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Biosynthesis of C_{20} and C_{22} Fatty Acids by Developing Seeds of *Limnanthes alba*

CHAIN ELONGATION AND Δ5 DESATURATION[1]

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ABSTRACT

The storage triacylglycerols of meadowfoam (*Limnanthes alba*) seeds are composed essentially of C_{20} and C_{22} fatty acids, which contain an unusual Δ5 double bond. When [1-^{14}C]acetate was incubated with developing seed slices, ^{14}C-labeled fatty acids were synthesized with a distribution similar to the endogenous fatty acid profile. The major labeled product was *cis*-5-eicosenoate, with smaller amounts of palmitate, stearate, oleate, *cis*-5-octadecenoate, eicosanoate, *cis*-11-eicosenoate, docosanoate, *cis*-5-docosenoate, *cis*-13-docosenoate, and *cis*-5,*cis*-13-docosadienoate. The label from [^{14}C]acetate and [^{14}C]malonate was used preferentially for the elongation of endogenous oleate to produce *cis*-[^{14}C]11-eicosenoate, *cis*-13-[^{14}C]docosenoate, and *cis*-5,*cis*-13-[^{14}C]docosadienoate and for the elongation of endogenous palmitate to produce the remaining C_{20} and C_{22} acyl species. The Δ5 desaturation of the preformed acyl chain and chain elongation of oleate and palmitate were demonstrated *in vivo* by incubation of the appropriate 1-^{14}C-labeled free fatty acids. Using [1-^{14}C]acyl-CoA thioesters as substrates, these enzyme activities were also demonstrated *in vitro* with a cell-free homogenate.

Meadowfoam (*Limnanthes alba*), a spring flower native to moist habitats in northern California and southern Oregon, has recently aroused interest as a potential new oilseed crop (10). The triacylglycerol fraction of the mature seed is composed principally of 20:1 (5c), 22:1(13c), and 22:2(5c13c) fatty acids (13, 20).[3] Our interest in this plant seed stems from the fact that the developing seed contains the enzymes necessary for the biosynthesis of C_{20} and C_{22} acids. The fatty acids also contain an unusual Δ5-*cis* double bond. This study complements our concurrent studies on long-chain fatty acid biosynthesis using developing seeds from nasturtium (17), jojoba (15, 16), and rapeseed.

The biosynthesis of 20:1(11c) and 22:1(13c) in oilseeds by chain elongation of preformed oleate rather than by a complete *de novo* biosynthesis is now a well-documented phenomenon (1, 5, 15–17). Our recent studies with cell-free extracts from developing jojoba cotyledons have shown that long-chain acyl-CoA thioesters, including oleoyl-CoA, and also stearoyl-ACP, are elongated in the presence of malonyl-CoA and NADPH (or NADH) (16). The probable pathway for erucate biosynthesis involves the synthesis of oleoyl-ACP from acetate by enzymes utilizing ACP-thioesters as substrates, the hydrolysis of oleoyl-ACP to oleic acid, its transfer to another compartment where reactivation to oleoyl-CoA, and the subsequent elongation of oleoyl-CoA occurs (15, 16).

This paper presents data from *in vivo* studies with immature meadowfoam seeds designed to elucidate the pathway to these unusual acids. Preliminary *in vitro* studies confirm this pathway and open the way to extensive characterization of the enzyme activities.

MATERIALS AND METHODS

[^{14}C]-labeled Substrates. [1-^{14}C]Acetate (58 Ci/mol), [2-^{14}C]pyruvate (3.7 Ci/mol), D-[U-^{14}C]glucose (333 Ci/mol), [^{14}C]bicarbonate (44 Ci/mol), [1-^{14}C]malonic acid (3 Ci/mol), [1-^{14}C]stearic acid (51 Ci/mol), and [1-^{14}C]oleic acid (56 Ci/mol) were purchased from New England Nuclear. [1-^{14}C]Palmitic acid (56 Ci/mol) and [1-^{14}C]linoleic acid (51 Ci/mol) were purchased from Amersham. [1-^{14}C]Arachidic acid (55 Ci/mol) was obtained from Applied Sciences Laboratories, Inc. (State College, Pa.). [2-^{14}C]Malonic acid (22 Ci/mol) was purchased from ICN (Albany, CA). *cis*-11-[1-^{14}C]Eicosenoic acid (48 Ci/mol), *cis*-13-[1-^{14}C]docosenoic acid (48 Ci/mol), [1-^{14}C]oleoyl-CoA (3.7 Ci/mol), [1-^{14}C]eicosenoyl-CoA (3.1 Ci/mol), and [1-^{14}C]docosenoyl-CoA (4.8 Ci/mol) were available from a previous study (16). [1-^{14}C]Palmitoyl-CoA (58 Ci/mol) and [1-^{14}C]stearoyl-CoA (57 Ci/mol) were purchased from Rosechem Products, Ltd. (Hollywood, CA). [1-^{14}C]Arachidyl-CoA (10.4 Ci/mol) was prepared from the corresponding acid as described previously (16).

