primrose plants were grown outdoors and the seeds as well as leaves harvested between 3 and 8 weeks after flowering.

Fatty acids and their methyl esters were purchased from Nu-Chek-Prep, Elysian, MN, and acyl lipids as well as column packings for gas chromatography from Applied Science Laboratories, Inc., State College, PA. All reagents and adsorbents for thin-layer chromatography were from E. Merck AG, D-6100 Darmstadt, Federal Republic of Germany. The enzyme preparations were obtained from Sigma Chemie GmbH, D-8000 München, Federal Republic of Germany.

Lipid Extraction and Analysis. The methods described previously (Mukherjee, 1983, 1986a,b) were used for the extraction of lipids from developing and mature seeds, fractionation of total lipids by thin-layer chromatography into classes of neutral lipids, phospholipids, and glycolipids, determination of the composition of lipids by gas chromatography of the methyl esters, and location of double bonds on the acyl moieties of the lipids by reductive ozonolysis and gas chromatography. Methyl esters of γ-linolenic acid, α-linolenic acid, and linoleic acid were separated and quantitated by gas chromatography on glass columns (1.8 m × 4 mm) packed with 10% (W/W) Silar 5 CP on Gas-Chrom Q (80–100 mesh).

Molecular species of triacylglycerols and phosphatidylcholines differing in the degree of unsaturation were fractionated by argentation thin-layer chromatography on Silica Gel G containing 20% (w/w) silver nitrate using chloroform–methanol (98.5:1.5, v/v) and chloroform–methanol–water (65:25:4, v/v), respectively, as developing solvents. The positional distribution of acyl moieties in triacylglycerols and phosphatidylcholines was determined by hydrolysis with porcine pancreatic lipase according to Luddy et al. (1964) and phospholipase A_2 from snake venom according to Van Golde and Van Deenen (1967), respectively. The products of lipolysis were analyzed as outlined elsewhere (Murphy et al., 1985).

RESULTS AND DISCUSSION

The data given in Table I show the changes in lipid content and the composition of fatty acids from total lipids of evening primrose seeds at various stages of development. Seed maturation and concomitant accumulation of lipids were accompanied by an increase in the relative proportions of linoleic and γ-linolenic acids in the total lipids,

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Table I. Lipid Content and Fatty Acid Composition of Total Lipids in Developing Seeds of Evening Primrose

weeks after flowering	lipid content, %	compn of fatty acids (mg/g seed tissue),[a] %					
		16:0	18:1	18:2	γ-18:3	α-18:3	others[b]
3	1.5	14.3 (2.2)	18.9 (2.8)	53.4 (8.0)	5.7 (0.9)	5.1 (0.8)	2.6
4	3.4	9.1 (3.1)	11.3 (3.8)	65.8 (22.4)	9.1 (3.1)	2.3 (0.8)	2.4
5	7.9	8.5 (6.7)	10.6 (8.4)	67.4 (53.3)	10.4 (8.2)	1.1 (0.9)	2.0
6	11.9	7.0 (8.3)	9.4 (11.2)	71.4 (85.0)	10.0 (11.9)	0.6 (0.7)	1.6
7	15.3	6.9 (10.6)	10.3 (15.8)	71.0 (108.6)	10.3 (15.8)	tr	1.5
8	16.5	7.3 (12.1)	10.8 (17.8)	69.9 (115.3)	10.4 (17.2)	tr	1.6
mature seed	24.9	6.6 (16.4)	13.4 (33.4)	70.0 (174.3)	8.1 (20.2)	tr	1.9

[a] Fatty acids are designated by number of carbon atoms:number of double bonds. γ-18:3 = γ-linolenic acid; α-18:3 = α-linolenic acid. [b] Including 18:0, 20:0, and 20:1.

