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INTERMEMBRANE TRANSFER OF LONG CHAIN FATTY ACID SYNTHESIZED BY ETIOLATED LEEK SEEDLINGS

PATRICK MOREAU, HÉLÈNE JUGUELIN, RENÉ LESSIRE and CLAUDE CASSAGNE

Institut de Biochimie Cellulaire et Neurochimie du C.N.R.S., 1, rue Camille Saint-Saëns, 33077 Bordeaux Cédex, France

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Key Word Index—*Allium porrum*; Liliaceae; leek seedlings; *in vivo* intermembrane transfer of fatty acids; very long chain fatty acids..

Abstract—Etiolated leek seedlings provided with [1-^{14}C]acetate *in vivo*, synthesized very long chain fatty acids (VLCFA) that were incorporated into the phospholipids (chiefly PC and PE) and the neutral lipids (including free fatty acids). After a labeling period and various chase times, the seedlings were homogenized and a 150000 *g* pellet was prepared. The latter was then subfractionated on a linear sucrose gradient (0.35–1.7 M). Four membrane fractions, banding at 0.65 ± 0.05 M (A), 0.85 ± 0.05 M (B), 1.05 ± 0.05 M (C) and 1.30 ± 0.10 M (D), were obtained. The lipids of each membrane fraction were extracted and their radioactivity determined. Nearly 60% of the total labeled lipids of the lightest membrane fraction (A) were transferred to heavier membranes during the chase. The fatty acid analysis of each membrane fraction showed that the labeled VLCFA of the membrane fraction A were integrally transferred to heavier membranes during the chase period.

INTRODUCTION

The study of VLCFA biosynthesis, *in vitro*, by leek epidermis and etiolated leek seedling microsomes [1, 2] has shown that the VLCFA are released from the microsomal elongase(s) as acyl-CoAs and then transferred to PC. This *in vitro* biosynthesis of VLCFA by the elongation of stearoyl-CoA has been shown to be localized in an endoplasmic reticulum (ER) enriched membrane fraction of leek epidermis [3] and of etiolated maize coleoptiles [4]. Indeed, the VLCFA biosynthesis is 3–4 times greater in this fraction than in the plasmalemma-enriched membrane fraction [3]. Paradoxically, VLCFA may represent up to 20% of the total fatty acids in the plasma membrane, whereas only a small amount of VLCFA is present in the ER-enriched membrane fraction. These results suggested an eventual transfer of the VLCFA from their site of synthesis (ER) to their sites of accumulation (plasma membrane and wax layer) [5]. The aim of the present study is to design a methodology capable of testing this hypothesis *in vivo*. For this, etiolated leep seedlings, which present a high and reproducible level of lipid synthesis [6], were incubated *in vivo*, with labeled acetate and, by 'pulse chase' experiments followed by membrane subfractionation, the eventual intermembrane transfer of the VLCFAs was studied.

RESULTS

In a first approach the conditions of 'pulse-chase' experiments were defined. Etiolated leek seedlings were first incubated with [1-^{14}C]acetate and then with unlabeled acetate as described in the experimental section. The dilution factor of [1-^{14}C]acetate by unlabeled acetate, determined as a function of the total moles of acetate, was about 1000 times. This study was limited to internal lipids, so wax lipids were discarded by extraction for 20 sec with chloroform. The internal lipids were then extracted by chloroform–methanol (2:1), and their radioactivity determined.

Table 1 shows the total radioactivity of the lipids after different labeling periods and chase times. In all experiments, the total incubation period (labeling + chase) was less than, or equal to 6 hr. In absence of added unlabeled acetate (chase time = 0), the incorporation of [1-^{14}C]acetate into the lipids was almost linear for 6 hr [6]. Whatever the time of incubation of seedlings with [1-^{14}C]acetate (labeling period), the increase of the total radioactivity of lipids was rapidly stopped after transfer of the seedlings in an unlabeled acetate medium (chase medium) demonstrating the efficiency of the chase. The analysis of the fatty acid methyl esters of the total lipids (Table 2) shows, as expected, that the radioactivity of C_{16}

Table 1. Total radioactivity in the lipids as a function of pulse and chase time

	Total lipids (cpm × 10^{-3}/g seedlings)		
Chase time (min)	Pulse 30 min	Pulse 60 min	Pulse 120 min
0	10	14	18.4
30	8.3	12	18.8
60	7.8	11.5	18
120	8.7	13.1	18.1
240	7.4	13.5	17.6

Etiolated leek seedlings were incubated with [1-^{14}C]acetate and unlabeled acetate as indicated in the Experimental. The lipids were extracted as previously reported [6] and their radioactivity determined in a liquid scintillation counter.

Table 2. Radioactivity in the C_{16}, C_{18} and very long chain fatty acids as a function of labeling periods and chase times

Chase time (min)	Radioactivity (cpm × 10^{-3}/g seedlings)								
	Labeling period 30 min			Labeling period 60 min			Labeling period 120 min		
	C_{16}	C_{18}	VLCFA	C_{16}	C_{18}	VLCFA	C_{16}	C_{18}	VLCFA
0	221	246	19	326	555	61	653	694	150
30	223	222	41	350	368	101	611	800	156
60	228	273	50	244	403	148	600	738	230
120	206	200	34	274	496	135	555	630	240
240	212	137	44	467	476	148	525	621	209

Experimental conditions as in Table 1. The fatty acid methyl esters were prepared and analysed as described in the Experimental.

and C_{18} fatty acids presents the same evolution as the total label of the lipids (Table 1).

The VLCFAs are considerably less labeled than C_{16} and C_{18} fatty acids, but their radioactivity increases faster than that of C_{16} and C_{18} fatty acids. Accounting for 4% of the total fatty acid label after a 30 min labeling period, the VLCFA label represents 10% of this total radioactivity after a 120 min labeling period. Whereas no significant change of the total radioactivity is observed during the chase (Table 1), the amounts of labeled VLCFA considerably increased. Whatever the length of the labeling period, this increase is observed during the first hr of chase. Moreover, the levels of radioactivity in the VLCFA reached after labeling periods of 30 and 60 min followed by 1 hr of chase are similar to those observed for labeling periods of 60 min and 120 min without chase period, respectively. These results show that, although the chase on total lipid synthesis was effective, the VLCFA formation was not immediately stopped. This enrichment in labeled VLCFA could be explained either by the elongation of newly synthesized substrates, or by the elongation of endogenous substrates with labeled malonyl-CoA accumulated during the labeling periods. Since the elongation yield is proportional to the length of the pulse, it could also be proportional to the amount of newly synthesized substrates of the elongase(s) and/or to the 'pool' of labeled malonyl-CoA in the vicinity of the site(s) of elongation.

In order to study the eventual intermembrane transfer of VLCFA *in vivo*, the level of labeling of these molecules should be as large as possible in the membrane fractions. Indeed, only half of the total label is recovered in the microsomal pellet. Hence, the amounts of labeled VLCFA after a 30 min or a 60 min labeling period being 19 000 cpm and 61 000 cpm per g of seedlings, respectively, the VLCFA label recovered in the microsomal pellet would be about 10 000 cpm and 30 000 cpm per g of seedlings. Moreover, after membrane subfractionation of the microsomal pellet, the labeling of the VLCFA in the different membrane fractions would be even lower. Consequently, a labeling period of 120 min was chosen for the subsequent experiments.

Since the phenomena under study are chiefly localized in the endomembrane system and the plasmalemma, the preparation of subcellular membrane fractions is required. For this, etiolated leek seedlings were homogenized and various pellets sedimenting at 1000 *g*, 12 000 *g* and 150 000 *g* were prepared.

In order to determine the nature of these pellets, succinodehydrogenase, as a mitochondrial marker enzyme, NADPH cyt-*c* reductase, as an endoplasmic reticulum marker enzyme, and Mg^{2+} ATPase stimulated by Na^+ and K^+ and glucan synthetase II, as plasmalemma marker enzymes, were measured in each of them. The results are given in the Table 3. NADPH cyt-*c* reductase, Mg^{2+} ATPase stimulated by Na^+ and K^+ and glucan synthetase II activities indicate that the 150 000 *g* pellet is enriched in endomembranes and plasmalemma. The study of the succinodehydrogenase marker enzyme shows that the activity in the 12 000 *g* and 150 000 *g* pellets represent

Table 3. Determination of some enzymic markers activities in the homogenate and 1000 *g*, 12 000 *g* and 150 000 *g* pellets

	Homogenate	1000 *g* pellet	12 000 *g* pellet	150 000 *g* pellet
Succinicdehydrogenase	12	3.4 (6.3)	80 (85)	4 (2)
NADPH cyt. *c* reductase	6.7	1.4 (3.4)	4.5 (6.4)	94 (81.8)
Mg^{2+} ATPase stimulated by Na^+ and K^+	0.8	0.8 (23.8)	1 (19)	7 (52.4)
Glucan synthetase II	0.9	0.4 (8.1)	1.1 (26.8)	8.4 (63.4)

Succiniodehydrogenase and NADPH cyt. *c* reductase activities are expressed as nmol/min/mg proteins. Mg^{2+} ATPase and glucan synthetase II activities are expressed as nmol/hr/mg proteins. Numbers in parentheses represent the percentage of the total activity found in the homogenate.

85% and 2% of the activity of the homogenate, respectively. The specific activity in the 12 000 *g* pellet was 80 nmol/min/mg and only 4 nmol/min/mg in the 150 000 *g* pellet. Consequently, the 150 000 *g* pellet is only very slightly contaminated by mitochondrial membranes. The distribution of the carotenoids between the various pellets was also determined and is as follows: 66% in the 1000 *g* pellet, 30% in the 12 000 *g* pellet and only 4% in the 150 000 *g* pellet.

In intermembrane transfer experiments, the 150 000 *g* microsomal pellet was loaded onto a linear sucrose gradient (see Experimental), and, after a 14 hr centrifugation at 130 000 *g*, four membrane fractions banding at 0.65 ± 0.05 M (A), 0.85 ± 0.05 M (B), 1.05 ± 0.05 M (C) and 1.30 ± 0.10 M (D) were obtained (Fig. 1). The lipids of these membrane fractions were extracted and their radioactivity was measured. The results are given in Table 4.

The efficiency of the chase found above (Table 1) was also observed for the 150 000 *g* pellet, since no significant change of its label was observed ($10.3 \pm 0.5 \times 10^5$ cpm/g of seedlings). The total radioactivity recovered from the four membrane fractions A, B, C and D ($9.6 \pm 0.11 \times 10^5$ cpm/g of seedlings) accounts for 93% ± 4% of the total radioactivity of the 150 000 *g* pellet. The variations of the radioactivity associated with the different membrane fractions could be considered in terms of a redistribution of the label between these membrane fractions. Interestingly, the radioactivity of the lipids of the fraction A (0.65 ± 0.05 M) decreased as a function of the chase time, whereas that of the other membrane fractions increased by an equivalent amount (table 4).

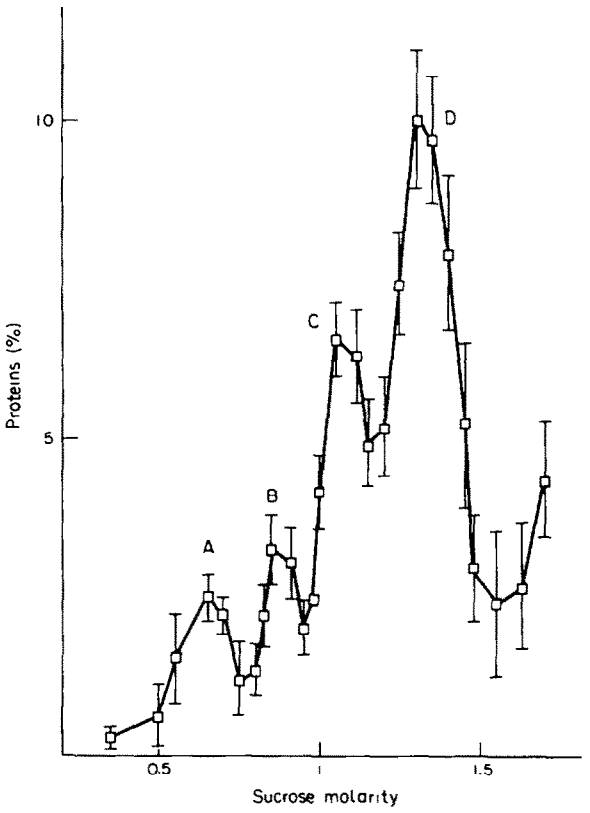

Fig. 1. Separation of the 150 000 *g* pellet on a linear sucrose gradient: protein distribution (%). Mean value ± s.d. of 12 experiments.

Table 4. