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Long Chain (C_{20} and C_{22}) Fatty Acid Biosynthesis in Developing Seeds of *Tropaeolum majus*

AN *IN VIVO* STUDY[1]

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ABSTRACT

The storage triacylglycerols of nasturtium (*Tropaeolum majus*) seeds are composed principally of *cis*-11-eicosenoate and *cis*-13-docosenoate. To investigate the biosynthesis of these C_{20} and C_{22} fatty acids, developing seed tissue was incubated with various ^{14}C-labeled precursors. Incubation with [1-^{14}C]acetate produced primarily *cis*-11-[1-^{14}C]eicosenoate and *cis*-13-[1,3-^{14}C]docosenoate in the triacylglycerol fraction, the odd-carbon [U-^{14}C]oleate also formed from [^{14}C] acetate was in the polar lipid fraction. Kinetic data showed that this oleate was not channeled into *cis*-11-eicosenoate nor *cis*-13-docosenoate over a 24-hour period. Under suitable conditions, nasturtium seed could also produce [^{14}C]stearate, [^{14}C]eicosenoate, and [^{14}C]docosenoate from [1-^{14}C]acetate. The results are discussed in terms of the number of pathways producing fatty acids. From pool size and other considerations, the results can be rationalized only in terms of different *de novo* systems for oleate biosythesis, one supplying oleate for incorporation into phospholipids and the other supplying oleate for chain elongation and subsequent esterification into triacylglycerols. Because of the probable heterogeneous nature of the seed tissue, it is not known if these two systems are operating in different cell types, in the same cell type at different stages of development, or in the same cell type concurrently.

Part of the study on wax ester biosynthesis in the jojoba [*Simmondsia chinensis* (Link)] seed has involved the investigation of the biosynthesis of *cis*-11-eicosenoate and *cis*-13-docosenoate (9, 11).[3] The fatty acids of the triacylglycerol fraction from mature nasturtium (*Tropaeolum majus*) seeds are rich in oleate (10%), *cis*-11-eicosenoate (25%), and *cis*-13-docosenoate (65%), with almost no linoleate or α-linolenate (13). Therefore, nasturtium seeds were chosen as a model system for elongation studies. Nasturtium can be readily grown all year round in the greenhouse, whereas developing jojoba seeds are available for only a limited period each year.

Working with *Brassica napus* L. and *Crambé abyssinica* seed, Downey and Craig (5) and Appleby *et al.* (2), respectively, have demonstrated that *cis*-11-eicosenoate and *cis*-13-docosenoate are produced by the elongation of *cis*-9-octadecenoate (oleate) rather than by total *de novo* biosynthesis. There is also evidence suggesting that the glycerol-3-P pathway for triacylglycerol biosynthesis is operative in *C. abyssinica* (6). Recent studies with cell-free extracts from developing jojoba cotyledons have shown that long chain acyl-CoAs, including oleoyl-CoA and also stearoyl-acyl carrier protein thioester, are elongated in the presence of malonyl-CoA and NADPH (or NADH) (11). Data from both *in vivo* (9) and *in vitro* (10, 11) experiments suggest that the probable pathway for *cis*-13-docosenoate biosynthesis involves first the synthesis of oleoyl-acyl carrier protein from acetate by enzymes utilizing acyl carrier protein thioesters as substrates, then the hydrolysis of oleoyl-acyl carrier protein to oleic acid, and lastly, reactivation to oleoyl-CoA, with subsequent elongation. The specific sites of *de novo* synthesis and subsequent elongation have not yet been defined.

This present investigation examines the biosynthesis of the major monoenoic fatty acids of the developing nasturtium seed under *in vivo* conditions.

MATERIALS AND METHODS

Plant Material. The dwarf cherry rose variety of nasturtium, *T. majus* (Northrup King Seeds, Fresno, Calif.), was grown in the greenhouse and the flowers were hand-pollinated. Fresh seeds, of age 13 to 18 days after pollination in spring and autumn and 17 to 22 days after pollination in winter, were generally used. During winter, flowering was stimulated by artificial lighting (14-h day length).

Substrates. [1-^{14}C]Acetate (58 Ci/mol), [2-^{14}C]pyruvate (3.7 Ci/mol), D-[U-^{14}C]glucose (333 Ci/mol), and [1-^{14}C]stearic acid (51 Ci/mol) were purchased from New England Nuclear. [2-^{14}C]Malonate (22 Ci/mol) was obtained from ICN. [1-^{14}C]Palmitic acid (56 Ci/mol) and [1-^{14}C]oleic acid (56 Ci/mol) were obtained from Amersham Searle.

Incubations. Developing nasturtium seeds were excised from their seed coats and cut into small pieces (about 1- to 2-mm cubes) with a razor blade. The tissue (150 mg) was incubated with ^{14}C-labeled substrate (1 - 5 μCi) in 0.1 M Na-phosphate buffer (0.5 ml) at pH 6.0. Incubations were generally for 6 h in open tubes in a reciprocating water bath at 26 to 27 C. Free 1-^{14}C-labeled fatty acids were added to the buffer as their ammonium salts in 20% aqueous ethanol (5 μl). In experiments where a high level of ^{14}C-labeled saturated fatty acid formation from [1-^{14}C]acetate was required, about 300 mg tissue was used per tube. The tube dead space (15 ml) then was flushed with N_2 for 1 min and the tube was tightly capped.

Incubations were terminated by adding isopropanol (1 ml) and heating at 80 C for several minutes. On cooling, 8 ml chloroform-methanol (2:1, v/v) were added, the tissue was homogenized, and

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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).

the mixture was left to stand overnight to facilitate extraction of the lipids. Shaking this monophasic mixture with 0.7% saline (2 ml) resulted in a chloroform phase containing extracted lipid. After acidification with a few drops of glacial acetic acid, the chloroform layer was dried under a stream of N_2, yielding the total lipid extract.