Table II. Content and Acyl Composition of Phospholipids Plus Glycolipids and Triacylglycerols in Developing Seeds of Evening Primrose

weeks after flowering	lipids[a]	content in total lipids, %	compn of major acyl moieties,[b] %				
			16:0	18:1	18:2	γ-18:3	α-18:3
3	PL + GL	17	17	29	28	tr	21
	TG	82	13	16	61	7	1
4	PL + GL	2	16	39	26	2	12
	TG	97	9	10	70	9	tr
5	PL + GL	2	22	17	45	3	6
	TG	97	9	11	70	9	tr
6	PL + GL	1	26	19	37	3	5
	TG	98	8	10	70	11	tr
7	PL + GL	1	19	23	45	2	3
	TG	98	7	11	71	10	tr
8	PL + GL	1	15	33	41	2	1
	TG	98	7	11	71	10	tr

[a] Key: PL = phospholipids; GL = glycolipids; TG = triacylglycerols. [b] See Table I for abbreviations of the acyl moieties.

whereas the relative proportions of oleic acid is barely altered from week 4 after flowering onward. These compositional changes are compatible with the following sequential desaturations:

oleic acid (oleoyl moieties) → linoleic acid (linoleoyl moieties) → γ-linolenic acid (γ-linolenoyl moieties)

The fatty acid composition of total lipids of evening primrose seeds (Table I) shows that formation of γ-linolenic acid is initiated at an early stage of seed maturation (3 weeks after flowering); at this stage the levels of γ-linolenic acid and α-linolenic acid are almost the same. With progressive seed maturation, α-linolenic acid virtually disappears from the seed lipids, while γ-linolenic acid is accumulated. It appears from these results that both α-linolenic acid and γ-linolenic acid are formed at an early stage of seed development by desaturation of linoleic acid/linoleoyl moieties mediated by Δ^6 desaturase and Δ^{15} desaturase, respectively, but at later stages the activity of the Δ^6 desaturase predominates in the seeds of evening primrose.

In contrast to the seeds, the leaves of evening primrose contained no γ-linolenic acid in the lipids. The leaf lipids contained about 63% α-linolenic acid, 14% linoleic acid, and 16% palmitic acid as the major constituents. Apparently, even in a plant that synthesizes substantial amounts of γ-linolenic acid in the seeds, none of this fatty acid is formed in the leaves. It seems therefore that α-linolenic acid is the only species of octadecatrienoic acid that is present as a major constituent of photosynthetic tissues.

The relative proportions of phospholipids plus glycolipids and those of triacylglycerols in developing seeds of evening primrose as well as the composition of acyl moieties of these lipids are given in Table II. These data taken together with that presented in Table I show that seed maturation proceeds with the accumulation of triacylglycerols and a concomitant reduction in the level of phospholipids plus glycolipids—a phenomenon generally observed in lipid-rich seeds. The relative proportions in triacylglycerols of palmitoyl, oleoyl, linoleoyl, and γ-linolenoyl moieties are barely altered between week 4 and week 8 after flowering, and hardly any α-linolenoyl moieties are detectable in the triacylglycerols throughout the entire period of seed development examined (Table II).

The data given in Table II also show that the composition of acyl moieties of phospholipids plus glycolipids is considerably altered during the development of evening primrose seeds. Especially, a distinct reduction in the relative proportion of oleoyl moieties between week 4 and week 5 and a concomitant increase in the level of linoleoyl moieties are observed in phospholipids plus glycolipids. During this period of seed development, extensive synthesis of γ-linolenic acid also occurs, as is evident from the data given in Table I. These findings are consistent with the sequential Δ^{12} desaturation of oleoyl to linoleoyl moieties, followed by Δ^6 desaturation of linoleoyl to γ-linolenoyl moieties. In addition, these results suggest the involvement of phospholipids and/or glycolipids as substrates in these desaturation reactions.

It can be seen from the data given in Table II that although α-linolenoyl moieties are barely detectable in the triacylglycerols throughout seed development, they constitute a major portion of phospholipids plus glycolipids at the early stage of seed development, e.g. at week 3 after flowering. With progressive seed development, however, the relative proportion of α-linolenoyl moieties in phospholipids plus glycolipids decreases sharply (Table II). These data support, as indicated before, that both Δ^{15} and Δ^6 desaturases are active in the seeds of evening primrose at an early stage of development; but, later the Δ^6 desaturase predominates.

