Biosynthesis of γ -linolenic acid in cotyledons and microsomal preparations of the developing seeds of common borage (*Borago officinalis*)

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The developing seeds of *Borago officinalis* (common borage) accumulate a triacylglycerol oil that is relatively rich in the uncommon fatty acid γ -linolenate (octadec-6,9,12-trienoic acid). Incubation of developing, whole, cotyledons with [14 C]oleate and [14 C]linoleate showed that the γ -linolenate was synthesized by the sequential desaturation of oleate $\rightarrow$ linoleate $\rightarrow$ γ -linolenate. Microsomal membrane preparations from the developing cotyledons contained an active Δ^6 -desaturase enzyme that catalysed the conversion of linoleate into γ -linolenate. Experiments were designed to manipulate the [14 C]linoleate content of the microsomal phosphatidylcholine. The [14 C]linoleoyl phosphatidylcholine labelled *in situ* was converted into γ -linolenoyl phosphatidylcholine in the presence of NADH. The substrate for the Δ^6 -desaturase in borage was, therefore, the linoleate in the complex microsomal lipid phosphatidylcholine, rather than, as in animals, the acyl-CoA. This was further confirmed in experiments that compared the specific radioactivity of the γ -linolenate, in acyl-CoA and phosphatidylcholine, that was synthesized when [14 C]linoleoyl-CoA was incubated with microsomal membranes, NADH and non-radioactive γ -linolenoyl-CoA. The Δ^6 -desaturase was positionally specific and only utilized the linoleate in position 2 of *sn*-phosphatidylcholine. Analysis of the positional distribution of fatty acids in the endogenous microsomal *sn*-phosphatidylcholine showed that, whereas position 1 contained substantial linoleate, only small amounts of γ -linolenate were present. The results shed further light on the synthesis of C_{18} polyunsaturated fatty acids in plants and in particular its relationship to the regulation of the acyl quality of the triacylglycerols in oilseeds.

INTRODUCTION

In animals the activity of the Δ^6 -desaturase enzyme that catalyses the conversion of linoleic acid (octadeca-9,12-dienoic acid) into γ -linolenic acid (octadeca-6,9,12-trienoic acid) is thought to be affected by certain dietary (Brenner, 1981) and clinical conditions (Holman & Johnson, 1981). The dietary application of vegetable oils rich in γ -linolenate is considered (Horrobin & Manku, 1983) to alleviate hypercholesterolaemia and many atopic disorders. Unfortunately, only a few plant species are known that produce γ -linolenate in sufficient quantities for commercial exploitation. The major sources of plant γ -linolenate are the seed oils of evening primrose (*Oenothera biennis*) (Hilditch & Williams, 1964) and the common borage (*Borago officinalis*) (Tetenyi *et al.*, 1974) and the fruits of *Ribes* spp. (currant) (Traitler *et al.*, 1984). To date nothing is known about the biosynthesis in plants of the Δ^6 isomer of linolenic acid, and yet its appreciation as an essential fatty acid and its popularity in the health-food industry has increased considerably in the last few years. It is important to establish, therefore, for plant-breeding programmes, how γ -linolenic acid is synthesized and the relationship of this to the formation of the seed triacylglycerols.

The occurrence of α -linolenic acid (octadeca-9,12,15-trienoic acid) is quite widespread in oilseeds and it is also a major component of photosynthetic membranes. It is thought that the substrate for the synthesis of α -linolenate in seeds (Browse & Slack, 1981) and leaves (Roughan

et al., 1979; Hawke & Stumpf, 1980; Jones & Harwood, 1980) is the linoleate in phosphatidylcholine and monogalactosyldiacylglycerol respectively. The evidence for this, particularly in oilseeds, is still rather poor, since this tissue has proved somewhat recalcitrant in providing consistently active membrane preparations for studies on the synthesis of α -linolenate. On the other hand, the evidence that the oleate (octadec-9-enoic acid) in microsomal phosphatidylcholine is the substrate for linoleate synthesis in plant tissues is overwhelming (Stymne & Appelqvist, 1978; Slack *et al.*, 1979; Stymne *et al.*, 1983; Murphy *et al.*, 1985). The synthesis of linoleate and, most likely, α -linolenate, occurs therefore in plants in complex lipid substrates, whereas γ -linolenate synthesis in animals (Holloway, 1983) utilizes the CoA ester directly. The reason why higher plants should favour complex lipid substrates for C_{18} polyunsaturated fatty acid synthesis is unclear. We have speculated (Stymne & Stobart, 1986), for experimental purposes, that the efficient insertion of a double bond between the methyl end of a C_{18} fatty acid and an already established centre of unsaturation, as occurs in plants, may require the intramolecular environment of a complex lipid. On the other hand the introduction of double bonds between an existing double bond and the carboxy end of a fatty acid molecule may necessitate, as in animals, the involvement of acyl-CoA. It has been previously proposed (Howling *et al.*, 1972) that the plant desaturase enzymes which catalyse the formation of linoleate from

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oleate may utilize both the acyl-CoA and phosphatidylcholine substrates. A study of the mode of γ -linolenate synthesis in plants was therefore undertaken to test these suggestions and also to increase our understanding of C_{18} polyunsaturated fatty acid formation for the possible manipulation of the acyl quality of the seed oils. We report here for the first time studies on the formation of γ -linolenic acid in plants and, in particular, its synthesis in microsomal membrane preparations from the maturing cotyledons of *Borago officinalis*.

