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TRIACYLGLYCEROL SYNTHESIS BY SUBCELLULAR FRACTIONS OF MATURING SAFFLOWER SEEDS*

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Key Word Index—*Carthamus tinctorius*; Compositae; safflower; biosynthesis; triacylglycerol.

Abstract—A subcellular particulate fraction (10^3 *g*, precipitate) prepared from maturing safflower seeds catalysed triacylglycerol synthesis from oleoyl-CoA. The addition of a *rac*-1,2-diacylglycerol emulsion stimulated triacylglycerol formation, which was depressed at a high concentration of oleoyl-CoA. Some properties of the reaction were investigated.

INTRODUCTION

The pathway for triacylglycerol biosynthesis in plant seeds has not been clearly defined. It has been generally assumed that plants utilize the same pathway as animals [1, 2]. It is clear that the CDP†-base pathway is the major route for the formation of phosphatidylcholine and phosphatidylethanolamine in plants [3–5]. Barron and Stumpf [6] and Gurr *et al.* [7] strongly suggested the presence of the glycerol phosphate pathway for triacylglycerol synthesis in avocado mesocarp and maturing *Crambe* seeds, respectively. However, it has not entirely been proved, while it is clear that diacylglycerol is the precursor of triacylglycerol in plants [6, 8, 9]. There are several papers on subcellular particles catalysing triacylglycerol synthesis in plants [6–8, 10, 11]. Up to the present, it has been believed that triacylglycerol synthesis in maturing seeds mainly occurs in so-called oil droplets [7, 10, 11]. On the other hand, avocado microsomes form triacylglycerol from acyl-CoA or acyl-ACP (acyl carrier protein) [12]. The nuclear/plastid and mitochondrial fractions of maturing *Crambe* seeds are able to incorporate not only stearoyl-CoA but also malonyl-CoA into triacylglycerol as effectively as the fat fraction is [8]. *In vivo* lipid synthesis in maturing safflower seeds has been reported in the first paper of this series [9]. The purposes of the present investigation are to find the main site(s) (subcellular fraction(s)) of triacylglycerol synthesis from diacylglycerol and acyl-CoA and to reveal some properties of the reaction.

RESULTS AND DISCUSSION

Triacylglycerol synthesis from different substrates by various enzyme preparations

Intact safflower seeds, 18 days after flowering, rapidly incorporated [^{14}C]acetate into lipids, and 65% of the total radioactivity was recovered in triacylglycerol (Table 1). The activity of triacylglycerol synthesis was higher in protoplasts isolated from maturing seeds of the same developing stage than in homogenate. However, *de novo* synthesis of fatty acids and triacylglycerol synthesis were depressed by homogenization of seeds. Triacylglycerol formation from acyl-CoA and diacylglycerol is one simple step. Nevertheless, oleoyl-CoA was a poorer substrate than acetate or malonate. In another section it is shown that this is due to the absence of an acyl acceptor, 1,2-diacylglycerol. In the homogenate of maturing *Crambe* seeds, both stearoyl-CoA and malonyl-CoA were efficiently utilized for triacylglycerol synthesis [8]. The homogenate prepared from safflower seeds 12 days after flowering was not able to synthesize triacylglycerol from acetate. The homogenate preparation from seeds 18 days after flowering seems to contain acyl-CoA synthetase, because a non-esterified fatty acid (NH_4 linoleate) added was readily incorporated into diacylglycerol and compound lipids. Why the free fatty acid does not serve as a suitable acyl donor for triacylglycerol synthesis is not clearly understood.

Lipid synthesis by subcellular fractions

It has been demonstrated that fat fractions from maturing *Crambe* seeds [8] and castor bean [10] primarily synthesize triacylglycerol from stearoyl-CoA and oleoyl-CoA, respectively. Unexpectedly, most of the radioactivity incorporated into lipids from [1-^{14}C]oleoyl-CoA by the fat layer of maturing safflower seeds was found in compound lipids and diacylglycerol (Table 2). The percentage of ^{14}C in triacylglycerol of the fat layer was not as high as expected, and was nearly equal to that for the unfractionated homogenate. The microsomal fraction (10^5 *g*, precipitate) actively synthesized compound lipids, mainly phospholipids, and the supernatant of centrifugation at 10^5 *g* showed a strong acyl-CoA hydrolase activity. Over 10% of the ^{14}C label incorporated into total lipids was recovered from triacylglycerol in the 10^3 *g* and 10^4 *g* particulate fractions (Table 2). As a large amount of precipitate was obtained by centrifugation at 10^3 *g*, the total activity of triacylglycerol synthesis (nmol/total protein) of this fraction was highest, in spite of its low specific activity of

* Part 3 in the series "Lipid Metabolism in Safflower". For Part 2 see Ichihara, K. and Noda, M. (1981) *Phytochemistry* **20**, 1023.