It is also obvious from the data given in Table II that α-linolenic acid is channeled almost exclusively to the phospholipids and glycolipids, which are the major constituents of the cellular membranes, whereas γ-linolenic acid is esterified mainly in the storage lipids, i.e. triacyl-

Table III. Distribution and Composition of Major Classes of Phospholipids and Glycolipids in Developing Seeds of Evening Primrose

weeks after flowering	lipid class[a]	rel proportion	compn of major acyl moieties,[b] %				
			16:0	18:1	18:2	γ-18:3	α-18:3
3	PI	10	36	17	23	tr	22
	PC	56	11	33	29	1	22
	PG	6	24	14	24	tr	34
	PE	25	21	20	27	tr	25
	MGDG	3	5	17	23	1	51
5	PI	17	27	13	36	1	12
	PC	54	11	16	48	5	14
	PG	4	31	14	31	2	14
	PE	22	24	18	33	2	15
	MGDG	3	6	14	35	9	31
7	PI		20	16	44	4	3
	PC		11	14	58	5	5
	PG		27	17	35	2	9
	PE		21	13	49	4	8
	MGDG		8	14	49	6	16

[a] Key: PI = phosphatidylinositols; PC = phosphatidylcholines; PG = phosphatidylglycerols; PE = phosphatidylethanolamines; MGDG = monogalactosyldiacylglycerols. [b] See Table I for abbreviations of the acyl moieties.

Table IV. Composition of Molecular Species of Triacylglycerols Containing γ-Linolenoyl Moieties in Seeds of Evening Primrose at Various Stages of Development

weeks after flowering	molec species/no. of double bonds	content, %	posn of acyl moieties	compn of acyl moieties,[a] %				
				16:0 + 18:0	18:1	18:2	γ-18:3	α-18:3
3	I/8	2	*sn*-1,2,3	12	6	27	42	13
			sn-2	43	28	12	11	5
	II/7	10	*sn*-1,2,3	2	2	60	36	tr
			sn-2	24	13	40	23	tr
	III/6	4	*sn*-1,2,3	9	22	36	27	4
			sn-2	6	74	11	2	7
5	I/8	3	*sn*-1,2,3	2	3	31	64	tr
			sn-2	59	21	10	10	tr
	II/7	19	*sn*-1,2,3	1	2	61	36	tr
			sn-2	21	7	44	28	tr
	III/6	3	*sn*-1,2,3	7	18	46	29	tr
			sn-2	38	23	19	18	tr
mature seed	I/8	2	*sn*-1,2,3	3	5	32	57	tr
			sn-2	33	19	24	21	tr
	II/7	17	*sn*-1,2,3	1	2	62	36	tr
			sn-2	7	5	56	32	tr

[a] See Table I for abbreviations of acyl moieties

glycerols, of evening primrose seeds.

Table III shows the distribution of the major classes of phospholipids and glycolipids in developing seeds of evening primrose and the composition of acyl moieties of these lipid classes. These data reveal, as an extension of the results presented in Table II, that α-linolenic acid is indeed a prominent constituent of each of the major classes of phospholipids and glycolipids at early stages of seed development (3–5 weeks after flowering). However, the level of α-linolenoyl moieties in each of these lipid classes decreases sharply with progressive seed maturation.

The data given in Table III show furthermore that γ-linolenoyl moieties appear first in phosphatidylcholines and monogalactosyldiacylglycerols and that the levels of γ-linolenoyl moieties in these two lipid classes remain consistently higher than in the other classes of phospholipids throughout the period of seed development examined. These findings may suggest the involvement of phosphatidylcholines and/or monogalactosyldiacylglycerols as substrates for desaturation of linoleoyl to γ-linolenoyl moieties. α-Linolenoyl moieties occur to the greatest extent in monogalactosyldiacylglycerols, which lends support to the desaturation of the linoleoyl moieties of this lipid class to the α-linolenoyl moieties. Earlier studies have provided evidence for the involvement of phosphatidylcholines (Browse and Slack, 1981; Stymne and Appelqvist, 1980) or monogalactosyldiacylglycerols (Heinz and Harwood, 1977; Jones and Harwood, 1980) as substrates for the Δ^{15} desaturation of linoleoyl moieties.