Lipid Analysis. Lipids were transmethylated by refluxing in methanol-benzene-sulfuric acid (20:10:1, v/v) for 3 h. When individual lipid classes were to be transesterified, the silica band from TLC was scraped directly into the transmethylation reagent.

GLC of the ^{14}C-labeled fatty methyl esters, with radioisotope monitoring by a gas flow proportional counter (referred to as radio-GLC) was as described previously (9). Packed columns with polar stationary phases (*i.e.* 10% DEGS-PS or 10% SP-2330, ex. Supelco) were used.

Neutral lipids were separated by TLC on Silica Gel G plates developed with petroleum ether (boiling point 40 to 60 C)-diethyl ether-acetic acid (80:20:1, v/v). The material remaining at the origin in this solvent system is termed the "polar lipid fraction" here. Polar lipid classes were usually separated by TLC using chloroform-methanol-H_2O (65:25:4, v/v). For TLC of monoacylglycerols, chloroform-methanol-acetic acid-H_2O (90:12:0.5:0.1, v/v) or petroleum ether-acetone-formic acid (76:24:0.2, v/v) solvent mixtures were used. For each solvent system, the appropriate authentic lipid standards were used to identify bands, which were located by staining with iodine vapor or by spraying with 2', 7'-dichlorofluorescin methanol solution (0.1% by weight) and then viewing under UV light. When recovery of material was not required, the TLC plates were sprayed with reagents for the detection of specific functional groups (3) or with 50% aqueous sulfuric acid followed by charring. To estimate the radioactivity associated with different bands on the TLC plate, the silica was scraped directly into PCS (ex. Amersham/Searle) -xylene (2:1, v/v) for liquid scintillation counting.

The mass of acyl residues in the total lipid extract or in lipid classes isolated by TLC was estimated by adding a known weight of heptadecanoic acid prior to transmethylation and using this as an internal standard to calculate the mass from the GLC trace. Corrections were made to allow for the different responses of the thermal conductivity mass detector to different acyl groups.

^{14}C-labeled Acyl Group Degradation. ^{14}C-labeled fatty methyl esters were separated into individual species by preparative GLC on a stainless steel 10% diethylene glycol succinate packed column (3 m × 6.4 mm o. d.).

Unsaturated esters were cleaved by reductive ozonolysis according to a microscale modification of the method of Stein and Nicolaides (15). The aldehyde and aldehyde-ester fragments were analyzed by radio-GLC.

For α-oxidation and controlled decarboxylation, the unsaturated methyl esters were first hydrogenated over a 10% palladium on charcoal catalyst at 40 p.s.i. H_2 gas pressure. Prior to saponification, an aliquot was examined by radio-GLC to check for complete reduction to the corresponding saturated ester.

Chemical α-oxidation of ^{14}C-labeled saturated acids was achieved by the method of Harris *et al.* (7). After α-oxidation with permanganate and extraction, the mixture of shorter chain acids was esterified with diazomethane and analyzed by radio-GLC.

Controlled chemical decarboxylation of ^{14}C-labeled saturated acids was done by a microscale adaption of the procedure of Dauben *et al.* (4). The ^{14}C distribution between the alkyl nitrile (C_{n-1}) and benzoic acid [containing C(1)] was routinely measured by TLC, using half and then full development in petroleum ether-diethyl ether-acetic acid (90:10:0.5, v/v). The figures obtained were in close agreement with measurements of the specific activities of the fragments by radio-GLC (10% SP-2330, 200 C). After base hydrolysis of the ^{14}C-labeled alkyl nitrile to yield the ^{14}C-labeled free acid, chain shortened by the loss of C(1), the entire procedure was repeated to give data on the per cent ^{14}C in C(2) and subsequently in C(3).

RESULTS

Lipid Composition and [1-^{14}C]Acetate Incorporation during Nasturtium Seed Development. Figure 1 shows the variation of seed weight, seed lipid content, and [1-^{14}C]acetate incorporation into the lipid extract during development. The seeds most actively synthesized lipid between 12 and 20 days after pollination, which is when they were routinely picked for these studies. This optimum period was a few days later for seeds harvested in winter. The profiles shown in Figure 1 are typical of most maturing oilseeds (1).

Details of the acyl composition during maturation are shown in Table I. The major lipid is triacylglycerol. There is a net increase in polar lipids during the early stages of lipid biosynthesis (12–16 days after pollination) which is associated with an increase in oleate in the polar lipid fraction. The levels of palmitate, linoleate, and α-linolenate remained largely unaltered over the entire period of seed development. For each of the three ages examined, phosphatidylcholine represented 50 to 60% of the total polar lipid fraction in terms of acyl groups present and had a fatty acid distribution similar to that for the total polar lipids. Phosphatidylethanolamine was also identified and was found to contain 10

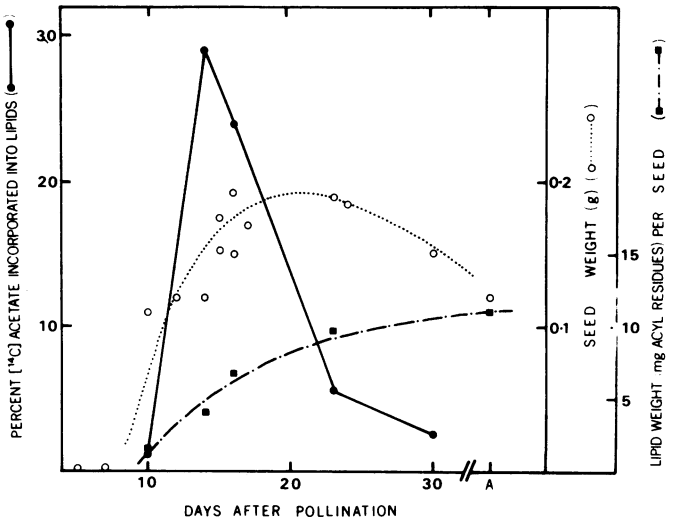

FIG. 1. Seed weight, mass of acyl lipid (mg) per seed, and [1-^{14}C]acetate incorporation into lipid extract in sliced tissue, as a function of seed development. Seeds were harvested on October 6. A, on the *x* axis, represents the mature, dehydrated seed.