Seed Tissue. *L. alba* seeds were the kind gift of Professor S. K. Jain, University of California, Davis. The plants were grown in the growth chamber, in a mixture of equal volumes of sand, soil, and peat moss. Germination and early growth was at 16 to 18 C with a 10-h photoperiod. This was altered to 24 C (daytime temperature) with a 15-h photoperiod after several weeks' growth. The flowers were hand-pollinated, and the immature seeds were used for experiments at the age of 13 to 19 days after pollination.

***In Vivo* Incubations.** Fresh, developing seeds were sliced in half with a razor blade and the green cotyledon (average weight, about 10 mg) was squeezed from the translucent seed coat between the fingertips. About 150 mg cotyledonous tissue was incubated with the ^{14}C-labeled substrate (1–5 μCi) in 0.5 ml 0.1 M Na-phosphate buffer (pH 6.0). Incubations were generally for 6 h, in open test tubes, in a reciprocating water bath at 26 C. Free 1-^{14}C-labeled fatty acids were added to the buffer as their ammonium salts in aqueous ethanol (5 μl). For the experiment where optimum ^{14}C-labeled saturated fatty acid formation from [1-^{14}C]acetate was

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[3] Fatty acid structures may be given in one of three ways: erucic acid (common name), *cis*-13-docosenoic acid (systematic name), or 22:1(13c) (abbreviation). Abbreviations: ACP, acyl carrier protein; DEGS, diethylene glycol succinate.

required, about 300 mg cotyledonous tissue was used/tube. The tube was flushed with N_2 and tightly capped. Incubations with [^{14}C]bicarbonate (20 μCi) were done at pH 8.0 in capped tubes under strong illumination.

Incubations were terminated by the addition of isopropanol (1 ml) and heating at 80 C for 5 min. The seeds were homogenized in chloroform-methanol (2:1, v/v) (8 ml), and the mixture was left to stand overnight to facilitate extraction of the lipids. Washing with 0.7% saline (2 ml) gave a chloroform layer containing the lipids which, after acidification with a drop of glacial acetic acid, was evaporated to dryness under a stream of N_2.

***In Vitro* Incubations.** A cell-free homogenate was made by grinding developing seed tissue in 2 volumes of buffer (80 mM Hepes, 0.3 M sucrose, 5 mM ascorbate, 2 mM DTT (pH 7.2), containing 4 mg/ml defatted BSA) in a chilled pestle and mortar. The resulting paste was filtered through Miracloth to give a cell-free preparation. Each incubation contained the following in a total volume of 0.5 ml (pH 7.2): sucrose, 0.3 M; Hepes, 80 mM; ascorbate, 2.5 mM; DTT, 1 mM; $MgCl_2$, 5 mM; $MnSO_4$, 5 mM; ATP, 2 mM; NADH, 0.5 mM; NADPH, 0.5 mM; malonyl-CoA, 0.2 mM; enzyme preparation, 0.25 ml; and ^{14}C-labeled substrate. Incubations were for 1 h in open tubes in a reciprocating water bath at 26 C. The reactions were usually terminated by the addition of 5% methanolic KOH (3 ml) and saponification was achieved by heating at 80 C for 1 h. After acidification, the fatty acids were extracted into petroleum ether (bp 30 to 60 C) and treated with an ethereal diazomethane solution to produce the methyl esters.

For analysis of the lipid classes, the incubation was terminated by the addition of isopropanol (1 ml) followed by heating at 80 C for 5 min. Lipids were extracted using petroleum ether-isopropanol (3:2, v/v) (7), leaving the acyl-CoA in the aqueous phase (16).

Lipid Analysis and Acyl Group Degradation. Lipid analysis by TLC and by GLC, acyl group degradation by reductive ozonolysis (21), chemical α-oxidation (8), and controlled, stepwise chemical decarboxylation (4) in order to locate the position of the ^{14}C label in the acyl chain are described in a companion paper (17).

Either 10% DEGS- or 10% SP-2330 (*ex* Supelco Inc., Bellefonte, PA)-packed columns were routinely used for GLC analysis, but samples were also run on a 10% SP-1000 column when the separation of 18:3 and 20:1 was required or as an aid to the identification of ozonolysis products. As base line separation of [^{14}C]20:0 and [^{14}C]20:1(5c) could not be achieved on the polar columns (Fig. 1) quantitation of [^{14}C]20:0 and [^{14}C]20:1(5c) was also done by argentation TLC or by ozonolysis.

Analysis of Acyl Composition of Triacylglycerols Separated by Degree of Unsaturation. A triacylglycerol rich fraction was obtained from mature seeds by exhaustive Soxhlet extraction with petroleum ether. Triacylglycerols were purified by Silica Gel G TLC [petroleum ether-diethyl ether-acetic acid (80:20:1, v/v), single development] and chromatographed on 5% (by weight) $AgNO_3$ in Silica Gel G TLC plates triply developed with benzene-chloroform (75:25, v/v). Triene (R_F, 0.62), tetraene (R_F, 0.43), and pentaene (R_F, 0.25) bands were located by spraying with 2′,7′-dichlorofluorescin methanolic solution (0.1% by weight) and viewing under UV light. The bands were scraped directly into transmethylation reagent (methanol-benzene-sulfuric acid (20:10:1, v/v/v) and, after the addition of heptadecanoic acid as an internal standard, the mixture was refluxed for 3 h. The methyl esters were analysed on a GC equipped with a thermal conductivity unit of mass detection. A 10% DEGS-PS on 80/100 Supelcoport stainless steel column (5.48 m × 0.65 cm) was used. The appropriate correction factors were used to convert area under the mass trace to moles of fatty acid.