MATERIALS AND METHODS

Chemicals

[1- 14 C]Stearic acid (octadecanoic acid), [1- 14 C]oleic acid (octadec-9-enoic acid) and [1- 14 C]linoleic acid (octadeca-9,12-dienoic acid) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Catalase (thymol-free, 11000 units/mg), bovine serum albumin (fraction V, fatty acid free), CoASH, NADH, phospholipase A_2 [from Indian cobra (*Naja naja*) venom] and various fatty acids were purchased from Sigma. SEP-PAK C_{18} silica-gel cartridges were from Waters Associates, Farmington, MA, U.S.A.

Heptadecanoyl-CoA, [1- 14 C]linoleoyl-CoA (sp. radioactivity 2597 d.p.m./nmol) and γ -linolenoyl-CoA (octadeca-6,9,12-trienoyl-CoA) were synthesized from their mixed anhydrides as described by Sanchez *et al.* (1973). The radioactive acyl-CoA had a purity greater than 97% as determined by t.l.c., g.l.c. and radio-g.l.c.

Plant material and microsomal preparations

Borago officinalis L. (common borage) seeds were purchased from Weibulls, Landskrona, Sweden, and for the experiments *in vivo* the plants were grown in a greenhouse during the 1985 summer period. The flowers were hand-pollinated and developing seeds harvested 15–20 days after flowering. For the experiments *in vitro*, however, it was found essential to cultivate the plants in a controlled environment. These plants were therefore grown in a 16 h photoperiod at 18 °C and an 8 h night at 13 °C.

Cotyledons (approx. 5 mg fresh weight/cotyledon pair) were removed from the developing seeds and stored in ice-cold 0.1 M-potassium phosphate buffer, pH 7.2, containing 0.3 M-sucrose. All further procedures were carried out at 1–4 °C. The cotyledons were ground in a mortar with 2 parts (v/w) of 0.1 M-potassium phosphate buffer, pH 7.2, containing 0.1% bovine serum albumin, 0.33 M-sucrose and 1000 units of catalase/ml. The homogenate was diluted 20-fold with fresh grinding medium and filtered through a double layer of Miracloth before centrifugation at 20000 *g* for 10 min. The supernatant was refiltered through Miracloth and centrifuged at 105000 *g* for 90 min. The resulting microsomal pellet was resuspended in the homogenization medium and either used directly or stored at –70 °C (for up to 3 days only) before use.

Enzyme assays

In the experiments *in vivo*, whole cotyledons were incubated (five pairs; approx. 25 mg fresh weight) for 30 min at 25 °C with the ammonium salt of either [14 C]oleate or [14 C]linoleate (15.9 nmol; sp. radioactivity 1.26×10^5 d.p.m./nmol in each case) in 0.25 ml of

0.1 M-phosphate buffer, pH 7.2. After that time the cotyledons were rinsed in buffer to remove residual radioisotope and extracted with an Ultra-Turrax homogenizer in the medium described by Bligh & Dyer (1959) or further incubated in fresh buffer for various times before extraction.

Microsomal assays were carried out at 25 °C with constant shaking. The incubation mixtures contained 10 mg of bovine serum albumin, 1000 units of catalase, 66 mM-sucrose, substrates and cofactors (as given in the Tables) and microsomal membranes in a final volume of 1 ml with 0.1 M-phosphate buffer. All the incubations were carried out in complete darkness, since light was found to have an adverse effect on the desaturase activity in the microsomes.

Analytical procedures

Reactions were terminated by the addition of 0.15 M-acetic acid and the lipids extracted by a modification of the medium described by Bligh & Dyer (1959). The lower chloroform phase, which contains the complex lipids and the non-esterified fatty acids, was removed and evaporated to dryness under N_2 . The residue was dissolved in a small volume of chloroform and the polar and neutral lipids were purified by t.l.c. on precoated silica-gel plates (Merck; silica-gel 60) with chloroform/methanol/acetic acid/water (170:30:20:7, by vol.) or hexane/diethyl ether/acetic acid (70:30:1, by vol.) respectively. Lipid areas, located by lightly staining with I_2 vapour, were removed from the plates and either assayed for radioactivity or methylated *in situ* with methanolic 2.5% (w/w) HCl (Kates, 1964) for the analysis of the fatty acids. The fatty acid methyl esters were analysed by g.l.c. with a glass column (2 m $\times$ 2 mm) containing 10% BDS on Chromosorb W (HP, 80–100 mesh) and quantified by using methylheptadecanoic acid as an internal standard or analysed by radio-g.l.c. for the determination of the radioactivity in the fatty acids.

