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FATTY ACID COMPOSITION AND LIPID SYNTHESIS IN DEVELOPING SAFFLOWER SEEDS*

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Key Word Index—*Carthamus tinctorius*; Compositae; safflower; biosynthesis; fatty acid composition; triacylglycerol; stereospecific analysis.

Abstract—Linoleic acid predominated in every lipid class during the whole period of seed development of safflower, while linolenic acid decreased with increasing maturation and it was not detected in mature seeds. Just before the initiation of triacylglycerol accumulation, the fatty acid composition of triacylglycerols changed more rapidly than those of phospholipids and glycolipids. Saturated fatty acids tended to accumulate at the 1- and 3-positions of the glycerol molecule and the more highly unsaturated acids at the 2-position. The fatty acid compositions at the 1- and 3-positions were similar in all cases investigated, but in none of the triacylglycerols was the distribution completely symmetrical. The positional distribution of linolenic acid in triacylglycerols prepared from the immature seeds 2 days after flowering and from the leaves was unusual; in spite of its highest degree of unsaturation, it was preferentially esterified at the 1- and 3-positions. When triacylglycerol was most rapidly accumulated (14–18 days after flowering), the incorporation of acetate-[U-^{14}C] into total lipids was also maximum and dienoic fatty acids were the principal acids labelled. Diacylglycerols and compound lipids reached the highest rate of synthesis 15 days after flowering, and then a maximum incorporation into triacylglycerol occurred 18 days after flowering. Incubation temperature affected the synthesis of individual lipid classes. Triacylglycerol was more rapidly synthesized at 32° than at 10°, while diacylglycerols and compound lipids were accumulated under the low-temperature condition. A rise of incubation temperature caused a depression in dienoic acid synthesis.

INTRODUCTION

The pathways of triacylglycerol biosynthesis in animal tissues are well documented [1]. In plants, the biosynthesis of triacylglycerols has been studied to a very minor extent [2], although fatty acid synthesis has been extensively investigated by many workers [3–7]. Recently, Gurr *et al.* [8–10] reported detailed data on the biosynthesis of fatty acids and triacylglycerols in maturing seeds of *Crambe abyssinica*. Other workers [2, 11] also investigated triacylglycerol synthesis connecting with subcellular particles in developing seeds. However, it seems that the information obtained up to the present is inadequate to explain the mechanism of triacylglycerol synthesis and its relationship to subcellular particles. Because of the lack of information on the lipid biochemistry of the developing seeds of oil plants, we have initiated a series of integrated studies to elucidate the nature of lipid metaboilsm in safflower, *Carthamus tinctorius* L., which is one of the most important economic oil plants.

Recent advances in the biosynthesis of fatty acids by maturing safflower seeds have been reviewed by Stumpf [12]. The fatty acid composition of safflower oil and the distribution of fatty acids between the α- and β-positions of triacylglycerol have already been reported [13, 14]. To complete these basic data, which are essential to future studies on lipid metabolism in developing safflower seeds, changes in the fatty acid compositions of triacylglycerols, phospholipids and glycolipids during seed development were investigated, and the positional distribution of fatty acids in triacylglycerols was determined by stereospecific analysis. In addition, the present paper also deals with acetate-[U-^{14}C] incorporation into lipids by developing safflower seeds *in vivo*.

RESULTS AND DISCUSSION

Triacylglycerol formation

The accumulation of triacylglycerol is initiated 12 days after flowering (Fig. 1). The lipid contents of the immature seeds were 2% or less at the early stage of development (0–10 days after flowering). The highest rate of triacylglycerol accumulation was observed between 14 and 18 days after flowering and was estimated to reach upward of 1.5 μmol per seed per day. The safflower seeds used in the present experiment produced oil more rapidly than those used by Sims *et al.* [15]; this may reflect differences in climate. It is interesting that the lag phase was constant (10 days) in both cases. The question posed by Stumpf [12] remains unsolved—why in the maturation of the seed does vigorous lipid synthesis begin on the 10th day after flowering? The triacylglycerol content increased to over 10 μmol per seed 20 days after flowering, and

* Part I in the series "Lipid Metabolism in Safflower".

then it was held constant at the late stage, in which an increase in the percentage of triacylglycerol, however, continued in consequence of loss of the moisture.

Fatty acid compositions of triacylglycerols, phospholipids and glycolipids

Sims *et al.* observed that the ratio of the concentrations of oleic and linoleic acids varied continuously in the total oil during the period of maturation [15]. This applies also for the individual lipid classes, viz. triacylglycerols, phospholipids and glycolipids (Fig. 2). In the first few days after flowering, the fatty acid composition of triacylglycerols changed little, and thereafter an increase in the proportion of oleic acid and a decrease of linolenic acid were observed between 6 and 10 days after flowering. The percentage of linoleic acid, which is a major fatty acid of safflower oil, increased rapidly during the period from 12 to 14 days after flowering, in which linoleic and oleic acids were complementary to each other in respect of proportion. The fatty acid composition of triacylglycerols at the period characterized by its rapid accumulation was almost the same as that in the mature seeds. In general, the fatty acid composition of phospholipids was similar to that of glycolipids, and it did not vary much during maturation. The fatty acids of triacylglycerols were more unsaturated than those of phospholipids and glycolipids. The decreases of oleic acid, which were observed between 12 and 15 days after flowering, were undoubtedly due to the rate of formation of oleic acid being less than that of linoleic acid [15]. It is interesting that the linolenic acid content of triacylglycerols at the early stage of development is *ca* 30% of the total fatty acids and that it is higher than those of phospholipids and glycolipids. All the lipid classes in the mature seeds comprised 4 fatty acids, palmitic, stearic, oleic and linoleic acids, but linolenic acid, the predominant fatty acid of glycolipids in safflower leaves, was not detected in mature seeds. The major classes of glycolipids in the seeds were sterol glycoside and esterified sterol glycoside, while

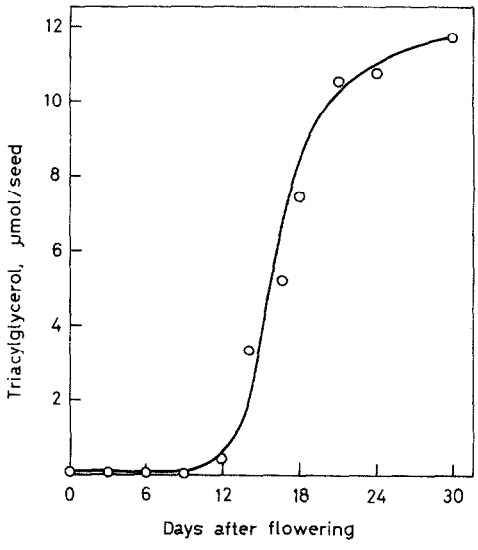

Fig. 1. Changes in triacylglycerol content of safflower seeds during maturation.

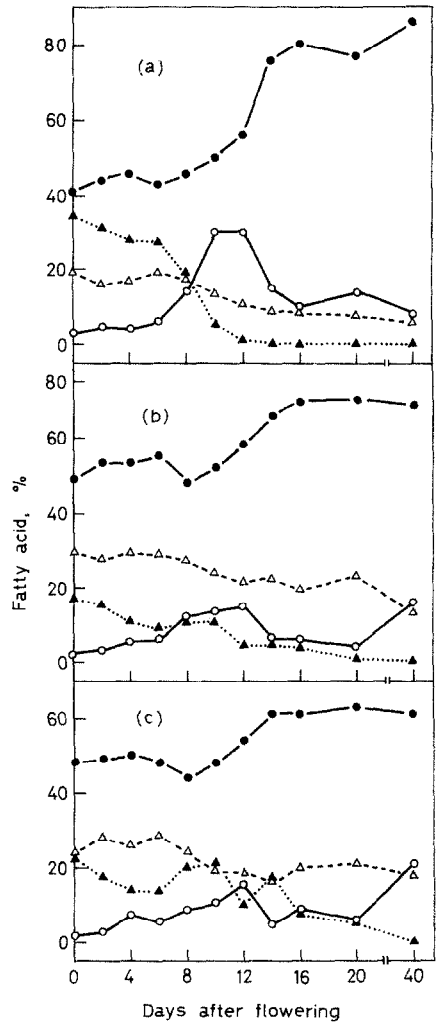

Fig. 2. Changes in fatty acid compositions of triacylglycerols(a), phospholipids(b) and glycolipids(c) in safflower seeds during maturation. Stearic acid is omitted because of its very low content (less than 3% of total fatty acids during the whole period of development). △---△, Palmitic acid; ○——○, oleic acid; ●——●, linoleic acid; ▲····▲, linolenic acid.

those in the leaves were monogalactosyldiacylglycerol and digalactosyldiacylglycerol (Ichihara, K. and Noda, M., unpublished data). This seems to cause the large difference in linolenic acid content between glycolipids of the two organs. Lauric, myristic and palmitoleic acids were found only in the immature seeds.

Positional distribution of fatty acids in triacylglycerols

During the whole period of development, palmitic and stearic acids occurred at the 1- and 3-positions, whilst oleic acid was randomly distributed among the 1-, 2- and 3-positions, and linoleic acid showed a preference for the 2-position (Table 1). It should be noted that linolenic acid in the triacylglycerols of the leaves and immature seeds (2 days after flowering) was preferentially located at the 1- and 3-positions in spite of its high degree of unsaturation. A similar tendency was observed with phospholipids of germinating

Table 1. Positional distribution of fatty acids in safflower triacylglycerols

Source of triacylglycerol	Position	Fatty acid (%) 16:0	18:0	18:1	18:2	18:3
Seed (2 DAF*)	1	29.7	5.7	4.6	38.2	21.6
	2	3.9	0.0	2.6	75.5	18.0
	3	24.0	2.5	5.2	39.3	29.1
	total	19.2	2.7	4.1	51.0	22.9
Seed (14 DAF)	1	13.7	2.1	13.8	70.3	0.0
	2	0.0	0.0	15.1	84.9	0.0
	3	11.8	0.0	15.2	72.8	0.0
	total	8.5	0.7	14.7	76.0	0.0
Seed (matured)	1	8.7	2.5	8.1	80.5	0.0
	2	0.0	0.0	8.1	91.9	0.0
	3	3.9	0.5	6.6	88.9	0.0
	total	4.2	1.0	7.6	87.1	0.0
Seedling (7 DAG†)	1	11.8	4.1	19.3	64.8	0.0
	2	1.6	0.0	20.8	77.6	0.0
	3	7.9	1.9	19.3	71.0	0.0
	total	7.1	2.0	19.8	71.1	0.0
Leaf	1,3	18.8	0.0	1.6	23.7	55.8
	2	0.0	0.0	1.7	49.7	48.6
	total	12.5	0.0	1.6	32.4	53.5

* Days after flowering.
† Days after germination.

safflower seedlings; linolenic acid was preferentially esterified at the 1-position of phosphatidylcholine and phosphatidylethanolamine (Ichihara, K. and Noda, M., unpublished data). On the basis of these data, the positional distribution pattern of fatty acids in saffllower triacylglycerols is summarized as follows; palmitic and stearic acids are exclusively esterified at the 1- and 3-positions, oleic acid is apparently non-specific for all positions, linoleic acid shows a preference for the 2-position; the general rule of fatty acid distribution is not applied to linolenic acid which shows a specificity for the 1- and 3-positions. The component triacylglycerols of safflower oil were estimated by Gunstone *et al.* [14]. But the question remained as to whether the fatty acids at the 1- and 3-positions were specifically or non-specifically distributed among these positions. This question can be resolved by stereospecific analysis. As shown in Table 1, there are some differences between the fatty acid compositions of the 1- and 3-positions. Regardless of seed age, saturated acids showed a preference for the 1-position while linoleic acid showed some preference for the 3-position.

Incorporation of acetate-[U-^{14}C] into total lipids and individual lipid classes

The incorporation of acetate-[U-^{14}C] into total lipids rapidly increased from 12 to 15 days after flowering. The maximum activity of incorporation was observed with immature seeds 18 days after flowering. The seeds at this stage of development have been found to accumulate triacylglycerols very rapidly. Thereafter, the incorporation rate decreased with proceeding maturation. These data were in reasonable agreement with those of McMahon *et al.* [16], who expressed the rate of incorporation in nmol per fr. wt.

Fig. 3 illustrates the distribution of radioactivity among the individual lipid classes and shows the variation with seed maturation. During the initial period of development, compound lipids, which mainly consisted of phospholipids, were primarily synthesized. During the period of rapid synthesis of lipids, i.e.