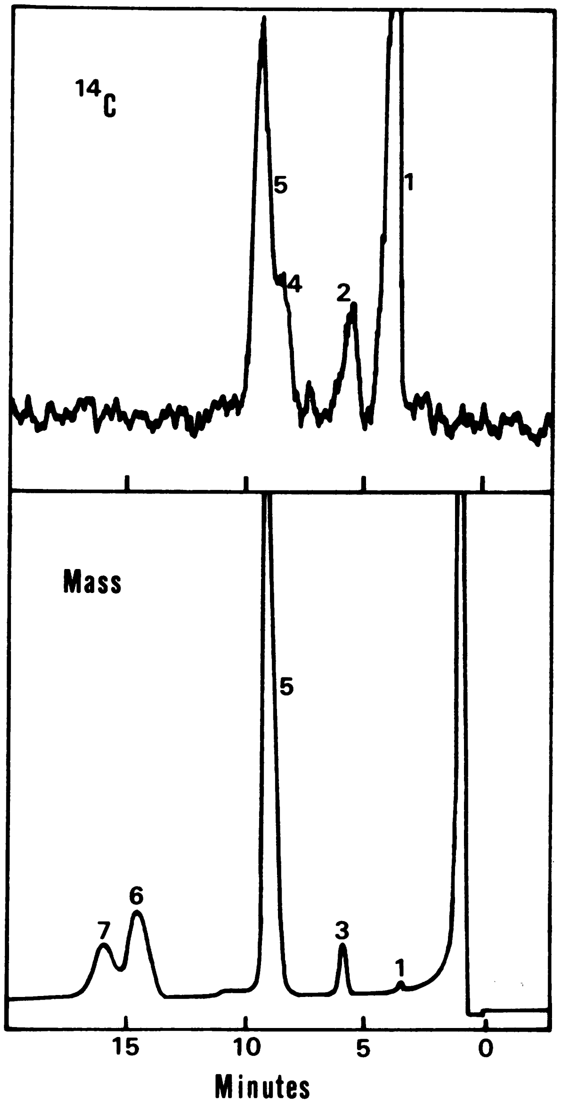

FIG. 1. Gas-liquid chromatographic pattern (10% SP-2330 packed column at 170 C) of the ^{14}C-labeled fatty methyl esters recovered after incubation of [1-^{14}C]palmitoyl-CoA with a cell-free homogenate from developing meadowfoam seeds in the presence of malonyl-CoA, NADH, and NADPH. The lower (mass) tracing shows the endogenous acyl groups, the upper tracing shows the ^{14}C activity. The peaks are labeled as follows: 1, 16:0; 2, 18:0; 3, 18:1; 4, 20:0; 5, 20:1(5c); 6, 22:1(13c); and 7, 22:2(5c13c).

RESULTS

Incorporation of [1-^{14}C]Acetate into Acyl Lipids. In *L. alba* seeds, the endogenous C_{20} and C_{22} fatty acids were confined to the triacylglycerols (the principal lipid present) and to diacylglycerols, whereas palmitate, oleate, linoleate, and linolenate are found in the polar lipids.[4] The band eluting with an R_F corresponding to that of phosphatidylethanolamine and of digalactosyl diglyceride after TLC in a chloroform-methanol-H_2O (65:25:4, v/v) solvent system probably contained both since a positive reaction was obtained with both phospholipid- and glycolipid-sensitive spray reagents (3). The relative amounts were not quantitated.

[1-^{14}C]Acetate was extensively incorporated into acyl lipids over a 6-h incubation with immature seed tissue. For the experiment reported in Table I, this incorporation was 62.5%. Table I shows the mass and ^{14}C-labeled acyl distributions for the major lipid classes, as well as the per cent ^{14}C distribution between these lipid

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[4] "Polar lipids" is a term used to describe the lipids remaining at the origin after TLC with Silica Gel G in a petroleum ether-diethyl ether-acetic acid (80:20:1, v/v) solvent system.

Table I. *Mass and [^{14}C]Acyl Distribution in Lipid Classes in Meadowfoam Seeds after Incubation with [1-^{14}C]Acetate*

Lipid Fraction	^{14}C in Lipid Fraction	Acyl Distribution								
		16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:1	22:2
	%					%				
Total										
^{14}C distribution	100	3.5	1	2			10	57[a]	18[b]	9
Mass distribution		1.5		5.5	1.5	4	2	60.5	15[b]	10
Triacylglycerols										
^{14}C distribution	83		1				10	61[a]	18.5[b]	9
Mass distribution				1[a]			1.5	70.5[a]	16.5[b]	10.5
Diacylglycerols (1,2 plus 1,3)										
^{14}C distribution	4.5		1				13	53[a]	21[b]	10.5
Mass distribution				5[a]			2	60.5[a]	22.5[b]	9.5
Total polar lipids										
^{14}C distribution	7.0	54	11	22[c]			1	11.5[d]		
Mass distribution		12	1	42.5[c, e]	6.5	37.5				
Phosphatidylcholine										
^{14}C distribution	3.6	43	15	27[c]			2	13		
Mass distribution		19	1	58[c, e]	5	16		1		
Phosphatidylethanolamine + digalactosyl diglyceride										
^{14}C distribution	1.3	52	20	11.5[c]			2	13.5		
Mass distribution		13.5	1	32.5[c, e]	9.5	43				

[a] Predominantly the $\Delta5$ isomer.