Table I. *Acyl Composition during Development of Nasturtium Seeds*

Time after Pollination[a]	Average Fresh Seed Weight	Weight of Acryl Residues					
		Total Triacylglycerol	Triacylglycerol Species[b]			Total Polar Lipid[c]	18:1 in Polar Lipid
			18:1	20:1	22:1		
days	*mg*	*mg/seed*					
12	120	0.3	0.03	0.11	0.16	0.45	0.11
16	196	4.15	0.17	0.87	3.07	0.9	0.55
29	225	7.2	0.07	0.94	6.13	0.75	0.48

[a] Seeds were harvested on November 28.

[b] The triacylglycerol fraction also contained traces of palmitate (<2%).

[c] Major acyl species present were palmitate, oleate, linoleate, and α-linolenate. Eicosenoate (20:1) and docosenoate (22:1) were not detected.

to 20% of the total polar lipid acyl residues. Since mono- and diacylglycerols, mono- and digalactosyl diglycerides, and free fatty acids were not detected by TLC, they could only be very minor components of developing nasturtium seed lipids.

During maturation, the level of *cis*-11-eicosenoate accumulation in triacylglycerols diminished, whereas *cis*-13-docosenoate synthesis continued (Table I). Whether this is due to a shift in the ratio of *cis*-11-eicosenoate to *cis*-13-docosenoate produced *de novo* or to a slow turnover of *cis*-11-eicosenoate in the triacylglycerols with its concurrent elongation to *cis*-13-docosenoate is not known.

In six separate experiments, [1-^{14}C]acetate incubation with chopped seed tissue (150 to 250 mg) in 0.5 ml buffer in open tubes gave 10 to 29% incorporation of radioactivity into the lipid extract after 6 h. The ^{14}C-labeled fatty acid distribution within total lipids (100%) was: palmitate, 3 to 8.5%; stearate, 1 to 14%; oleate, 11 to 41.5%; eicosenoate, 0.5 to 5%; *cis*-11-eicosenoate, 10.5 to 17%; docosenoate, 2 to 11%; and *cis*-13-docosenoate, 30.5 to 65.5%. Label was incorporated mainly into triacylglycerol and polar lipid fractions as indicated by TLC (Table II), with less than 2% of the radioactivity in total lipids being found as free fatty acids and less than 5% as diacylglycerol. The C_{20} and C_{22} labeled acids were incorporated predominantly into triacylglycerols. By contrast, C_{16} and C_{18} acids were found mainly in the polar lipid fractions. This fraction also contained small amounts of C_{20} and C_{22} acids. Phosphatidylcholine was always the principal labeled polar lipid (>50%), whereas phosphatidylethanolamine was also present (about 10%). Other polar lipid ^{14}C-labeled bands were not identified. Immature seeds did not produce labeled linoleate or linolenate from [1-^{14}C]acetate.

Some General Considerations with Nasturtium Seeds. Apart from the developing seed, several other tissues from nasturtium were examined for [1-^{14}C]acetate incorporation *in vivo*.

The seed coat, 15 days after pollination, incorporated 3% of the ^{14}C-labeled precursor into lipids, with only C_{16} and C_{18} acyl groups labeled. Endogenous lipids from the seed coat did not contain eicosenoate or docosenoate.

Nasturtium seeds were germinated for 6 days, by which time distinct embryonic growth was observed. A 4% incorporation of [1-^{14}C]acetate into lipids by the cotyledons was observed but, again, only C_{16} and C_{18} acids were labeled.

Radwan (13) has reported that nasturtium petals contain considerable amounts of eicosenoate and docosenoate. However, incubation of developing petals with [1-^{14}C]acetate did not produce labeled eicosenoate or docosenoate, nor could these acyl groups be detected as constituents of the endogenous lipids. For petals, the acyl composition was (mass; ^{14}C): palmitate (31%; 32%), stearate (4%; 4%), oleate (5%; 55%), linoleate (37%; 9%), and α-linolenate (9%; 0%).

To determine whether sliced tissue was representative of the normal metabolism of nasturtium tissue, [1-^{14}C]acetate (5 μCi in 5 μl) was injected directly into maturing seeds still attached to the plant. This resulted in a 60% incorporation of label into lipid after 4.5 h and 85% after 24 h. Triacylglycerols contained about 60 to 70% of the label at either time point. In each case, the total ^{14}C-labeled fatty acid distribution was: palmitate, 2%; oleate, about 15%; eicosenoate, about 17%; docosanoate, 2%; and docosenoate, about 65%. Labeled palmitate and oleate were found almost entirely in the polar lipid fraction, and labeled eicosenoate, docosanoate, and docosenoate were found in the triacylglycerol fraction. The labeling pattern was quite similar to that obtained with sliced tissue.

An acetate concentration of 2 mM gave the maximal rate of incorporation into lipid, equivalent to about 0.1 to 0.2 μmol acetate incorporated into lipid/seed·day. The average rate of endogenous lipid biosynthesis during the period of maximum biosynthetic activity is 2 to 3 μmol acyl groups/seed·day (Fig. 1). Thus, even at its saturating level, the rate of utilization of exogenous acetate is only a small fraction of the rate of synthesis endogenous lipid. The incubation concentration of [1-^{14}C]acetate normally used was about 0.2 mM.

Incubation of ^{14}C-labeled substrates other than acetate *in vivo* did not yield useful data. Incorporation into ^{14}C-labeled acyl groups was low for D-[U-^{14}C]glucose (<0.5%), [2-^{14}C]pyruvate (<1.5%), and [2-^{14}C]malonate (2–3%) under the standard incubation conditions and was not improved by pulse-chasing with cold substrate. The ^{14}C-labeled fatty acid distributions in total lipids for all three substrates were fairly similar to that for [1-^{14}C]acetate. Free ^{14}C-labeled fatty acids were also treated as substrates. [^{14}C]-Oleic acid was incorporated into triacylglycerols only to a negligible extent (<0.5%), with longer chain acids in this fraction being barely detectable. As chain length of the substrate decreased, incorporation into triacylglycerols increased (*e.g.* 5% for myristate, 19% for laurate, 37% for decanoate), although chain elongation of these acids was not observed either.

Production of ^{14}C-labeled Saturated Fatty Acids. Nasturtium seeds contain only traces of C_{18} to C_{22} saturated acids (13). However, under suitable conditions, incubations with [1-^{14}C]acetate could give rise to appreciable amounts of these labeled acids (Table III). Conditions that favored formation of saturated acids were purging of the tube dead space (15 ml) with N_2, followed by immediate capping, and using greater amounts of seed tissue/0.5 ml buffer.

Distribution of Label along Acyl Chain from Incubation of Nasturtium Seeds with [1-^{14}C]Acetate. Table IV shows the distri-

Table II. *[^{14}C]Acyl Distribution in Lipid Classes of Developing Nasturtium Seeds after Incubation with [1-^{14}C]-Acetate*

The data are expressed as a percentage of the total ^{14}C incorporated into the total lipid extract.

Lipid Class	^{14}C in Lipid Class[a]	[^{14}C]Acyl Distribution						
		16:0	18:0	18:1	20:0	20:1	22:0	22:1
	%							
Total	100	3	1	11	0.5	15.5	3.5	65.5
Triacylglycerols	70.3			0.6	0.4	8.4	2.6	57.9
Free fatty acids	0.8							
1,3-Diacylglycerols	1.2			0.7		1.4		1.7
1,2-Diacylglycerols	2.6							
Total polar lipids	21.5	3.7	0.9	10.7		2.6	0.8	2.6
Phosphatidylcholine	13.8	1.0	0.3	8.7		1.8	0.3	1.4
Phosphatidylethanolamine	2.4	0.4	tr	0.8		0.5	tr	0.5
Remaining polar lipids	5.3	3.0	tr	0.8		tr	tr	0.2

[a] The experiment reported is for seeds harvested in autumn 20 days after pollination. Experiments with younger seeds (data not shown) gave a higher percentage of label in oleate and polar lipids.

Table III. *^{14}C-labeled Saturated Acid Formation From Incubations of [1-^{14}C]Acetate with Nasturtium Seed Slices*

Incubation Conditions[a]	Weight of Seed Tissue/Incubation	^{14}C Incorporated into Total Lipid	^{14}C-labeled Fatty Acid Composition							
			16:0	18:0	18:1	20:0	20:1	22:0	22:1	(18:0 + 20:0 + 22:0)
	mg	%					%			
Air	50	7	0.5	0	40	0	14	0	46	0
Air	150	23	5	5	41	3	11	5	30	13
Air	270	29	7	14	15	5	12	11	36	30
Air	170	27	4	2	24	1	14	3	52	6
O_2	150	19	4	2	20	0	14	2	58	4
N_2	160	14	9	17	7	4	17	8	34	29
Air	150	16	5.5	0.5	34	0.5	12	2	45.5	3
N_2	310	11.5	4	28	6	7	12	12	31	47

[a] O_2, a steady flow of oxygen was maintained through the open tube; N_2, the dead space in the tube (15 ml) was purged with nitrogen prior to capping.

Table IV. *^{14}C Distribution along the Acyl Chain of Lipids Produced from Incubation of Nasturtium Seeds with [1-^{14}C]Acetate*

Data for three separate experiments are shown. In Experiment 2, a [1-^{14}C]acetate solution was injected directly into the developing seeds on the plant.

Acyl Group	Reductive Oxonolysis		α-Oxidation[a]					Controlled Decarboxylation[b]		
	Aldehyde	Aldehyde-Ester	C_n	$C_{(n-1)}$	$C_{(n-2)}$	$C_{(n-3)}$	$C_{(n-4)}$	C(1)	C(2)	C(3)
	% ^{14}C							% ^{14}C released		
Experiment 1										
16:0			1	0.8	1.05	1.05				
18:0			1	1.05	1.0	0.9	0.8			
18:1	(C_9) 45	(C_9) 55	1	0.9	0.8	0.7	0.7			
20:0			1	0.6	0.5	0.45	0.5			
20:1	(C_9) 4	(C_{11}) 96	1	0.1	0.1	0.1				
22:0			1	0.8	0.7	0.65	0.65			
22:1	(C_9) 2	(C_{13}) 98	1	0.65	0.5	0.1				
Experiment 2										
18:1	(C_9) 42	(C_9) 58								
20:1	(C_9) 11	(C_{11}) 88								
22:1	(C_9) 7	(C_{13}) 92								
Experiment 3										
16:0								10		
18:0								8		
18:1	(C_9) 43	(C_9) 56						9		
20:0								28	1	35
20:1	(C_9) 11	(C_{11}) 89						81	<1	2
22:0								21	1	27
22:1	(C_9) 4	(C_{13}) 95						47	2	45

[a] The specific radioactivities of chain-shortened acids are relative to the original acid (C_n).