Methyl esters of γ -linolenic acid were authenticated by co-chromatography with standard methyl γ -linolenate in the g.l.c. system given above. The retention times of methyl α -linolenate and methyl γ -linolenate, relative to the internal standard methyl heptadecanoate, were 1.96 and 1.80 respectively. Further authentication was achieved by argentation t.l.c. in which the methyl γ -linolenate from seed lipid samples co-chromatographed with trienoate standards. Trace amounts of $C_{20:1}$, $C_{22:1}$ (erucic acid) and $C_{24:1}$ (nervonic acid), present in the seed triacylglycerol, were tentatively identified according to their retention times during g.l.c. relative to authentic standards.

Positional analysis of the fatty acids in phosphatidylcholine was performed by treatment of the lipid with phospholipase A_2 as previously described (Griffiths *et al.*, 1985).

Where the analysis of the acyl-CoA was required, the incubation mixture was supplied with heptadecanoyl-CoA (100 nmol), as an internal standard, immediately before the reactions were terminated and the lipids extracted. The methanol/water phase from the Bligh & Dyer (1959) extraction, which contained all the acyl-CoA, was freed from protein by passing it through a C_{18} silica-gel cartridge as previously described (Stymne & Glad, 1981). The acyl-CoA fraction was further purified by t.l.c. in butan-1-ol/acetic acid/water (5:2:3, by vol.) (Stymne & Stobart, 1985). The gel containing the

Table 1. Fatty acid composition of the major complex lipids in the developing cotyledons of borage

The results are the means of two determinations $\pm$ S.E.M.

Lipid	Fatty acid . . .	Composition (%)					
		C _{16:0}	C _{18:0}	C _{18:1}	C _{18:2}	C _{γ-18:3}	C _{20:1}
Phosphatidylcholine		20 $\pm$ 1	5 $\pm$ 2	20 $\pm$ 1	39 $\pm$ 1	15 $\pm$ 1	1 $\pm$ 0.5
Phosphatidylethanolamine		25 $\pm$ 2	3 $\pm$ 1	7 $\pm$ 1	55 $\pm$ 4	9 $\pm$ 1	1 $\pm$ 0.5
Diacylglycerol		19 $\pm$ 1	6 $\pm$ 1	21 $\pm$ 2	37 $\pm$ 1	16 $\pm$ 2	1 $\pm$ 0.5
Triacylglycerol*		16 $\pm$ 1	6 $\pm$ 1	28 $\pm$ 3	33 $\pm$ 2	17 $\pm$ 1	Trace

* Trace amounts of C_{20:1}, C_{22:1} (erucic acid) and C_{24:1} (nervonic acid) were also present.

acyl-CoA was removed and the fatty acids methylated *in situ* with sodium methoxide. The methyl esters were analysed by g.l.c. and radio-g.l.c. as described above. When the specific radioactivity of the methyl esters from the acyl-CoA was required methyl [¹⁴C]stearate of known specific radioactivity (2045 d.p.m./nmol) was used as an internal standard and co-chromatographed with the sample during the analysis by g.l.c. and radio-g.l.c. It should be noted that no methyl stearate was originally present in the sample.

Lipid samples were assayed for radioactivity in PCS (Amersham/Searle)/xylene (2:1, v/v) scintillant in a Beckman LS-230 liquid-scintillation counter with an efficiency of 94% for ¹⁴C. All radioactivity counts were corrected for background and quenching.

RESULTS

The acyl composition of the major complex lipids in the developing cotyledons of plants grown under greenhouse conditions is given in Table 1. All the lipids examined contained substantial quantities of γ -linolenate. It is noteworthy that the relative distribution of fatty acids in phosphatidylcholine was almost identical with that of the diacylglycerol, and this supports the view that these lipids are in equilibrium during the synthesis of triacylglycerols, as observed in oilseeds of some other species (Slack *et al.*, 1983; Stobart & Stymne, 1985a). The relative abundance of γ -linolenate in triacylglycerol was also similar to that in phosphatidylcholine and diacylglycerol. This would indicate that some γ -linolenate is being utilized in the third acylation step in the final formation of the triacylglycerol. It is also of interest that the triacylglycerol also contained small quantities of erucic acid (C_{22:1}) and nervonic acid (C_{24:1}) (results not shown).

Utilization of [¹⁴C]oleate and [¹⁴C]linoleate in the synthesis of γ -linolenate in cotyledons of borage

The intact and developing cotyledons (batches of five pairs; approx. 25 mg) were incubated with the ammonium salt of either [¹⁴C]oleic acid or [¹⁴C]linoleic acid for 30 min. After that time the cotyledons were thoroughly washed in phosphate buffer, pH 7.2, to remove residual isotope and either extracted immediately for the analysis of lipids or resuspended in fresh buffer for further incubation. At regular intervals after the removal of the excess isotope samples were again taken for analysis. The incorporation of radioactive substrates into the lipid

fraction varied slightly between samples (Table 2), as would be expected in experiments *in vivo* that utilize whole tissues. Because of this difference the results for the distribution of radioactivity in the major lipids are presented as relative values (Table 2). The results show that, even after 30 min incubation in either substrate, the major lipids under investigation (phosphatidylcholine, diacylglycerol and triacylglycerol) contained most of the radioactivity, with the major part being present in the triacylglycerols at all times. After removal of the exogenous [¹⁴C]substrate and subsequent transfer to buffer, similar general trends of radioactive incorporation were observed in both the oleate- and linoleate-fed tissues. The relative amount of radioactivity in triacylglycerol generally increased, whereas that in phosphatidylcholine dramatically declined. The relative radioactivity in the diacylglycerol, on the other hand, remained constant. Complex lipids, other than those given in Table 2, contained 4% or less of the total radioactivity found in the lipid fraction at all incubation times.