† CDP = cytidine diphosphate.

total lipid synthesis; namely, 47% of the total activity of triacylglycerol synthesis was localized in this fraction. According to an optical microscopic observation, this fraction consisted mainly of homogeneous particles and was hardly contaminated with cell debris or other cell organelles. From maturing *Crambe* seeds, Appleby *et al.* [8] obtained three fractions catalysing triacylglycerol synthesis; one of the fractions consisted of organelles with the greatest density. McMahon and Stumpf [13, 14] showed that a particulate fraction (3000 g, precipitate) from maturing safflower seeds had the highest specific activity for incorporation of [1-^{14}C]acetate into fatty acids and that the fraction efficiently converted oleoyl-CoA to linoleic acid. On the basis of these data, it is concluded that the subcellular particles with the greatest density in maturing safflower seeds are, at least, one of the sites for triacylglycerol synthesis from acyl-CoA and 1,2-diacylglycerol and that they play very important roles not only in fatty acid synthesis and desaturation, but also in triacylglycerol formation. This fraction was not further purified in the present work, and these particles were not identified. Tentatively, we think that they may be protein bodies (aleurone grains) or plastids. Their ultrastructure and chemical composition are now under investigation.

Effect of rac-1,2-*diacylglycerol on triacylglycerol synthesis*

The incorporation of oleoyl-CoA into triacylglycerol by the 10^3 *g* particulate fraction was 10% of the total at

Table 1. Lipid biosynthesis from different substrates by various enzyme preparations from safflower seeds

Enzyme preparation	Substrate	Distribution of ^{14}C (%)			
		TG	FA	DG	CL
Homogenate from seeds 18 DAF*	[1-^{14}C]Oleoyl-CoA†	5.8	21.4	26.0	46.7
	NH_4 [1-^{14}C]linoleate‡	0.9	11.9	47.9	39.3
	Na [2-^{14}C]malonate§	14.3	18.8	12.3	54.6
	Na [U-^{14}C]acetate‖	14.5	12.1	12.0	61.4
Homogenate from seeds 12 DAF	Na [U-^{14}C]acetate ¶	0.7	3.7	0.3	95.3
Protoplasts from seeds 18 DAF	Na [U-^{14}C]acetate**	38.4	7.6	32.6	21.5
Protoplasts from cotyledons 5 DAG*	Na [U-^{14}C]acetate**	8.0	10.4	24.5	57.0
Intact seeds 18 DAF††	Na [U-^{14}C]acetate	64.8	4.9	17.5	12.9

* DAF = Days after flowering, DAG = days after germination.

† The reaction mixture contained 50 mM K-Pi(pH 7), 0.4 M sorbitol, 1.6 μM [1-^{14}C]oleoyl-CoA and 0.26 mg protein of the homogenate.

‡ The incubation with NH_4 [1-^{14}C]linoleate (4.2 μM) was carried out in 0.1 M K-Pi buffer (pH 7) containing 0.4 M sorbitol and 3.75 mg protein of the homogenate, but no cofactor was added to the reaction mixture.

§ The reaction mixture contained 50 mM K-Pi(pH 7), 0.4 M sorbitol, 5 mM ATP, 0.2 mM NADPH, 0.2 mM CoA, 8.0 μM Na [2-^{14}C]malonate and 4.6 mg protein of the homogenate.

‖ The reaction mixture contained 50 mM K-Pi (pH 7), 0.4 M sorbitol, 2 mM ATP, 0.1 mM NADH, 0.1 mM NADPH, 5 mM $KHCO_3$, 0.5 mM $MnCl_2$, 0.1 mM CoA, 20.3 μM Na [U-^{14}C]acetate and 4.6 mg protein.