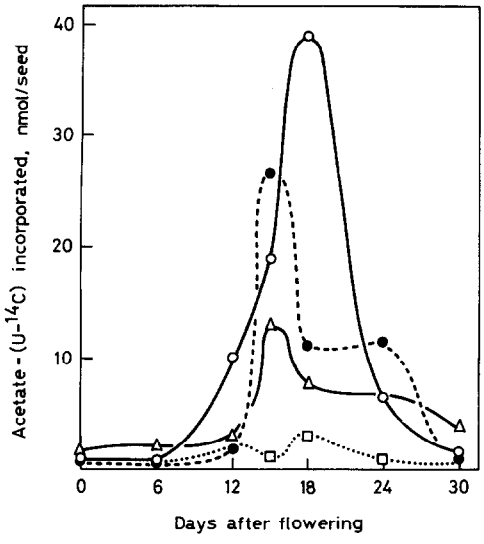

Fig. 3. Incorporation of acetate-[U-^{14}C] into individual lipid classes by developing safflower seeds. The data on incorporation into sterol esters and free sterols are omitted. The maximum values for sterol esters and free sterols were 3.6 and 3.4 nmol/seed, respectively, 12 days after flowering. ○——○, Triacylglycerol; ●---●, diacylglycerol; □···□, free fatty acid; △——△, compound lipids.

12–24 days after flowering, most of the ^{14}C label appeared in triacylglycerols, diacylglycerols and compound lipids, with less in sterol esters, free fatty acids and free sterols. The low incorporation rate of ^{14}C for free fatty acids eliminated the possibility that diacylglycerols might be formed by lipase hydrolysis during the period of incubation or lipid extraction. Both diacylglycerols and compound lipids reached the highest rate of synthesis 15 days after flowering, and then the maximum incorporation into triacylglycerols occurred 18 days after flowering. These results may suggest that diacylglycerol is the precursor of triacylglycerol, and that compound lipids also participate in triacylglycerol formation. The results of labelling studies with the developing cotyledons of linseed, soya bean and safflower by Slack *et al.* suggested that oleate, initially esterified to phosphatidylcholine, was first desaturated, and then polyunsaturated fatty acids transferred to triacylglycerol via diacylglycerol [17]. The incorporation curves in Fig. 3 are consistent with the operation of the biosynthetic route proposed by Slack *et al.* The distribution pattern of radioactivity 30 days after flowering resembled those at the early stage of development, with minor exceptions. The immature seeds harvested during the period 15–18 days after flowering will be most suitable for the experimental materials for studies on triacylglycerol biosynthesis.

Biosynthesis of fatty acids

Saturated and monoenoic fatty acids, probably palmitic, stearic and oleic acids, were effectively synthesized at the early stage of development (Table 2), although their absolute amounts of incorporation were small. When lipid biosynthesis proceeded most rapidly (15 and 18 days after flowering), dienoic fatty acids were the principal acids labelled. This is expected because linoleic acid comprises over 70% of total fatty acids in safflower oil. In mature seeds that had completed the synthesis and accumulation of triacylglycerols, most of the ^{14}C label incorporated into fatty acids appeared in saturated and monoenoic fatty acids, while only 8% of the label was present in the dienoic acid fraction.

Effect of temperature on lipid synthesis

There was no difference in incorporation of acetate-[^{14}C] into total lipids between the seeds incubated at 10° and 32° (Table 3). Both these incorporation rates were half the value measured at 20° (Fig. 3). Therefore, the incubation temperatures, 10° and 32°, seem to be too low and too high, respectively, for lipid synthesis in safflower seeds.

The biosynthesis of each lipid class was considerably affected by incubation temperature. Triacylglycerol synthesis was stimulated under the high-temperature condition and depressed under the low-temperature condition. In view of seed maturation, this result is predictable because maturation occurs early in summer. In contrast with triacylglycerols, diacylglycerols and compound lipids were synthesized more effectively at 10° than at 32°. The accumulation of diacylglycerols under the low-temperature condition seems to be due to the depression of their conversion into triacylglycerols.

Both sterol and sterol ester comprised *ca* 10% of total lipids synthesized at 32°. As the temperature was changed to 10°, the incorporation of acetate-[^{14}C] into sterol decreased to 25% of that at 32° while sterol ester increased in radioactivity by 50%. The ratio between free sterol and sterol ester synthesized may be of significance for the maintenance of membrane structures in the seed cells at the different temperatures.