To ascertain whether γ -linolenate was synthesized from [¹⁴C]oleate and [¹⁴C]linoleate, the distribution of radioactivity in the C₁₈ unsaturated fatty acids present in the complex lipids was determined (Table 3). The results show that the [¹⁴C]oleate was efficiently desaturated to linoleate and γ -linolenate and that the γ -linolenate accumulated in all the complex lipids that were analysed. The [¹⁴C]linoleate-fed tissues accumulated even greater quantities of radioactive γ -linolenate, and again this was found to accumulate in the complex lipids. The results strongly suggest that the γ -linolenate was synthesized in a sequential fashion from oleate and linoleate, but gave, however, no indication of the true substrate(s) involved.

An analysis of the intramolecular positional distribution of the ¹⁴C-labelled fatty acids in the *sn*-phosphatidylcholine obtained from the cotyledons that had been incubated with [¹⁴C]linoleate showed that [¹⁴C]linoleate was present at the 1 and 2 positions of *sn*-phosphatidylcholine (26 and 56% of the total radioactivity respectively). Radioactive γ -linolenate was, however, only found in position 2 (18% of the total radioactivity) of *sn*-phosphatidylcholine. The observation that the Δ^6 -desaturase did not appear to utilize substrate at position *sn*-1 of *sn*-phosphatidylcholine was given further support from positional analysis of the relative distribution of fatty acid species present in the endogenous microsomal complex lipid. γ -Linolenate was found in abundance at position *sn*-2 of phospho-

Table 2. Incorporation of radioactivity into various lipids in the intact cotyledons of borage incubated in either [¹⁴C]oleate or [¹⁴C]linoleate

Intact cotyledons (fresh weight 25 mg) were incubated with the ammonium salt of either [¹⁴C]oleate or [¹⁴C]linoleate (15.9 nmol; sp. radioactivity 1.26×10^5 d.p.m./nmol in each case) for 30 min. The cotyledons were then rinsed to remove residual label and resuspended in fresh buffer. At regular intervals thereafter the cotyledons were extracted and the radioactivity determined in the individual lipids. For experimental details, see the Materials and methods section.

Substrate	Incubation time (h)	¹⁴ C incorporation into total complex lipids (nmol)	Radioactive distribution among the complex lipids (%)		
			Phosphatidylcholine	Diacylglycerol	Triacylglycerol
[¹⁴ C]Oleate	0.5	6.4	22	25	36
	1	4.5	15	22	49
	2	7.6	10	19	52
	3	7.8	6	20	62
	4	6.8	4	19	64
[¹⁴ C]Linoleate	0.5	8.1	17	23	46
	1	5.7	14	20	44
	2	8.8	8	18	65
	3	9.4	5	20	65
	4	7.2	4	19	68

tidylcholine (32%), with only trace amounts (< 2%) at the *sn*-1 position. On the other hand, both positions of *sn*-phosphatidylcholine contained substantial oleate (15 and 21% in positions *sn*-1 and *sn*-2 respectively) and linoleate (39 and 47% in positions *sn*-1 and *sn*-2 respectively).

Desaturation of [¹⁴C]linoleate in cell-free preparations from developing cotyledons

The previous experiments *in vivo* failed to give an indication of the true substrate (acyl-CoA versus complex lipid) for the synthesis of γ -linolenate. In order to study, therefore, the kinetics of γ -linolenate formation in greater detail, it was essential to obtain cell-free fractions from the developing cotyledons of borage that were active in producing this fatty acid. A number of experiments with seeds of different age and different homogenization and assay conditions were carried out to optimize, as far as possible, the linoleate Δ^6 -desaturase activity in the microsomal preparations. Treatments that enhanced and stabilized the desaturase (see the Materials and methods section) were the inclusion of catalase in the homogenization buffer and in the assay mixtures, the use of bovine serum albumin in the reaction mixtures and the enzyme assays carried out in the absence of light. The addition of NADH was essential for the desaturation of linoleate in the microsomal preparations. Microsomal preparations from the developing cotyledons at stages older than 20 days after flowering (> 6 mg fresh weight/cotyledon pair) gave poor and irreproducible desaturase activity.