[b] Predominantly the $\Delta13$ isomer.

[c] $\Delta9$ isomer.

[d] Free fatty acids contained 3% of the label.

[e] May include 16:3 as well as 18:1.

classes.

[1-^{14}C]Acetate was incorporated into every fatty acid with the exceptions of linoleate and linolenate, *i.e.* 16:0, 18:0, 18:1(5c), 18:1(9c), 20:0, 20:1(5c), 20:1(11c), 22:1(5c), 22:1(13c), and 22:2(5c13c). Labeled C_{20} and C_{22} acyl groups accounted for 94% of the total radioactivity and were found principally in triacylglycerols. Labeled palmitate, stearate, and oleate were confined to the polar lipids. Although 20:1(5c) is not found in the endogenous polar lipids, a small amount of this labeled fatty acid was detected in this fraction (about 1% of the total ^{14}C-labeled acyl lipid).

The use of anaerobic incubation conditions increased the percentage of ^{14}C-labeled saturated fatty acids from 13.5% to 80% (Table II). The bulk of this change came from the disappearance of 20:1(5c) and the appearance of 20:0.

Incubation with ^{14}C-labeled Precursors other than Acetate. Labeled acetate, malonate, pyruvate, D-glucose, and bicarbonate were incorporated into acyl lipids with decreasing efficiency (Table III). However, as low concentrations of high specific activity substrates were used in order to obtain enough labeled lipid for analysis, the figures quoted in Tables III and IV for per cent incorporation are not necessarily synonymous with saturating levels of incorporation. [^{14}C]Bicarbonate was incorporated into the tissue to 9.5% in 8 h, of which 9% was H_2O-soluble metabolites and only 0.5% was ^{14}C-labeled lipid. The ^{14}C-acyl distributions for all the precursors were similar to [1-^{14}C]acetate, the single exception being that [2-^{14}C]malonate did not produce much [^{14}C]palmitate (Table III).

Table II. *[^{14}C]Acyl Distribution in Meadowfoam Seeds after Aerobic and Anaerobic Incubation with [1-^{14}C]Acetate*

Incubation Condition	^{14}C Incorporated into Lipid	Acyl Distribution							
		16:0	18:0	18:1	20:0	20:1	22:0	22:1	22:2
	%				%				
Air, 150 mg tissue	62.5	3.5	1	2	10	57		18	9
N_2, 300 mg tissue	50	14.5	11		53	11	1.5	9	

Table IV shows the metabolism of free 1-^{14}C-labeled fatty acids *in vivo*. Double-bond position of the products was confirmed by ozonolysis. The chain elongated and $\Delta5$-desaturated products were incorporated preferentially, but not exclusively, into triacylglycerols. The data in Table IV clearly demonstrate that the pathways of elongation and desaturation of palmitate and oleate shown in Figure 2 are indeed operative. [1-^{14}C]Linoleic acid was also tested *in vivo*, and label was incorporated into triacylglycerols (3.5%). This fraction contained labeled 18:2(9c12c) (20%), 20:1(5c) (65%), 22:1 (8%), and 22:2 (7%), but not the products of a direct chain elongation or $\Delta5$ desaturation of linoleate. These results suggest extensive degradation of [^{14}C]linoleate and use of the label released for the synthesis of long-chain acids.

Distribution of Label along Acyl Chain of ^{14}C-labeled Lipids Produced from Various ^{14}C-labeled Precursors. The ^{14}C-labeling patterns along the acyl chain were analyzed by ozonolysis, controlled decarboxylation, and α-oxidation (Table V). Interpretations are complicated by the presence of two positional isomers in the 20:1 and 22:1 fractions, but the data are consistent with 20:1(11c), 22:1(13c), and 22:2(5c13c) being produced by an elongation of oleate and with 18:1(5c), 20:0, and 20:1(5c) being produced by an elongation of palmitate, although, in this latter case, a very small contribution by an elongation of stearate cannot be ruled out. For example, decarboxylation of [^{14}C]20:0 produced from [1-^{14}C]acetate shows C(1) and C(3) to be predominantly and equally labeled, with 16% of the total label in the remainder of the chain (Table V, Experiment 1). [^{14}C]Eicosenoate derived from [1-^{14}C]acetate is composed of 5-*cis* and 11-*cis* isomers. The 11-*cis* isomer, from its degradation by ozonolysis, is known to account

Table III. *Incorporation of ^{14}C-labeled Precursors (Other Than Free Fatty Acids) into ^{14}C-labeled Acyl Lipids in Meadowfoam Seeds*

^{14}C-labeled Substrate	^{14}C Incorporated into Lipid	^{14}C as		[^{14}C]Acyl Distribution in Total Lipids[a]							
		Triacylglycerols	Polar lipids	16:0	18:0	18:1	20:0	20:1	22:0	22:1	22:2
	%	%		%							
[1-^{14}C]Acetate	62	47	29.5	18.5	10.5	4.5	24.5	35.5	2	3.5	0.5
[2-^{14}C]Malonate	20	56.5	22	2	10	3	39	40	2	3.5	0.5
[2-^{14}C]Pyruvate	5	48.5	32.5	23.5	9	9	25	30	2	3	0.5
[U-^{14}C]Glucose	2[b]	23.5	62.5	25	12	4	31	26.5		1.5	
[$^{1}\alpha$4C]Bicarbonate[c]	0.5			1	2	2	5	64	1	10	6

[a] The higher amounts of ^{14}C-labeled saturates result from a larger amount of tissue being used (300 mg) than normal (150 mg).