Table 2. Incorporation of acetate-[U-^{14}C] into individual fatty acid classes by developing safflower seeds

Seed age (DAF*)	Distribution of ^{14}C incorporated (%) Saturated	Monoenoic	Dienoic
0	30.5	24.0	45.5
6	44.4	47.9	7.7
12	23.1	37.7	39.1
15	12.6	10.9	76.5
18	15.4	13.2	71.5
24	12.9	42.6	44.6
30	30.1	62.2	7.6

* Days after flowering.

The ^{14}C label was also recovered as trienoic acids in appreciable proportions, but the data on these acids are omitted because of contamination by unknown substances. The sum of the radioactivities of saturated, monoenoic and dienoic acids is set at 100%. Although fatty acids in each fraction were not identified, the saturated, monoenoic and dienoic acid fractions were presumed to be comprised of palmitic plus stearic, oleic and linoleic acids, respectively, from the data shown in Fig. 2.

It is known that some plant species, when grown in different environments, produce seed oils differing in unsaturation. The highest content of linoleic acid (or linolenic acid in the case of linseed oil) and the lowest content of oleic acid are attained when the plant is grown in the coolest climate [13]. Hilditch *et al.* [13] noted that these variations were generally confined to oleic, linoleic and linolenic acids, but that climatic temperature had little effect on the proportion of saturated fatty acids. It was observed by Canvin [18] that the fatty acid compositions of the seed oils of rape, sunflower and flax depended on temperature when the plants were grown under laboratory conditions, while safflower and castor oils were not affected. He also showed that the proportion of saturated fatty acids in these seed oils was independent of environmental temperature. In brief, the fatty acid composition of safflower oil is almost independent of growth temperature. In our experiment using developing safflower seeds, a rise of incubation temperature caused a significant decrease of the proportion of dienoic acids synthesized, a moderate increase of monoenoic acids and a marked increase of saturated acids (Table 4). Thus, the fatty acid composition was dependent on temperature when fatty acids were synthesized from acetate in a flask by the naked seeds separated from the intact plant of safflower, while it was independent of temperature when fatty acids were synthesized by the intact plant. This obvious discrepancy can be partly explained by the following hypothesis. The fatty acid composition of safflower oil is essentially temperature-dependent, but it is controlled by the desaturase level which is governed by the genes. When a change in environmental temperature

Table 3. Effect of temperature on lipid synthesis in developing safflower seeds

Incubation temperature	Acetate-[U-^{14}C] incorporated		Distribution of ^{14}C (%)					
	nmol/seed	nmol/mg fr. wt	SE	TG	FA	ST	DG	CL
10°	33.4	2.98	13.5	43.5	0.6	2.3	20.2	20.1
32°	34.9	3.17	9.4	54.8	1.6	9.6	10.5	14.1

SE, sterol esters; TG, triacylglycerols; FA, free fatty acids; ST, free sterols; DG, diacylglycerols; CL, compound lipids.

occurs, the desaturase level varies to keep the fatty acid composition constant. However, transient changes in the composition may be unavoidable. Consequently, the fatty acid composition of safflower oil appears to be independent of growth temperature although it changes transiently. The temperature dependence of the fatty acid compositions of sunflower and flax seeds [18] may be due to a lack of such a long-term control

Table 4. Effect of temperature on fatty acid synthesis in developing safflower seeds

Incubation Tempetature	Distribution of ^{14}C incorporated (%)		
	Saturated	Monoenoic	Dienoic
10°	9.3	23.9	66.8
32°	29.4	28.8	41.8

Data on trienoic acid synthesis are omitted.

system. Knowles [19] showed that the fatty acid composition of an experimental type of safflower with *ca* equal amounts of oleic and linoleic acids in the seed oil was responsive to temperature. This observation supports the above hypothesis that the fatty acid composition of safflower oil is essentially temperature-dependent, and it suggests the participation of the long-term control system in fatty acid synthesis and desaturation in the high linoleic type of safflower. Furthermore, the presence of physical control systems must also be taken into account to give an explanation of the discrepancy described above. One of the systems is the control of oxygen concentration which is the rate-limiting factor for desaturation [20]. As a change in temperature brings about a change in the solubility of oxygen, the oxygen concentration in the naked seeds (without seed coat) used in this experiment must have been directly affected by incubation temperature. This change in oxygen concentration in the naked seeds might cause the differences between the compositions of fatty acids synthesized under the different temperature conditions.