Preliminary experiments showed that the microsomal preparations were active in transferring substantial amounts of the [¹⁴C]linoleate in acyl-CoA to phosphatidylcholine. Other observations indicated that a major proportion of synthesized γ -[¹⁴C]linolenate also resided in the microsomal phosphatidylcholine (results not shown). These preliminary results bore a close resemblance to the utilization of the [¹⁴C]oleoyl-CoA and its desaturation to radioactive linoleate observed in

microsomal phosphatidylcholine in preparations from the oilseeds of other species (Stymne & Appelqvist, 1978; Stobart *et al.*, 1983; Stymne & Stobart, 1984b). Experiments were therefore carried out to establish whether [¹⁴C]linoleoyl phosphatidylcholine labelled *in situ* could be desaturated to γ -[¹⁴C]linolenoyl phosphatidylcholine. Microsomal preparations from borage cotyledons were incubated with [¹⁴C]linoleoyl-CoA in the presence of free CoA and bovine serum albumin under non-desaturating conditions (minus NADH) for 20 min. Free CoA and bovine serum albumin were included in the reaction mixture, since they promote the reversible transfer of fatty acids between position 2 of *sn*-phosphatidylcholine and the pool of acyl-CoA, a process that is probably catalysed by an acyl-CoA:lysophosphatidylcholine acyltransferase (Stymne & Stobart, 1984a, 1985). The transfer of [¹⁴C]linoleate in acyl-CoA to the microsomal phosphatidylcholine was terminated after 20 min incubation by diluting the incubation mixture with cold homogenization buffer. The microsomal membranes were recovered by a further centrifugation. The washing of the membranes in fresh buffer removed almost totally the [¹⁴C]acyl-CoA substrate and any non-esterified fatty acids that had been generated during the incubation period. Some 70% of the radioactivity in the pretreated and washed microsomes resided in phosphatidylcholine, of which all was [¹⁴C]linoleate (Table 4). Aliquots of the [¹⁴C]linoleate-labelled microsomes (equivalent to 86 nmol of phosphatidylcholine) were incubated further for 20 and 80 min under both non-desaturating and desaturating (plus NADH) conditions. At these times the radioactive linoleate and γ -linolenate were determined in phosphatidylcholine (Table 4). The results show that appreciable [¹⁴C]linoleate was present in phosphatidylcholine, with no γ -[¹⁴C]linolenate in any of the incubations minus NADH. On the addition of NADH, however, γ -[¹⁴C]linolenate appeared in the phosphatidylcholine and this accumulated with further incubation. In fact 20 and 80 min after the addition of NADH some 14 and 28% of the

Table 3. Relative distribution of radioactivity in the fatty acid components of the major lipids
Intact cotyledons of borage were incubated in either [^{14}C]oleate or [^{14}C]linoleate as described in Table 2. For further experimental details, see the Materials and methods section.

Substrate	Incubation time (h)	[^{14}C]Fatty acid distribution (% in each lipid)							
		Phosphatidylcholine		Diacylglycerol		Triacylglycerol			
		C _{18:1}	C _{18:2}	C _{γ-18:3}	C _{18:1}	C _{18:2}	C _{γ-18:3}	C _{18:1}	C _{γ-18:3}
[^{14}C]Oleate	0.5	66	31	3	65	32	3	Not analysed	—
	1	56	38	6	—	Not analysed	—	78	18
	2	54	34	12	53	38	9	Not analysed	4
	3	66	25	9	63	29	8	56	34
	4	—	Not analysed	—	57	33	10	59	31
[^{14}C]Linoleate	0.5	—	89	11	—	87	13	92	8
	1	—	85	15	—	80	20	93	7
	2	—	76	24	—	85	15	94	6
	3	—	81	19	—	76	24	Not analysed	—
	4	—	83	17	—	74	26	90	10

Table 4. Desaturation of [^{14}C]linoleoyl phosphatidylcholine labelled *in situ* in microsomal preparations from the developing cotyledons of borage

Microsomal preparations (equivalent to 860 nmol of phosphatidylcholine) were prelabelled with [^{14}C]linoleate by incubation for 20 min at 25 °C with 400 nmol of [^{14}C]linoleoyl-CoA, 400 nmol of CoA and 20 mg of bovine serum albumin in 0.1 M-phosphate buffer, pH 7.2, in a total volume of 2 ml. The reaction mixture was then diluted to 25 ml with cold homogenization buffer and the microsomes collected by centrifugation at 105000 *g* for 60 min. After resuspension of the microsomes, aliquots (equivalent to 86 nmol of phosphatidylcholine) were incubated with (2 μmol) or without NADH for the times stated in the Table. Results are, where indicated, the means $\pm$ S.E.M. for two incubations.

Incubation time (min)	NADH	[^{14}C]Radioactivity (nmol)					
		Phosphatidylcholine		Non-esterified fatty acids	Other complex lipids		
		C _{18:2}	C _{γ-18:3}		C _{18:2}	C _{γ-18:3}	
0	—	12.7	0	0.1*	4.7		0
20	—	11.8 $\pm$ 0	0	0.2 $\pm$ 0*		5.3 $\pm$ 0*	
80	—	9.7 $\pm$ 0.3	0	0.4 $\pm$ 0.1*		5.7 $\pm$ 0*	
20	+	10.1 $\pm$ 0.1	1.7 $\pm$ 0.1	0.2 $\pm$ 0*		5.3 $\pm$ 0*	
80	+	7.6 $\pm$ 0.1	3.0 $\pm$ 0.1	0.5 $\pm$ 0*	5.5 $\pm$ 0.2		1.1 $\pm$ 0.1

* Distribution of radioactivity not determined.

radioactivity in phosphatidylcholine was present in γ -linolenate respectively. After 80 min incubation with NADH, 16% of the radioactivity in the remaining complex lipids was also in γ -linolenate.