[b] Only half of this activity was recovered as ^{14}C-labeled acyl groups after transmethylation.

[c] A separate, 8-h incubation with 150 mg tissue. [^{14}C]Linoleate (9%) was noted in the acyl lipids.

Table IV. *Incubation of Free 1-^{14}C-labeled Fatty Acids with Immature Meadowfoam Seeds*

1-^{14}C-labeled Fatty Acid	^{14}C Incorporated into[a]		[^{14}C]Acyl Distribution in Triacylglycerols[b]								
	Triacylglycerols	Polar Lipids	16:0	18:0	18:1 (5c)	18:1 (9c)	20:0	20:1 (5c)	20:1 (11c)	22:1 (13c)	22:2 (5c13c)
	%		%								
16:0[b]	37.5	17.5	14	4.5	3.5		10.5	67.5			
18:0[b]	14.5	3.0		9	3.5		11.5	75			
20:0[b]	1.5	0.5					52	48			
18:1(9c)	9.5	7.5				40.5			27.5	19.5	12.5
20:1(11c)	13.0	3.5							69.5	18.5	12.0
22:1(13c)	4.5	0.5								62.5	37.5

[a] Incubations were for 24 h.

[b] Traces of 22:1(5c) were also detected in triacylglycerols.

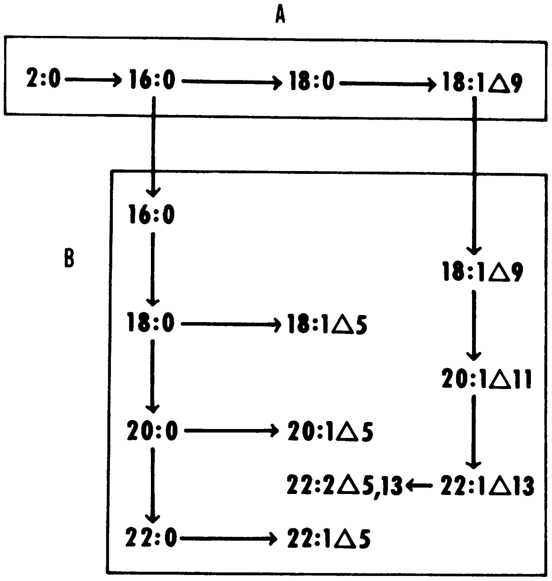

FIG. 2. Pathways to the long-chain fatty acids found in the triacylglycerols of meadowfoam seeds. Compartment A, containing the enzymes responsible for the "ACP-track" *de novo* biosynthesis of palmitate, stearate and oleate, is largely impermeable to exogenous acetate or malonate. The chain-elongating enzyme(s) in compartment B, which utilize either palmitate or oleate from A, can readily utilize exogenous acetate or malonate.

for 10% of the total label, which can be assumed to reside exclusively in C(1), as previous studies have shown that this isomer is produced by elongation of oleate (5, 15, 17). If the biosynthesis of 20:1(5c) occurs via an elongation of preformed palmitate, the ozonolysis data can be used to calculate that 34% of the total ^{14}C in the 20:1 fraction resides in C(5) to C(19) of the Δ5 *cis* isomer, with 28% each in C(1) and C(3). That is, successive decarboxylations of the 20:1 5-*cis* and 11-*cis* isomer mixture should give 38% of the total label in C(1), 0% in C(2), and 28% in C(3). If 20:1(5c) was derived solely from an elongation of preformed stearate, the predicted pattern for successive decarboxylations would be: C(1), 61.5%; C(2), 0%; and C(3), 4.5%. This pattern is consistent with 20:1(5c) being produced principally by an elongation of palmitate.

Ozonolysis of the [^{14}C]20:1 fraction derived from [2-^{14}C]malonate shows that fatty acids with chain lengths shorter than C_{16} are insignificant as precursors for 20:1(5c) (Table V, Experiment 2). If such shorter chain acids were precursors, C(6), C(8),...etc., carbon atoms would have appreciable amounts of label, and the C_{15} aldehyde fragment would be much more extensively labeled than 2%. [1-^{14}C]- and [2-^{14}C]malonate label only the chain elongated portion of the acyl chain, and, unlike [1-^{14}C]acetate, were essentially excluded from the *de novo* portion of the chain. α-Oxidation of the 20:1 fraction from [1-^{14}C]malonate also confirms the conclusion that 20:1(5c) is derived principally from the elongation of palmitate (Table V, Experiment 3).