¶ The composition of the reaction mixture was the same as that of the mixture described above (‖), except that protein was 3.8 mg.

** Protoplasts were isolated from seeds 18 days after flowering and from cotyledons 5 days after germination by the conventional method [20], and incubated in 0.1 M K-Pi buffer (pH 7) containing 0.7 M sorbitol and 20.3 μM Na [U-^{14}C]acetate. Protein of protoplasts added was 0.095 mg in the case of protoplasts from maturing seeds and 0.66 mg in the case of protoplasts from germinating cotyledons, respectively.

†† Data from ref. [9].

Incubation was carried out at 30° for 1–3 hr (total vol. 1 ml), but intact seeds were incubated at 20° for 4 hr. Data on sterol and sterol ester syntheses are omitted, because only small amounts of these compounds were synthesized by the homogenate and protoplast preparations. TG, Triacylglycerol; FA, free fatty acid; DG, diacylglycerol; CL, compound lipids.

Table 2. Incorporation of [1-^{14}C]oleoyl-CoA into lipid classes by various subcellular fractions of maturing safflower seeds 18 days after flowering

Fraction	Total protein (mg)	Incorporation of [1-^{14}C]oleoyl-CoA into total lipids (nmol/mg protein)	Distribution of ^{14}C(%)				Total incorporation of [1-^{14}C]oleoyl-CoA into TG (nmol)
			TG	FA	DG	CL	
Homogenate	225.0*	1.00	5.9	11.6	21.5	61.1	13.3
10^3 g, ppt.	86.1	0.60	10.9	23.3	26.0	39.8	5.6
10^4 g, ppt.	6.3	1.67	10.3	17.3	15.4	57.0	1.1
10^5 g, ppt.	7.5	2.40	6.0	8.1	7.4	78.5	1.1
10^5 g, sup.†	35.3	1.30	3.0	65.6	10.9	20.5	1.4
Fat layer	35.3	1.24	6.2	7.9	18.7	67.2	2.7
(Recovery)	(75.8%)						(89.5%)

* Corresponding to 72 grains of seed.
† Supernatant fluid.
Each reaction mixture contained 0.3–1.3 mg protein.

most, in contrast to *de novo* synthesis of triacylglycerol from acetate (Table 1). We attempted to raise the activity of incorporation of oleoyl-CoA into triacylglycerol *in vitro*. Dybing and Craig [15] revealed the presence of small phospholipid and diacylglycerol pools and their active turnover in developing flax seeds. Various preparations from safflower seeds incorporated labelled substrates into diacylglycerol which was a very small pool, except for the homogenate from seeds 12 days after flowering (Table 1). An *in vivo* study on triacylglycerol synthesis in maturing safflower seeds suggested that diacylglycerol is the precursor of triacylglycerol [9]. The addition of *rac*-1,2-diacylglycerol emulsified without Triton X-100 was the most effective method for triacylglycerol formation (Table 3). When the particles were sonicated before incubation, triacylglycerol formation was inhibited. Triton X-100 also inhibited the reaction. Although avocado microsomes rapidly transferred acyl moieties of acyl-ACPs to endogenous diacylglycerol, exogenously added *rac*-1,2-dioleoylglycerol did not serve as an acyl acceptor [12]. It is not clear to what this difference in utilization of exogenous diacylglycerol between avocado and safflower may be attributed.

Properties of in vitro *syntheses of triacylglycerol and other lipids from oleoyl-CoA*

[1-^{14}C]Oleoyl-CoA was most rapidly incorporated into compound lipids (Fig. 1). The label in these lipids decreased slowly during the middle and later periods of incubation, while the label in diacylglycerol gradually increased during the same periods. This suggests that part of the phospholipids may be converted to diacylglycerol by phospholipase(s). Slack *et al.* [16] showed the important roles of phosphatidylcholine for oleate desaturation and triacylglycerol formation. Triacylglycerol formed appears not to be destructively metabolized in this system.