A quantitative analysis of the fatty acids in the microsomal phosphatidylcholine in the incubations was also carried out (Table 5). The results show that the major fatty acid in phosphatidylcholine in the untreated microsomal preparations was linoleate and that oleate and γ -linolenate amounted to 15 and 18% of the acyl species present respectively. The mass of linoleate in phosphatidylcholine increased in microsomes pretreated with [^{14}C]linoleoyl-CoA. After the addition of NADH, however, there was an increase in the mass of γ -linolenate from 16 to 19% after 20 min incubation and this reached 24% after 80 min. The increase in the mass of γ -linolenate was concomitant with the desaturation of [^{14}C]linoleate to yield radioactive γ -linolenate (see Table 4). It should be noted that little change was observed in the fatty acid pattern of phosphatidylcholine in incubations lacking NADH and that no significant change in the total mass of the microsomal phosphatidylcholine had occurred. It is also of interest that, upon the addition of NADH, the oleate in phosphatidylcholine decreased from 14% at zero time to 6% after 80 min incubation (Table 5). Under the conditions of these experiments, little change was observed in the mass of linoleate in phosphatidylcholine during desaturation. A proportion of the γ -[^{14}C]linolenate (approx. 25%) that was produced in the microsomes upon the addition of NADH and at the prolonged incubation time was also associated with complex lipids other than phosphatidylcholine (Table 4). It is also of interest that the total radioactivity in phosphatidylcholine declined somewhat after 80 min incubation under both desaturating and non-desaturating conditions (Table 4).

The kinetics of the desaturation *in situ* (see above) were consistent with the activity of a linoleoyl phosphatidylcholine Δ^6 -desaturase enzyme in the microsomal membranes. It could still be argued, however, that the [^{14}C]linoleate, in these experiments, may have been transferred from the phosphatidylcholine to acyl-CoA

and there desaturated to γ -[^{14}C]linolenate, which, in its turn, was rapidly re-incorporated back into the complex lipid. In order to assess this possibility, experiments were designed in which active microsomal membranes were incubated with [^{14}C]linoleoyl-CoA and non-radioactive γ -linolenoyl-CoA in the presence of NADH. The rationale for this was that, if the linoleate Δ^6 -desaturase enzyme utilized the acyl-CoA substrate, then the newly formed γ -[^{14}C]linolenoyl-CoA would be rapidly diluted by the large amount of added non-radioactive γ -linolenoyl-CoA. Hence the subsequent incorporation of γ -[^{14}C]linolenate from acyl-CoA to the phosphatidylcholine would be from a pool of γ -linolenate with a lowered specific radioactivity. The specific radioactivity of the γ -[^{14}C]linolenate transferred to phosphatidylcholine would be even further decreased by the endogenous γ -linolenate present in this complex lipid. The specific radioactivity of the γ -linolenate would therefore be greater in the acyl-CoA fraction than in the phosphatidylcholine. If, on the other hand, the γ -linolenate was synthesized in phosphatidylcholine, then its specific radioactivity would be greater in the complex lipid than in the pool of acyl-CoA.

In a typical experiment a microsomal preparation (equivalent to 192 nmol of phosphatidylcholine) was incubated with 65 nmol of [^{14}C]linoleoyl-CoA and 100 nmol of non-radioactive γ -linolenoyl-CoA under desaturating conditions. After 40 and 90 min incubation the radioactive distribution of [^{14}C]linoleate and γ -[^{14}C]linolenate and their specific radioactivity were determined in the acyl-CoA fraction, phosphatidylcholine and the total remaining complex lipids. The results (Table 6) showed that, after 40 min incubation, some 14% of the radioactivity in the phosphatidylcholine was present as γ -[^{14}C]linoleate and this was equivalent to 2 mol of desaturated [^{14}C]linoleate substrate. At that incubation time no γ -[^{14}C]linolenate was detected in the acyl-CoA fraction, although some 60 nmol of the non-radioactive γ -linolenoyl-CoA was still present (Table 6). At the longer incubation time the γ -[^{14}C]linolenate in phosphatidylcholine had increased to 23% (equivalent to 5.5 nmol of desaturated

Table 5. Fatty acid composition of the phosphatidylcholine in microsomal preparations prelabelled with [14 C]linoleate and incubated in the absence or presence of NADH

Microsomal preparations from the developing cotyledons of borage, pre-labelled with [14 C]linoleate by incubation with [14 C]linoleoyl-CoA as described in the legend to Table 6, were incubated for various time periods in the presence (2 μ mol) or absence of NADH. The incubations are the same as those reported in Table 6. The total mass of the fatty acids in phosphatidylcholine at each incubation is calculated on the average fatty acid content in that lipid for all incubations (171 ± 15 nmol). For experimental conditions, see the Materials and methods section. Results, where indicated, are the means $\pm$ s.e.m. for two incubations. Values in parentheses are the relative (%) concentrations of the fatty acids in the lipid.