With [2-^{14}C]malonate as substrate, the ozonolysis of [^{14}C]22:2(5c,13c) confirms that only C(2) and C(4) are labeled (Table V, Experiment 3), *i.e.* 22:2(5c13c) is produced from the elongation of a preformed C_{18} acid. This is to be expected, as previous studies have shown that 22:1(13c) is produced by an elongation of oleate (5, 15, 17).

Table V. *Distribution of Label along the Acyl Chain of ^{14}C-labeled Fatty Acids from Incubation of ^{14}C-labeled Precursors with Meadowfoam Seeds*

Experiment	Substrate	Fatty Acid Product	Ozonolysis Data[a]		^{14}C Released/Decarboxylation[b]			α-Oxidation[c]					
			Aldehyde	Aldehyde-ester	C(1)	C(2)	C(3)	C_{20}	C_{19}	C_{18}	C_{17}	C_{16}	C_{15}
			% ^{14}C		*%*			*specific activity*					
1	[1-^{14}C]Acetate	16:0			9								
		18:0			23								
		18:1	15(C_{13}) 32(C_9)	17(C_5) 35(C_9)									
		20:0			42	2	40						
		20:1	30(C_{15}) <1(C_9)	60(C_5) 10(C_{11})	46	1	33						
		22:1	5.5(C_{17}) 2(C_9)	14(C_5) 78.5(C_{13})	46								
		22:2	2(C_9) 4(C_8 dialdehyde)	94(C_5)									
2	[1-^{14}C]Acetate	18:1	10(C_{13}) 35(C_9)	9(C_5) 43(C_9)									
		20:1	28(C_{15}) ≤1(C_9)	56(C_5) 16(C_{11})									
	[2-^{14}C]Malonate	20:1	2(C_{15}) ≤1(C_9)	71(C_5) 26(C_{11})									
	[2-^{14}C]Pyruvate	20:1	55.5(C_{15}) 5(C_9)	28.5(C_5) 11(C_{11})									
	[U-^{14}C]Glucose	20:1	70(C_{15}) 2(C_9)	23(C_5) 5(C_{11})									
3	[1-^{14}C]Malonate	20:1	≤2(C_{15}) ≤1(C_9)	87(C_5) 10(C_{11})				1[d]	0.4	0.4	≤0.1		
	[2-^{14}C]Malonate	20:1	5(C_{15}) <1(C_9)	88(C_5) 7(C_{11})									
		22:1	<1(C_{17}) <1(C_9)	35(C_5) 65(C_{13})									
		22:2	<1(C_9) <1(C_8 dialdehyde)	≥98(C_5)									
4	H^{14}CO$_3^-$	20:1	15(C_{15}) <1(C_9)	80(C_5) 5(C_{11})				1[d]	0.5	0.45	0.2	0.15	<0.1
		22:1	3(C_{17}) <2(C_9)	30(C_5) 65(C_{13})									
		22:2	≤2(C_9) ≤2(C_8 dialdehyde)	≥96(C_5)									
	[1-^{14}C]Malonate	20:1(5c) derived from 16:0						1[e]	0.47	0.47	0.04	0.04	0.04
		20:1(5c) derived from 18:0						1[e]	0.04	0.04	0.04	0.04	0.03

[a] Reductive ozonolysis according to Stein and Nicolaides (21). Aldehydic fragments were analyzed by radio-GLC using a 10% DEGS column. Many of the fractions contained two positional isomers.

[b] Successive, controlled decarboxylations of the hydrogenated acids according to Dauben *et al.* (4).

[c] Chemical α-oxidation of the hydrogenated acids (8). This produces a series of chain shortened acids, whose specific activities, relative to the parent C_{20} acid, are measured by radio-GLC.

[d] Specific activities are experimental values. Compare with calculated values below.

[e] Specific activities are calculated values.

An interesting observation for [^{14}C] acyl groups derived from [1-^{14}C]acetate is that the ratio of ^{14}C/elongated carbon atom to ^{14}C/preformed carbon atom differs depending on whether the long chain fatty acid is derived from palmitate or oleate. For 18:1(5c), 20:1(5c) and 22:1(5c), which are all derived from palmitate, this ratio falls in the range of 1:0.14 to 0.21. For 20:1(11c), 22:

1(13c), and 22:2(5c13c), which are all derived from oleate, the ratio is 1:0.015 to 0.025. This order of magnitude difference is not consistent with the assumption that the two classes of long chain fatty acids are produced concurrently within the same cell type, utilizing the same two pools of acetate. However, an explanation may lie in the fact that the tissue slices used represent a heterogeneous cell population.

Within the series of ^{14}C-labeled substrates tested, malonate, acetate, pyruvate, and glucose, there is an increasing tendency for the ^{14}C label to be uniformly distributed over the whole acyl group. This is shown by the ozonolysis of the 20:1 fraction (Table V, Experiment 2). At one extreme, the label from malonate is used almost exclusively for chain elongation of endogenous palmitate or oleate, whereas, at the other extreme, glucose produces uniformly labeled species.

[^{14}C]Bicarbonate incorporation into 20:1(5c) was an unexpected result. Developing meadowfoam seeds are green and, therefore, it seemed plausible that there was a photosynthetic contribution to storage lipid biosynthesis. However, degradation (Table V, Experiment 4) suggests that the origin of the label is nonphotosynthetic, as the label is located principally at C(1) and C(3).

***In Vitro* Incubations with Cell-free Homogenates.** The incubation of [1-^{14}C]acyl-CoA thioesters with a cell-free homogenate from developing meadowfoam seeds is reported in Table VI. The elongation of [1-^{14}C]palmitoyl-CoA to stearate and eicosanoate, the Δ5 desaturation of eicosanoate, and the formation of triacylglycerols (Table VI; Fig. 1) show that the individual steps proposed (Fig. 2) for *cis*-5-eicosenoate biosynthesis in developing meadowfoam seeds are all operative *in vitro.* For the novel desaturation of [1-^{14}C]eicosanoyl-CoA, the product was identified by GLC, argentation TLC, and ozonolysis. *cis*-5-[1-^{14}C]Eicosenoate was found almost exclusively in the triacylglycerol fraction, with very minor amounts in diacylglycerols, phosphatidylcholine, free fatty acids, or acyl-CoA. However, as the 1-h period probably represents an end point for the incubation, the exact nature of the true acyl substrate for Δ5 desaturation is still unknown.

Distribution of Acyl Groups Separated by Degree of Unsaturation. The distribution of acyl groups in total triacylglycerols and in trienoic, tetraenoic, and pentaenoic triacylglycerol species isolated from mature seeds is given in Table VII. The observed distribution in total triacylglycerols can be used to calculate the distribution of acyl groups in the individual polyenoic triacylglycerol species, assuming either a completely random distribution or a distribution whereby fatty acids derived by the elongation of palmitate [*i.e.* 20:1(5c)] or oleate [*i.e.* 22:1(13c) and 22:2(5c13c)] are esterified on separate glycerol molecules. These represent the two possible extremes in acyl group distribution. The calculation ignores the very small contributions by acyl species such as 18:1, 20:2, etc. [each representing less than 1% of the total acyl groups present (13c)], and assumes that 20:1 and 22:1 are composed entirely of the Δ5-*cis* and Δ13-*cis* isomers, respectively, when intact traces of the Δ11-*cis* and Δ5-*cis* isomers, respectively, are present.

Table VI. *Incubation of ^{14}C Acyl-CoA Thioesters with a Cell-free Homogenate from Meadowfoam Seeds*

1-^{14}C-labeled Acyl-CoA Substrate[a]	^{14}C-labeled Fatty Acid Produced	
	Product	Amount
		nmol
16:0 (10)	18:0	0.9
	20:0	1.0
	20:1(5c)	3.1
18:0 (10)	20:0	1.0
	20:1(5c)	2.6
20:0 (13)	20:1(5c)	4.3

[a] Values in parentheses are nmol/incubation.

Table VII. *Distribution of Endogenous Acyl Groups in Various Triacylglycerols Separated by Degree of Unsaturation*

For an explanation of this table, see "Distribution of Acyl Groups Separated by Degree of Unsaturation" under "Results."

Triacylglycerol Fraction	Total Acyl Groups Present	Percent Acyl Composition		
		$20:1^{\Delta5}$	$22:1^{\Delta13}$	$22:2^{\Delta5,13}$
	%		*mol*	
Total	100	62.7	12.0	24.2
Trienoic				
Experimental	49	79.9	16.8	1.9
Calculated				
Random	41.7	83.9	16.1	0
Separated	64.0	98	2	0
Tetraenoic				
Experimental	36	54.8	10.6	33.1
Calculated				
Random	40.5	55.1	10.7	32.8
Separated	7.9	0	66.7	33.3
Pentaenoic				
Experimental	14	33.7	6.0	59.0
Calculated				
Random	14.1	26.0	6.4	64.9
Separated	16.1	0	33.3	66.7
Hexaenoic				
Experimental	<1[a]			
Calculated				
Random	1.4	0	0	100
Separated	10.9	0	0	100

[a] A distinctive hexaenoic triacylglycerol band was not observed by Ag^{+} TLC.

When the experimental distribution of acyl groups for each polyenoic triacylglycerol band is compared with the two calculated modes, it is clear that the principal acyl groups present [20:1(5c), 22:1(13c), and 22:2(5c13c)] are distributed on each glycerol molecule in a completely random fashion.

DISCUSSION

The preferential incorporation of [1-^{14}C]acetate *in vivo* into C(1) of 20:1(11c) and C(1) and C(3) of 22:1(13c) is a general phenomenon observed in oilseeds rich in these acids. It has been demonstrated in three very different plant species: *Tropaeolum majus* (17), *Simmondsia chinensis* (15), and *Brassica napus* (5). Thus, 20:1(11c) and 22:1(13c) are derived from the chain elongation of oleate, which is a reaction metabolically distinct from the *de novo* biosynthesis of oleate. Different pools of acetate supply each process. The labeling patterns of ^{14}C-labeled acyl groups derived from [^{14}C]acetate and [^{14}C]malonate show that in developing *L. alba* seeds 20:1(11c) (a minor component), 22:1(13c), and 22:2(5c13c) are once again produced by a chain elongation of oleate. This work also demonstrated that 22:2(5c13c) is produced *in vivo* from 22:1(13c) by a direct Δ5 desaturation. However, the major fatty acid present in meadowfoam seeds is 20:1(5c) (13, 20). Both *in vivo* and *in vitro* experiments show that this acid results from a direct Δ5 desaturation of 20:0. Degradation data for [^{14}C]20:0 and [^{14}C]20:1(5c) produced from [1-^{14}C]acetate or [1-^{14}C]malonate indicate that biosynthesis of these acids occurs predominantly by a chain elongation of palmitate as the initial substrate, not stearate.

Figure 2 summarizes the pathways of acyl group biosynthesis in the triacylglycerol fraction of developing meadowfoam cotyledons. It should be made clear that the "compartments" in Figure 2 are a rationalization of the fatty acid chemistry. Such a scheme should not be taken to imply that there is necessarily just one elongase or one Δ5 desaturase nor that further compartmentation of the pathways on a physiological basis does not occur. These questions are still to be addressed. From a consideration of the amounts of the various fatty acids in the mature seed (13, 20), the relative rates of synthesis of long-chain acids by palmitate elongation and by oleate elongation are of the order of 70:30. Data on the acyl composition of individual triacylglycerol species (Table VII) show that fatty acids from palmitate and oleate chain elongations are distributed in a random, or at least near-random, fashion. Both elongation pathways must be operating concurrently within the cell and must be supplying the same apparatus for triacylglycerol biosynthesis.

An unexpected observation was that the ratio of ^{14}C/elongation carbon atom to ^{14}C/preformed carbon atom varies depending on whether the [^{14}C] acyl group labeled from [1-^{14}C]acetate is derived from palmitate or oleate. It is important to evaluate whether this is due to separate compartmentation of the two elongation pathways within the cell or to cellular heterogeneity within the tissue. This problem of interpretation is related to uncertainties about the cell population in the tissue and has already been encountered in *in vivo* studies on C_{20} and C_{22} fatty acid biosynthesis in nasturtium seeds (17). There is a close correlation between the developing seed tissues of nasturtium and meadowfoam. Under the appropriate *in vivo* conditions, developing nasturtium seeds can produce [^{14}C]20:0 and [^{14}C]22:0 from [1-^{14}C]acetate by elongation of palmitate, and there is 1 order of magnitude discrepancy between the ratios of ^{14}C/elongation carbon atom to ^{14}C/preformed carbon atom for acyl groups produced by palmitate or oleate elongation (17).

Different H_2O-soluble precursors can be used by the intact tissue for lipid biosynthesis (Table III). The more distant the substrate is from malonyl-CoA, *viz.* sucrose → glucose → pyruvate → acetate → malonate, the more likely it is to be used for *de novo* lipid biosynthesis. Only glucose produced uniformly labeled C_{20} and C_{22} acyl groups. C_{20} and C_{22} acyl groups labeled from [^{14}C]bicarbonate were not produced by a photosynthetic utilization of this substrate, but presumably by an acetyl-CoA carboxylation followed by a C(1) to C(3) CoA transfer. The incorporation of 1-^{14}C-labeled fatty acids into triacylglycerols and polar lipid was dependent on chain length and unsaturation. Since the acids were elongated, desaturated, and incorporated into triacylglycerols, they were being activated. As no long-chain fatty acid:ACP ligase or acyl-CoA:ACP transacylase has been found in higher plants, the metabolism of these acids in meadowfoam seeds probably occurs on the "CoA-track" (22).

Δ5 Desaturation is an essential transformation in the metabolism of polyunsaturated acids in animals (6). It is also found in certain species of bacteria (18). Although oilseeds which contain fatty acids with either Δ5-*cis* or Δ5-*trans* unsaturation have been known for some time (9), this work contains the first demonstration of the Δ5 desaturation of fatty acids in oilseeds.

The nature of the acyl substrate for Δ5 desaturation is unknown. Although added acyl-CoA is Δ5 desaturated *in vitro*, it cannot be concluded that this thioester must be the immediate substrate. An interesting parallel can be made with the oleoyl desaturase which produces linoleate in developing safflower seeds. From *in vitro* time-course studies Stymne and Appelqvist (23) concluded that oleoyl phosphatidylcholine produced from added oleoyl-CoA is, in fact, the substrate. This conclusion was also reached by Slack *et al.* (19) on the basis of *in vivo* time-course studies. This contradicted the earlier work of Vijay and Stumpf (24), who proposed that oleoyl-CoA was the true substrate. However, the latter authors used the Barron and Mooney $NaBH_4$ reduction as a test for thioesters (2), a method which was subsequently shown to have an inbuilt error (14). Careful time-course studies must be made with developing meadowfoam seeds to determine the exact nature of the acyl substrate for Δ5 desaturation. Since endogenous phosphatidyl choline and ethanolamine contain very low levels of 20:1(5c), these results would suggest that the Δ5 desaturation does not occur with these polar lipids as substrates. Such studies are underway. The *in vivo* incubations (Table II) give a preliminary indication that this Δ5 desaturation requires molecular oxygen, as do the other plant desaturations studied (11, 12).

That acyl-CoA thioesters are substrates for chain elongation has been clearly demonstrated for the developing jojoba seed (6). This also holds for meadowfoam seeds.

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