Although the data presented in Tables I–III argue strongly for sequential desaturation of oleoyl to linoleoyl and γ-linolenoyl moieties of the lipids of evening primrose seeds, in a similar manner as in animal tissues (Jeffcoat and James, 1984) and algae (Nichols and Wood, 1968), alternative modes of desaturation are also conceivable. For example, *cis*-6- or *cis*-12-octadecenoyl moieties, derived by Δ^6 desaturation or Δ^{12} desaturation, respectively, of stearoyl moieties, could undergo sequential Δ^9 and Δ^{12} desaturations or Δ^9 and Δ^6 desaturations, respectively, to yield γ-linolenoyl moieties. If such pathways were operative, one might expect to detect *cis*-6- or *cis*-12-octadecenoyl moieties in the seed lipids of evening primrose. In order to check the operation of such pathways, the C_{18}-monounsaturated fatty acids were isolated as their methyl esters from the total lipids of developing and mature seeds of evening primrose by argentation thin-layer chromatography and preparative gas chromatography. Subsequent analysis of this methyl ester fraction by reductive ozonolysis and gas chromatography revealed that in all samples examined the constituent monounsaturated fatty acids are composed of 92–94% *cis*-9-octadecenoic (oleic) acid and 6–8% *cis*-11-octadecenoic (vaccenic) acid only. Neither *cis*-6-octadecenoic acid nor *cis*-12-octadecenoic acid was detectable. These data suggest that the sequential

Δ^{12} desaturation of oleoyl to linoleoyl, followed by Δ^6 desaturation to γ-linolenoyl moieties, is the major, if not the sole, pathway of biosynthesis of γ-linolenic acid in seeds of evening primrose and possibly in other higher plants.

Since most of the γ-linolenic acid formed is found to be esterified in the triacylglycerol fraction of evening primrose seeds (Table II), it is of interest to examine the biogenesis of triacylglycerols containing γ-linolenoyl moieties. Molecular species of triacylglycerols at three stages of seed development were fractionated according to the degree of unsaturation by argentation thin-layer chromatography. The major molecular species of triacylglycerols containing γ-linolenoyl moieties were isolated and analyzed. The data given in Table IV show that three molecular species (I–III) containing substantial proportions of γ-linolenoyl moieties are detectable in the seed lipids, of which the species II is the most predominant constituent. This fraction is composed of about two-thirds linoleoyl and one-third γ-linolenoyl moieties, and it can thus be identified as dilinoleoyl-γ-linolenoylglycerols. The relative proportion of this molecular species increases with progressive seed maturation (Table IV). It can also be seen from the data given in Table IV that, in the molecular species I and III, both linoleoyl and γ-linolenoyl moieties are preferentially esterified at the *sn*-1,3-positions than at *sn*-2-position.

In order to examine whether phosphatidylcholines are possible metabolic precursors of triacylglycerols containing γ-linolenoyl moieties, as suggested for triacylglycerols containing polyunsaturated acyl moieties (Slack and Browse, 1984), structural analogies between these two lipid classes in developing evening primrose seeds were investigated. The phosphatidylcholines were fractionated by argentation thin-layer chromatography into molecular species differing in the number of olefinic bonds. Examination of two major molecular species of phosphatidylcholines containing γ-linolenoyl moieties showed that the γ-linolenoyl moieties occurred almost exclusively at the *sn*-1-position (data not shown), which exhibits some analogy with the relative abundance of γ-linolenoyl moieties at the *sn*-1,3-positions of the triacylglycerol species I and III (Table IV). However, no resemblance between the compositions of acyl moieties at the *sn*-2-position of the major species of phosphatidylcholines containing γ-linolenoyl moieties and those of triacylglycerols was observed.

Minor proportions (<1%) of diacylglycerols were also detected in the lipids of developing evening primrose seeds. Fatty acid composition of the diacylglycerols had some resemblance with that of triacylglycerols, but it was totally different from the fatty acid composition of phosphatidylcholines (data not shown). These findings do not seem to support an equilibrium between phosphatidylcholines and diacylglycerols during the synthesis of triacylglycerols, as suggested for several oilseeds (Slack and Browse, 1984).

Registry No. 16:0, 57-10-3; 18:1, 112-80-1; 18:2, 60-33-3; α-18:3, 463-40-1; γ-18:3, 506-26-3.

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