[b] The theoretical release of ^{14}C on C(1) decarboxylation of a *de novo* labeled chain (*i.e.* even distribution of label) is 12.5% for C_{16}, 11% for C_{18}, 10% for C_{20}, and 9% for C_{22}.

bution of label along the acyl chains of palmitate, stearate, oleate, eicosanoate, *cis*-11-eicosenoate, docosanoate, and *cis*-13-docosenoate derived from [1-^{14}C]acetate incubations, as determined by three degradative procedures: α-oxidation, controlled decarboxylation, and reductive ozonolysis. The degradation data for palmitate, stearate, and oleate are consistent with their *de novo* synthesis from [1-^{14}C]acetate. That is, each odd-numbered carbon atom is equally labeled. Ozonolysis of *cis*-11-eicosenoate and *cis*-13-docosenoate showed that most of the label was in the aldehyde-ester (*i.e.* carboxyl end) fragment. Successive decarboxylations, either by α-oxidation (7) or by the method of Dauben *et al.* (4), indicated that C(1) of *cis*-11-eicosenoate, and C(1) and C(3) of *cis*-13-docosenoate are the highly labeled carbon atoms. The production of [^{14}C]eicosenoate and [^{14}C]docosenoate is therefore by elongation of endogenous oleate with [^{14}C]acetate. Separate pools of acetate supply the *de novo* biosynthesis of this oleate and the subsequent chain elongation. Ozonolysis data showed that 9 to 25% of the label was in the *de novo* derived portion of eicosenoate [(*i.e.* C(3) to C(20)] and 4.5 to 9% in the *de novo* derived portion of docosenoate [(*i.e.* C(5) to C(22)]. These results are consistent with the results from the stepwise chemical decarboxylation of docosenoate, where C(1) and C(3) are essentially equally labeled and

demonstrate that *cis*-13-docosenoate biosynthesis has occurred *via* the addition of two [1-^{14}C]acetate molecules to endogenous oleate and not via the elongation of *cis*-11-eicosenoate from an endogenous pool.

A somewhat different distribution of ^{14}C label was observed for docosanoate and eicosanoate (Table IV). Successive decarboxylations of [^{14}C]eicosanoate suggested that this acyl moiety was obtained by chain extension of palmitate since C(1) and C(3) were approximately equally labeled. Careful examination of the data in Table IV reveals that the ratio of specific activity of carbon atoms in the *de novo* portion of the acyl chain to the specific activity of carbon atoms in the elongated portion is much higher (by approximately a factor of 5) for the ^{14}C-labeled saturated acids than for the ^{14}C-labeled monounsaturated acids. Such observations are common to meadowfoam (*Limnanthes alba*) seeds, where the elongation of palmitate to eicosanoate is an important reaction (12).

Time Course and Pulse-Chase Studies for [1-^{14}C]Acetate Incubation with Nasturtium Seeds. The incorporation of label from [1-^{14}C]acetate into total lipids, triacylglycerols, polar lipids, and acyl species over a 22-h period, with and without a pulse chase of cold acetate at 3.5 h, is given in Figure 2. The tissue remained viable over the entire period. Although a relative diminution in incorporation of [1-^{14}C]acetate into oleate and into the polar lipid fraction occurred over 22 h, the pulse chase demonstrated that there was negligible transfer of label from oleate, in the polar lipid fraction, into eicosenoate and docosenoate in triacylglycerols. This was confirmed by ozonolysis of the individual acyl species. The ^{14}C distribution along the acyl chain remained constant over 22 h for each species. Ozonolysis of [^{14}C]oleate indicated a *de novo* biosynthesis from [1-^{14}C]acetate, whereas, for [^{14}C]eicosenoate, 69 to 76% of the label was calculated as being at C(1) and, for [^{14}C]docosenoate, a similar calculation gave 84 to 90% of the label at C(1) + C(3). Ozonolysis of ^{14}C-labeled acyl species from the pulse chase also indicated no detectable increase in label in the *de novo* portion of eicosenoate or docosenoate over the 3.5- to 22-h pulse chase period. The distribution of ^{14}C-labeled acyl groups within triacylglycerols or the polar lipid fraction did not alter during either the time course or the pulse chase.

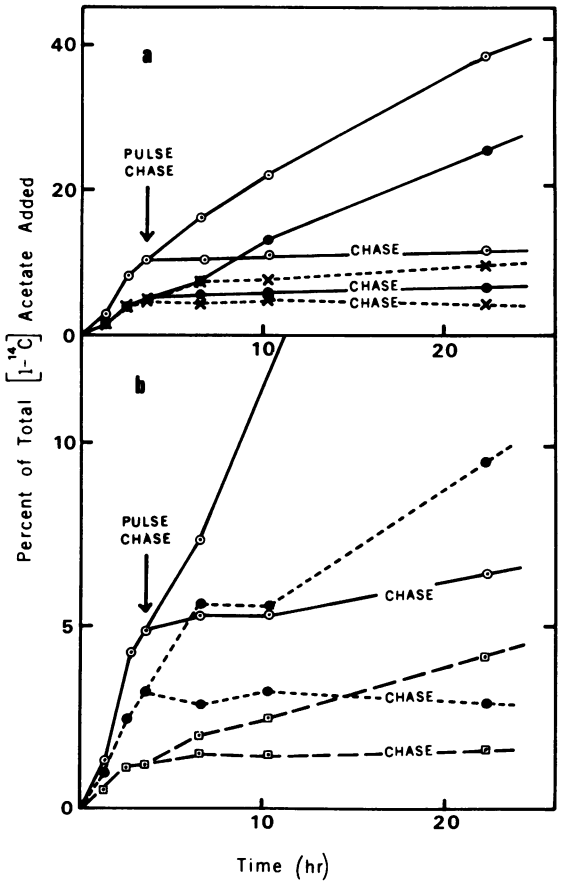

FIG. 2. Time course and pulse chase for [1-^{14}C]acetate incorporation into ^{14}C-labeled monounsaturated acyl lipids in nasturtium seeds. For the pulse chase at 3.5 h the seed slices were rinsed with buffer (3 ml, three times) to remove [1-^{14}C]acetate, and 5 mM unlabeled acetate was added. a: ⊙——⊙, total lipid extract; ●——●, triacylglycerols; X- - -X, polar lipids. b: ●- - -●, oleate; ⊡- - -⊡, *cis*-11-eicosenoate; ⊙——⊙, *cis*-13-docosenoate.

The time course and pulse chase for ^{14}C-labeled saturated fatty acid production (Fig. 3) showed a similar pattern to that for ^{14}C-labeled monoene production (Fig. 2b). Pulse chasing with acetate did not result in a noteworthy transfer of label from palmitate or stearate to eicosenoate or docosenoate (Fig. 3a). Figure 3b shows the early part of the time course for synthesis of saturated fatty acids. There is a time lag for the appearance of [^{14}C]docosanoate.

Action of Inhibitors on [1-^{14}C]Acetate Incorporation into Lipids in Nasturtium Seeds. Trichloroacetic acid has been observed to inhibit erucic acid biosynthesis *in vivo* in *C. abyssinica* prior to the inhibition of total acyl lipid biosynthesis (2). A similar experiment was conducted for nasturtium (Fig 4a), where identical trends were observed. The synthesis of [^{14}C]docosenoate was inhibited more effectively by trichloroacetic acid (50% inhibition at 0.25 mM) than was the synthesis of [^{14}C]eicosenoate (50% inhibition at 4 mM), whereas [^{14}C]oleate production was halved only at a much higher trichloroacetic acid concentration (15 mM). Triacylglycerol labeling fell concomitantly with the inhibition of [^{14}C]eicosenoate and [^{14}C]docosenoate biosynthesis (50% inhibition at 0.5 mM), whereas the decrease in polar lipid labeling followed the inhibition of [^{14}C]oleate biosynthesis (50% inhibition at 15mM). The distribution of ^{14}C-labeled acyl groups within triacylglycerols (mainly eicosenoate and docosenoate) and polar lipids (mainly palmitate and oleate) was not affected by trichloroacetic acid. In a separate experiment where conditions were set to favor ^{14}C-labeled saturated acyl formation (Fig. 4b), [^{14}C]eicosanoate and [^{14}C]docosanoate inhibition by trichloroacetic acid mirrored the decrease in [^{14}C]eicosenoate and [^{14}C]docosenoate, respectively, whereas [^{14}C]stearate inhibition occurred at a higher trichloroacetic acid concentration.

2,4-Dinitrophenol also inhibited lipid biosynthesis *in vivo* at low concentrations. Again, [^{14}C]docosenoate biosynthesis was inhibited before [^{14}C]eicosenoate biosynthesis (50% inhibition at 0.08 and 0.3 mM 2,4-dinitrophenol, respectively), whereas [^{14}C]-oleate formation was reduced by half at a higher concentration (0.8 mM). The incorporation of [1-^{14}C]acetate into triacylglycerols was halved at 0.1 mM, and that into polar lipids was halved at 0.6 mM.

DISCUSSION

The preferential incorporation of [1-^{14}C]acetate *in vivo* into C(1) of *cis*-11-eicosenoate and C(1) plus C(3) of *cis*-13-docosenoate appears to be a general phenomenon for oilseeds containing these acids. It has been demonstrated in *B. napus* L. (5), *S. chinensis* (9), *Limnanthes alba* (12), and *T. majus*, four very different plant species. Eicosenoate and docosenoate appear to be derived from a chain elongation of oleate, which is a process metabolically separate from the *de novo* biosynthesis of oleate from acetate.

Although data obtained from intact tissue or tissue slices provide important leads for the establishment of biosynthetic pathways, interpretation of such data should be conservative. For example, incubation of a ^{14}C-labeled substrate with intact seed tissue followed by isolation of ^{14}C-labeled products from the total tissue reflects the total capacity of different cell types to utilize the ^{14}C-

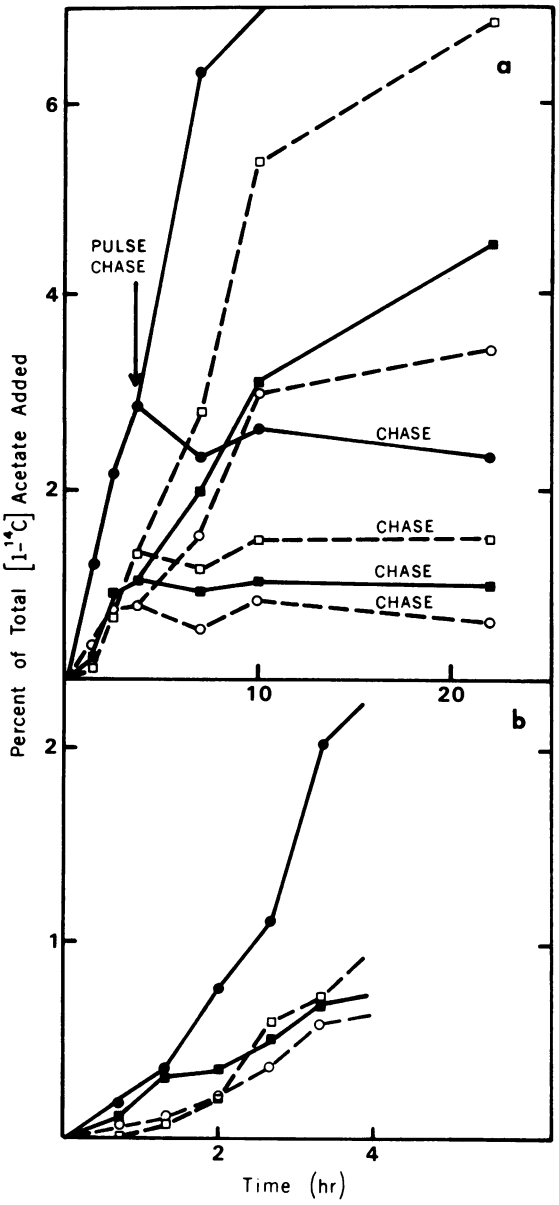

FIG. 3. Time course and pulse chase for [1-^{14}C]acetate incorporation into ^{14}C-labeled saturated acyl lipids in nasturtium seeds. For the pulse chase (a), the seed slices were rinsed with buffer (3 ml, 3 times) after 3.75 h to remove [1-^{14}C]acetate, and 5 mM unlabeled acetate was added. b shows an expansion of the early part of the time-course, the data being taken from a separate experiment to that for a. O- - -O, palmitate; ●——●, stearate; ■——■, eicosanoate; □- - -□, docosanoate.

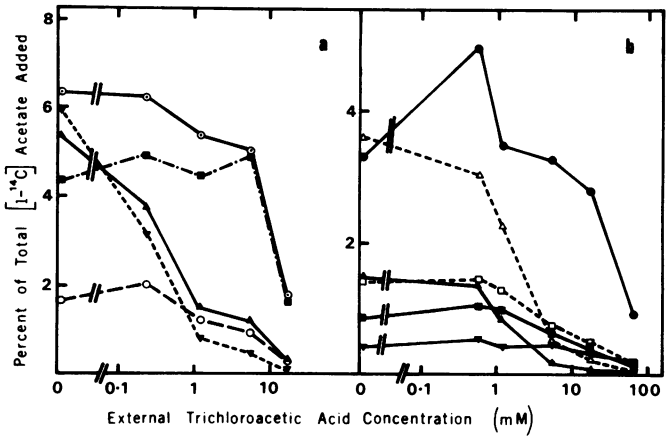

FIG. 4. Trichloroacetic acid inhibition of [1-^{14}C]acetate incorportion into lipids in nasturtium seeds. a, experimental conditions favoring monounsaturated acyl formation; ⊙——⊙, polar lipids; ▲——▲, triacylglycerols; ■-·-·-■, oleate; O- - -O, *cis*-11-eicosenoate; ▼- - -▼, *cis*-13-docosenoate. b, experimental conditions favoring saturated acyl formation; ▼——▼, palmitate; ●——●, stearate; ■——■, eicosenoate; □- - -□, *cis*-11-eicosenoate; ▲——▲, docosanoate; △- - -△, *cis*-13-docosenoate.

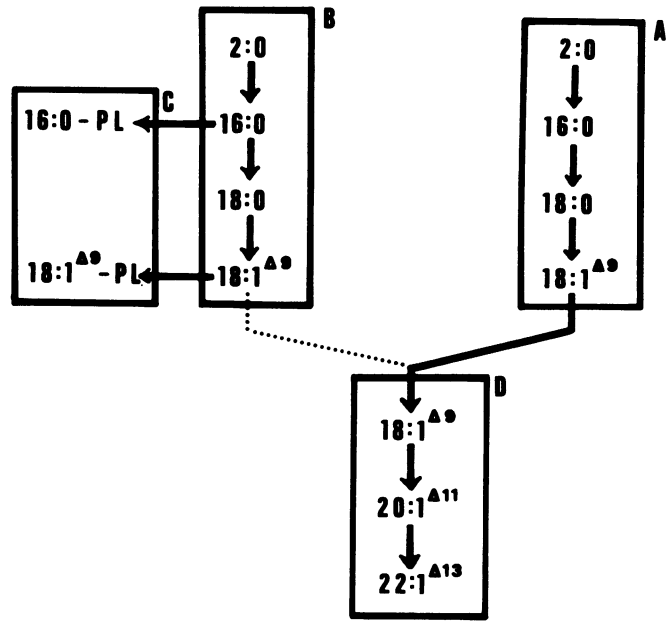

FIG. 5. Summary of the *in vivo* pathways of lipid biosynthesis in maturing seed tissue from nasturtium, as deduced from *in vivo* labeling experiments from [1-^{14}C]acetate. There are apparently two independent *de novo* fatty acid synthesis systems (A and B). One (A) supplies oleate for chain elongation (D) and subsequent storage lipid biosynthesis (triacylglycerols), whereas the other (B) supplies palmitate and oleate for phospholipid (PL) biosynthesis (C). It is not yet clear whether A to D are all operative within a single cell type within the seed but, if so, it is possible that B supplies a small contribution of oleate to D (·····).

labeled substrate. Furthermore, for each cell type there will probably be a range of developmental stages. Unfortunately, experimentalists often tend to view such data as a reflection of the biochemistry of a single cell type. With these comments in mind, judicious interpretation of the data obtained here will now be proposed.

Degradation data for the ^{14}C-labeled acyl groups produced when incubating developing seed tissue from nasturtium with [1-^{14}C]acetate, coupled with time course and pulse-chase studies, strongly suggest that there are two distinct sites of oleate biosynthesis. One site supplies oleate for chain elongation, whereas the other supplies oleate for phospholipid biosynthesis (Fig. 5). Whether or not they co-exist within the same cell type cannot yet be answered. The label in ^{14}C-labeled oleoyl polar lipids could not be observed moving through into [^{14}C]eicosenoate and [^{14}C]docosenoate over a 24-h period (Fig. 2b). Also, the ratio of ^{14}C as oleate in the polar lipids to ^{14}C in the *de novo* portion of eicosenoate and docosenoate (*i.e.* the first 18 carbon atoms, counting from the methyl end) remained constant at about 3:1. However, the ratio of endogenous oleate channeled into phospholipids and into triacylglycerol biosynthesis *in vivo* must be the reverse. If it is assumed that there is only a single site for oleate biosynthesis

within the tissue, the majority of the oleate produced would have to move through the polar lipid pool in order to be channeled towards triacylglycerol biosynthesis. Over the period of maximum lipid biosynthesis (14 to 16 days after pollination; Fig. 1), about 0.3 to 0.5 mg eicosenoate plus docosenoate are produced/seed·day, whereas total endogenous oleoyl phospholipids represent about 0.5 mg oleoyl residues/seed (Table I). If oleate were moving through to the site of chain elongation via total endogenous polar lipids, this pool should be turned over in about 1 day, and [^{14}C]oleate would move from polar lipids into [^{14}C]eicosenoate and [^{14}C]docosenoate. This was not observed. Nevertheless, the seed must have a mechanism to produce oleoyl phospholipids, as these increase during the early development of the seed (Table I).

Inasmuch as a single "site" of oleate biosynthesis cannot explain these data, the question then arises as to whether two sites exist in seeds within the same cell-type at the same developmental stage. If such is the case, then seed cells differ from leaf cells with respect to their sites for lipid biosynthesis because it has recently been shown (8) that only one site of fatty acid synthesis (acyl carrier protein-dependent) occurs in the leaf cell (*Spinacea oleracea* leaf protoplasts) and that this site is associated exclusively with the chloroplast. However, there are other explanations for the data presented here. Heterogeneity of cell type within the tissue (*i.e.* cotyledon, endosperm, and embryo), could give rise to a composite ^{14}C-labeled lipid pattern. Or the cotyledon cells may mature at very different rates. Thus, young cells, actively synthesizing membranes, would produce ^{14}C-labeled oleoyl phospholipids from [1-^{14}C]acetate, whereas older cells, actively synthesizing triacylglycerols, would produce mainly [1-^{14}C]eicosenoate and [1,3-^{14}C]docosenoate (Fig. 5).

The above results and discussion do not rule out the possibility of a small, specific polar lipid pool being involved in the biosynthesis of triacylglycerols containing *cis*-11-eicosenoate and *cis*-13-docosenoate nor even a small polar lipid pool transporting oleate from A to D (Fig. 5). The incorporation of radioactivity, as acyl groups from [1-^{14}C]acetate, into diacylglycerols and phosphatidic acid was very low in nasturtium seeds and, hence, not followed with time. However, the glycerol-3-P pathway to triacylglycerols may be active in nasturtium inasmuch as it has been demonstrated in *C. abyssinica* (6), an oilseed which produces large amounts of *cis*-13-docosenoate. The labeling pattern of the lipid classes in nasturtium differed markedly from that reported by Slack *et al.* (14) for oilseeds containing linoleate and α-linolenate, where high levels of diacylglycerol and phosphatidylcholine were labeled and where a rapid turnover of these lipids was suggested as being linked to desaturation and triacylglycerol production.

Although *cis*-11-eicosenoate and *cis*-13-docosenoate are labeled extensively at the chain-extended carbons, there is a small amount of label in the *de novo* portion. The relative specific radioactivities for carboxyl end and *de novo* portion carbon atoms were about 1: 0.03. This may represent a small "leakage" of exogenous [1-^{14}C]acetate, or its metabolites, into site A (Fig. 5) for oleate biosynthesis. Conversely, if two sites of oleate biosynthesis are operative concurrently within the cell, and since exogenous [1-^{14}C]acetate readily supplies site B (Fig. 5), site B may supply some [^{14}C]oleate for chain elongation.

The difference between the two types of lipid biosynthetic activity (A + D and B + C in Fig. 5) is highlighted by inhibition studies with trichloroacetic acid or 2,4-dinitrophenol. Inhibition of [1-^{14}C]acetate incorporation into [1-^{14}C]eicosenoate and [1,3-^{14}C]docosenoate, esterified into triacylglycerols, preceded inhibition of [^{14}C]oleate biosynthesis and polar lipid formation. [^{14}C]-Docosenoate biosynthesis was inhibited more strongly than [^{14}C]eicosenoate biosynthesis. This trend is reminiscent of the fatty acid patterns in breeding an erucate-free rapeseed (5). While approaching the zero-erucate rapeseed variety, the oil content/seed remained constant, as did the level of *cis*-11-eicosenoate, whereas the level of *cis*-13-docosenoate dropped concomitantly with oleate increase. Only when a zero-erucate variety was achieved did the level of *cis*-11-eicosenoate drop drastically. Two genes are believed to control the level of *cis*-13-docosenoate in rapeseed (5). From *in vivo* experiment presented here, it cannot be ascertained whether such an inhibition of elongation occurs only at the elongase(s) or at the level of cofactor and substrate supply, or both.

Under anaerobic or near-anaerobic incubation conditions, nasturtium seeds could synthesize labeled stearate, eicosanoate, and docosanoate from [1-^{14}C]acetate. These acids are not found *in vivo*. Pulse-chase studies suggested that [^{14}C]stearate and [^{14}C]palmitate (in polar lipids) were not transferred to [^{14}C]eicosanoate and [^{14}C]docosanoate (Fig. 3a). Also, the inhibition of [^{14}C]stearate and [^{14}C]palmitate biosynthesis by trichloroacetic acid occurred at higher concentrations than those which caused the inhibition of [^{14}C]eicosanoate and [^{14}C]docosanoate biosynthesis (Fig. 4b). Thus, the behavior of [^{14}C]palmitate and [^{14}C]stearate in polar lipids mirrors that of ^{14}C-labeled oleoyl polar lipids. The time course for saturated acyl production (Fig. 3b) indicates that it takes several hours for steady state levels to build up for the pathway palmitate → stearate → eicosanoate → docosanoate. Trichloroacetic acid inhibition seems to act at the "elongation system," the concurrent inhibition of [^{14}C]eicosanoate with [^{14}C]eicosenoate and [^{14}C]docosanoate with [^{14}C]docosenoate (Fig. 4b) would argue for a common pathway for the elongation of saturated and monounsaturated fatty acids. The saturated acid chain elongation pathway has relevance to fatty acid biosynthesis in meadowfoam. (*L. alba*) seeds, and its significance is discussed in a companion paper (12).

In summary, two sites of oleate biosynthesis may exist within nasturtium seed tissue, one which supplies oleate for incorporation into phospholipids and another which supplies oleate for chain elongation and subsequent triacylglycerol biosynthesis. It would not be surprising if, during cellular differentiation, the seed establishes an apparatus specific for the synthesis, from sucrose, of large amounts of "storage" fatty acids. However, the possible heterogeneity of the seed issue prevents further extrapolation of the data to ascertain whether there are two sites of *de novo* fatty acid biosynthesis operative (*a*) in the same cell type concurrently, (*b*) in the same cell type at different stages of development, or (*c*) in different cell types. The full answer to this important question must await techniques for the isolation of homogeneous cell populations from seeds and also the *in vitro* localization of the sites of fatty acid synthesis in these seeds.

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