	Incubation time (min)	NADH	Fatty acid . . .	Distribution in phosphatidylcholine (nmol)										
				C _{16:0}	C _{18:0}	C _{18:1}	C _{18:2}	C _{γ-18:3}	C _{20:1}					
Untreated microsomes	—	—	26.4	(15)	8.9	(5)	25.2	(15)	76.5	(45)	31.0	(18)	3.4	(2)
Pre-labelled microsomes	0	—	24.0	(14)	8.9	(5)	23.8	(14)	82.8	(48)	28.1	(16)	3.6	(3)
	20	—	21.2 ± 2.0	(12)	8.6 ± 0.4	(5)	23.9 ± 0.6	(14)	84.2 ± 1.2	(49)	29.3 ± 0.7	(17)	4.2 ± 1.1	(3)
	80	—	23.6 ± 0.6	(14)	8.4 ± 0	(5)	23.6 ± 0.1	(14)	83.9 ± 0.4	(49)	27.5 ± 0.1	(16)	4.1 ± 0	(2)
	20	+	23.4 ± 0.1	(14)	8.7 ± 0.2	(5)	16.0 ± 0.1	(9)	87.1 ± 0.3	(51)	32.1 ± 0.1	(19)	3.7 ± 0.2	(2)
	80	+	24.3 ± 0.6	(14)	8.6 ± 0.2	(5)	10.9 ± 0.1	(6)	83.1 ± 0.3	(49)	40.6 ± 0.2	(24)	3.6 ± 0	(2)

Table 6. Desaturation of [¹⁴C]linoleate in a microsomal preparation incubated with [¹⁴C]linoleoyl-CoA in the presence of non-radioactive γ -linolenoyl-CoA

Microsomal preparations from the developing cotyledons of borage (equivalent to 192 nmol of phosphatidylcholine) were incubated with 65 nmol of [¹⁴C]linoleoyl-CoA, 100 nmol of non-radioactive γ -linolenoyl-CoA and NADH (2 μ mol) at 25 °C for 40 and 90 min. For incubation conditions and analytical procedures, see the Materials and methods section. Results are, where indicated, the means $\pm$ S.E.M. for two incubations.

Lipid fraction	Incubation time (min)	Linoleate			γ -Linolenate		
		¹⁴ C* (nmol)	Mass (nmol)	10 ⁻² $\times$ sp. radioactivity (d.p.m./nmol)	¹⁴ C* (nmol)	Mass (nmol)	10 ⁻² $\times$ sp. radioactivity (d.p.m./nmol)
Acyl-CoA	0†	74.2	66.3	29	0	98.9	0
	40	36.9 $\pm$ 0.9	43.0 $\pm$ 0.4	22 $\pm$ 1	0 (< 0.2)	59.2 $\pm$ 0.1	0
	90	15.6 $\pm$ 1.2	26.2 $\pm$ 0.5	15 $\pm$ 1	1.0 $\pm$ 0.1	40.9 $\pm$ 0.7	0.6 $\pm$ 0.1
Phosphatidylcholine	0†	0	161	0	0	62.2	0
	40	12.6 $\pm$ 0.1	173 $\pm$ 1	2.1 $\pm$ 0.1	2.1 $\pm$ 0.0	88.9 $\pm$ 0.6	0.7 $\pm$ 0.1
	90	19.3 $\pm$ 0	168 $\pm$ 0	2.8 $\pm$ 0.1	5.6 $\pm$ 0.3	99.2 $\pm$ 0.5	1.4 $\pm$ 0.1
Other complex lipids	0†	0	n.a.‡	—	0	n.a.	—
	40	5.7 $\pm$ 0.1	n.a.	n.a.	0.3 $\pm$ 0.0	n.a.	n.a.
	90	9.0 $\pm$ 0.1	n.a.	n.a.	0.9 $\pm$ 0.1	n.a.	n.a.

* nmol of ¹⁴C is calculated on the basis of the original sp. radioactivity of the [¹⁴C]linoleoyl-CoA substrate (26 $\times$ 10² d.p.m./nmol).

† About 5 s.

‡ n.a., not analysed.

[¹⁴C]linoleate substrate) and small amounts were now present in the acyl-CoA fraction (equivalent to 1 nmol of desaturated substrate). The mass of γ -linolenate in the acyl-CoA pool also decreased substantially during the times of incubation. The specific radioactivity of the γ -linolenate in phosphatidylcholine was twice that observed in the acyl-CoA. The results therefore strongly support the findings of the previous experiments that the synthesis of γ -linolenate in the developing cotyledons of borage utilizes the substrate linoleoyl phosphatidylcholine and not the acyl-CoA. It is also of interest to note that small amounts of γ -[¹⁴C]linolenate (12% of the total γ -[¹⁴C]linolenate, Table 6) were present in lipids other than phosphatidylcholine after 40 min incubation and at a time when no radioactive γ -linolenate was found in the acyl-CoA fraction.