Table 3. Effect of *rac*-1,2-diacylglycerol, Triton X-100 and sonication treatment on incorporation of [1-^{14}C]oleoyl-CoA into lipids by the 10^3 g particulate fraction

Concn of *rac*-1,2-DG (mg/ml)	Concn of Triton X-100 (%)	Time of sonication (sec)	Incorporation of [1-^{14}C]oleoyl-CoA (nmol/mg protein)				
			Total lipids	TG	FA	DG	CL
0	0	0	1.28	0.15	0.36	0.18	0.59
0	0	30*	0.83	0.05	0.43	0.12	0.22
0	0.02	0	0.57	0.08	0.32	0.08	0.09
0	0.02	30*	0.58	0.05	0.38	0.08	0.07
2.5†	0	0	1.30	0.35	0.34	0.21	0.40
2.5†	0	30*	0.83	0.21	0.21	0.15	0.26
2.5†	0.02	0	0.72	0.16	0.23	0.07	0.26
2.5†	0.02	30*	0.46	0.07	0.19	0.09	0.11

*The reaction mixtures including the particulate fraction were sonicated at 0° just before incubation.
† *rac*-1,2-Diacylglycerol was previously emulsified with or without Triton X-100 by sonication.
Each reaction mixture contained 0.33 mg protein of the 10^3 g particulate fraction.

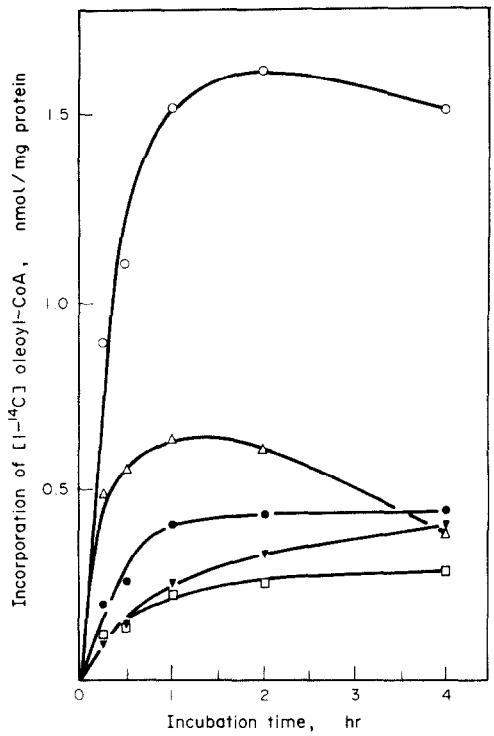

Fig. 1. Time course of lipid synthesis from [1-^{14}C]oleoyl-CoA by the 10^3 g particulate fraction. The reaction mixture contained 0.1 M K-Pi (pH 7), 0.4 M sorbitol, 0.25 mg *rac*-1,2-diacylglycerol, 1.6 μM [1-^{14}C]oleoyl-CoA and 0.48 mg protein of the fraction. ○———○, Total lipids; ●———●, triacylglycerol; ▼———▼, diacylglycerol; □———□, free fatty acid; △———△, compound lipids.

At the highest concentration of oleoyl-CoA (0.1 mM), the incorporation of the oleoyl moiety into triacylglycerol and compound lipids decreased, while the radioactivity of free fatty acid increased (Table 4). A similar inhibition for triacylglycerol formation has been observed in avocado microsomes [12]. The hydrolase, acyl-CoA hydrolase, lipase or phospholipase that caused the ^{14}C accumulation in free fatty acid was not examined. It is most likely that acyl-CoA hydrolase is activated by acyl-CoA as a self-defensive cell function, because long-chain acyl-CoA is a surfactant and may be harmful to cells. The radioactivity in diacylglycerol also increased at the highest concentration of oleoyl-CoA and that in compound lipids decreased. This may be due to the activation of phospholipase C and/or phosphatidate phosphatase by oleoyl-CoA.

Table 4. Effect of oleoyl-CoA concentration on lipid biosynthesis from [1-^{14}C]oleoyl-CoA by the 10^3 g particulate fraction

Concn of oleoyl-CoA (μM)	Incorporation of [1-^{14}C]oleoyl-CoA (nmol/mg protein)				
	Total lipids	TG	FA	DG	CL
2	1.56	0.44	0.27	0.26	0.60
5	4.11	1.36	0.69	0.77	1.29
20	8.19	1.54	4.09	0.89	1.66
100	11.69	0.36	8.14	2.19	1.00

Each incubation mixture contained 0.1 M K-Pi (pH 7), 0.4 M sorbitol, 0.25 mg *rac*-1,2-diacylglycerol, 0.48 mg protein of the fraction and [1-^{14}C]oleoyl-CoA.