EXPERIMENTAL

Safflower plants were grown in the University field, and the developing seeds were obtained at desired stages of development.

Lipid extraction. Seeds were homogenized ×3 with $CHCl_3$–MeOH(1:1), the homogenates combined, filtered and conca under red. pres. The crude lipids obtained were dissolved in Et_2O and dried (Na_2SO_4). After removal of Na_2SO_4 and insoluble non-lipid contaminants by filtration, the solvent was removed by distillation and the lipids dissolved in a minimum vol. of $CHCl_3$.

Fractionation, determination and analyses of seed lipids. The $CHCl_3$ soln of seed lipids was applied to a Si gel column. Neutral lipids were eluted with $CHCl_3$, glycolipids with Me_2CO, and phospholipids with MeOH. Triacylglycerols were isolated from the neutral lipid fraction by TLC on Si gel plates with *n*-hexane–Et_2O–HOAc (85:15:1). Triacylglycerols were determined by the hydroxamate method of ref. [21]. Fatty acid Me esters were prepared from the individual lipid classes by methanolysis with NaOMe–MeOH and analysed by GLC (15% DEGS). The procedure of ref. [22] was used to determine the positional distribution of fatty acids in triacylglycerols.

Incorporation of acetate-[U-^{14}C]. Safflower seeds were harvested at the desired stages of development. They were cut in half with a razor and their seed coats removed. Immediately, 5 grains for each developing stage were immersed in 0.1 ml 10 mM Na acetate-[U-^{14}C] (2 μCi) at 20°. After incubation for 4 hr, the seeds were rinsed with H_2O and homogenized with a mixture of 1 ml $CHCl_3$, 2 ml MeOH and 0.8 ml H_2O. Insoluble materials were removed by filtration, and $CHCl_3$ and H_2O were added to give a final proportion of $CHCl_3$–MeOH–H_2O, 2:2:1.8 [23]. The $CHCl_3$ was evapd to dryness under red. pres. and the lipids were stored at −20° as a C_6H_6 soln. The radioactivity of the total lipids was measured in 10 ml of toluene scintillation fluid containing 20 mg PPO and 0.5 mg POPOP.

Separation of labelled lipids into lipid classes. The radioactive lipids were separated into sterol esters, triacylglycerols, free fatty acids, free sterols, diacylglycerols and compound lipids by TLC. A TLC plate of Si gel was developed twice with *n*-hexane–C_6H_6–HOAc (25:75:2) to separate free fatty acids, free sterols and diacylglycerols. A mixture of the partial hydrolysate of olive oil with pancreatic lipase and cholesterol was co-chromatographed on the plate. The chromatogram was sprayed with a 1% soln of I_2 in EtOH. Each band of Si gel was scraped into a scintillation vial containing 40 mg PPO, 1 mg POPOP, 750 mg naphthalene and 3.3 ml each of toluene, 1,4-dioxane and ethylene glycol monoethyl ether for the measurement of radioactivity.

Incorporation of acetate-[U-^{14}C] *into fatty acids.* An aliquot of the total lipids labelled with ^{14}C was heated with 5% BF_3 in MeOH under reflux for 20 min. $CHCl_3$ was added to the reaction mixture and then washed ×3 with H_2O. After removal of $CHCl_3$ by evapn, the fatty acid Me esters were applied to a TLC plate coated with $AgNO_3$–Si gel (1:9). The plate was developed with *n*-hexane–Et_2O (75:25) in the dark. The separated Me esters were visualized under UV after being sprayed with a 0.2% EtOH soln of 2′,7′-dichlorofluorescein. Bands corresponding to individual classes of esters were scraped off and transferred to counting

vials. The chemical composition of the scintillation fluid was the same as that used for the measurement of the radioactivity of lipid classes.

Effect of incubation temperature on incorporation of acetate-[U-^{14}C] into lipids. Developing safflower seeds, 16 days after flowering, were incubated at 10° and 32°. The other incubation conditions and the methods of lipid analysis were the same as those described above.

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