DISCUSSION

The developing seed cotyledons of *Borago officinalis* contained substantial quantities of γ -linolenate in their storage oils and polar lipids (Table 1). In experiments *in vivo* the cotyledons carried out a sequential desaturation of oleate and linoleate to yield γ -linolenate (Table 3). It was not possible, however, to ascertain the substrate for the Δ^6 -linoleate desaturase in these experiments with whole tissue. The maturing cotyledons from the seeds, however, yielded, when grown under suitable conditions, consistently active microsomal membrane preparations that were capable of synthesizing γ -linolenate from exogenously supplied linoleoyl-CoA. The desaturation of [¹⁴C]linoleoyl phosphatidylcholine *in situ* resulted in the formation of radioactive γ -linolenoyl phosphatidylcholine and indicated that the substrate for the linoleate Δ^6 -desaturase was probably the complex lipid phosphatidylcholine (Table 4). That phosphatidylcholine was in fact the major substrate for the Δ^6 -desaturase was confirmed in experiments in which the specific radioactivity of the γ -[¹⁴C]linolenate in acyl-CoA and

phosphatidylcholine was measured during the desaturation of [¹⁴C]linoleate in the presence of non-radioactive γ -linolenoyl-CoA (Table 6). It is also interesting to note the changes in the mass of the fatty acids in phosphatidylcholine during desaturation (Table 5). There appears to be an inverse relationship between the desaturation of oleate and the appearance of γ -linolenate, whereas little change was observed in the linoleate content during this period of incubation. Since the γ -linolenate is being synthesized from oleate via linoleate (Table 3), this would indicate that, in the microsomal preparations, the activities of the oleate Δ^{12} -desaturase and linoleate Δ^6 -desaturase enzymes are similar and hence, in the presence of NADH, the pool of linoleate is kept at a constant, steady-state, level. It is also of interest, in this context, that the fatty acid composition of the seed oils from a large number of different borage populations also appeared to exhibit an inverse relationship with the content of oleate and γ -linolenate (U. Nyman, personal communication). It would seem, therefore, that the regulation in borage of the oleate Δ^{12} -desaturase and linoleate Δ^6 -desaturase enzymes is closely related and that any induction in one is closely paralleled in the other.

Some of the γ -linolenate that was produced in the microsomes incubated with NADH was, certainly at the longer times, associated with complex lipids other than phosphatidylcholine (Table 4). Furthermore, a decrease in the radioactivity in phosphatidylcholine was also observed, and this occurred even under non-desaturating conditions (Table 4). These observations indicate that the γ -linolenate in phosphatidylcholine is being made available, perhaps via acyl exchange with a small pool of acyl-CoA (Stymne *et al.*, 1983), for transfer to other lipids. It is of course a possibility that the linoleate Δ^6 -desaturase can utilize, to some extent, the fatty acid in complex lipids other than phosphatidylcholine. It is also noteworthy that radioactive γ -linolenate appeared in complex lipids before it was detected in acyl-CoA

(Table 6), a result that is probably due to the reverse reaction of a CDP-choline:diacylglycerol choline-phosphotransferase, an enzyme known to operate in microsomal preparations from other oilseed species (Slack *et al.*, 1985; Stobart & Stymne, 1985a). Although acyl exchange between acyl-CoA and phosphatidylcholine was not measured *per se* in these experiments, it was nonetheless strongly evident, particularly in the final experiment in which there was a dramatic fall in the specific radioactivity of the linoleate in the acyl-CoA fraction (Table 6).

The Δ^6 -desaturase appears only to utilize as substrate the linoleate in position 2 of *sn*-phosphatidylcholine. The intramolecular specificity of the linoleate Δ^6 -desaturase was also evident from the fact that γ -linolenate was largely present in position 2 of endogenous, microsomal, *sn*-phosphatidylcholine, whereas linoleate was present in both positions, *sn*-1 and *sn*-2. The oleate Δ^{12} -desaturase in a high-linoleate variety of safflower (*Carthamus tinctorius*) was found to act on the oleate in positions *sn*-1 and -2 of microsomal phosphatidylcholine (Slack *et al.*, 1979; Stobart & Stymne, 1985b). In view of the distinction made by the linoleate Δ^6 -desaturase for the linoleate in positions *sn*-1 and -2 of phosphatidylcholine, it is possible that different desaturase enzymes exist that are positionally specific for substrate.

Contrary to our original speculation (see the Introduction) on the direct substrate for the linoleate Δ^6 -desaturase in plants, the experimental evidence unequivocally illustrates that the major substrate is linoleoyl phosphatidylcholine and that the acyl-CoA is a most unlikely candidate. It would seem, therefore, that perhaps all the desaturase enzymes in higher plants, and in particularly their seeds, that utilize oleate and linoleate, do so on a complex lipid substrate irrespective of where the centres of unsaturation are being inserted into the acyl chain. The formation of γ -linolenate in plants is particularly interesting since it is the only C_{18} polyunsaturated fatty acid whose synthesis can be compared directly with that of an animal counterpart. An explanation of why higher plants have evolved desaturase enzymes that utilize complex lipids would not appear to be based on a direct mechanistic reason. If one accepts teleological argument then one might conject that perhaps complex lipid substrates are more efficient in plants in that the desaturase enzymes are not then competing directly with other major reactions that utilize acyl-CoA.

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