One grain of seed 18 days after flowering corresponds to 1.2–2.4 mg protein of the 10^3 g particles. A linear relationship between the amount of protein and total lipid synthesis was obtained at low concentrations of protein (<0.5 mg/ml). In a preliminary experiment with unfractionated homogenate, it was found that protein concentration does not affect the percentage of incorporation of oleoyl-CoA into triacylglycerol.

Triacylglycerol and diacylglycerol syntheses were markedly depressed under lower pH conditions (Fig. 2), while compound lipid synthesis proceeded significantly even under these conditions. The pH profile of triacylglycerol synthesis represents that of diacylglycerol acyltransferase, and the pH optimum is nearly equal to those of animal acyltransferases (pH 7.4–9.0) [2].

EXPERIMENTAL

Preparation of subcellular fractions. Maturing safflower seeds were harvested 16–18 days after flowering and homogenized in 0.1 M K-Pi buffer (pH 7) containing 0.4 M sorbitol with a Potter–Elvejhem homogenizer. The homogenate was filtered through two layers of cotton cloth. The filtrate was centrifuged at 10^3 g for 10 min, and the supernatant centrifuged at 10^4 g for 10 min. Floating fat layers obtained by these centrifugations were combined. The microsomal and final supernatant fractions were obtained by centrifugation at 10^5 g for 1 hr. The pellets and the fat layer obtained were suspended in the same buffer used for homogenization.

Incubation and determination of radioactivity. The standard assay system contained 0.05–0.1 M K-Pi (pH 7), 0.4 M sorbitol, 1.6 μM [1-^{14}C]oleoyl-CoA and an aliquot of the suspension of each subcellular fraction. The final vol. was 1 ml and incubation was carried out at 30° for 1 hr, unless otherwise stated. Lipids

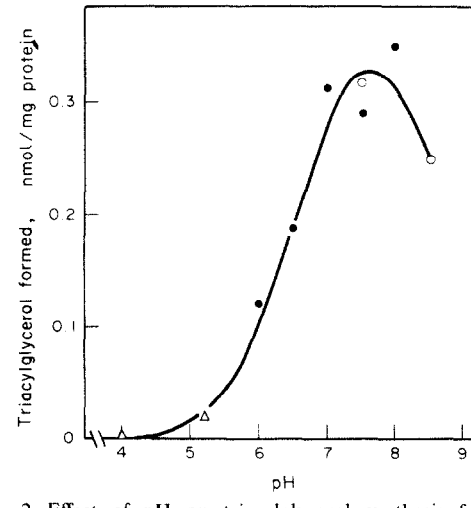

Fig. 2. Effect of pH on triacylglycerol synthesis from [1-^{14}C]oleoyl-CoA by the 10^3 g particulate fraction. The reaction mixture contained 0.4 M sorbitol, 0.25 mg *rac*-1,2-diacylglycerol, 1.6 μM [1-^{14}C]oleoyl-CoA, 0.15 mg protein of the fraction and 0.1 M buffer soln. △, K citrate; ●, K-Pi; ○, Tris–HCl.

were extracted by the method of ref. [17] and were separated into triacylglycerol, free fatty acid, diacylglycerol and compound lipids by TLC on Si gel G(hexane–Et_2O–HOAc, 70:30:1). The radioactivity of each lipid class was measured by the procedure described in ref. [9].

Preparation of rac-1,2-*diacylglycerol.* Olive oil was partially hydrolysed by pancreatic lipase. The hydrolysate was extracted with Et_2O and washed with 5% Na_2CO_3 to remove free fatty acid. *rac*-1,2-Diacylglycerol was isolated from the lipid mixture by TLC on Si gel G(C_6H_6–Et_2O, 17:3). The fatty acid composition was 5.4% palmitic acid, 0.9% stearic acid, 83.0% oleic acid and 10.8% linoleic acid.

Determination of protein. Protein was determined by the methods of refs. [18] or [19].

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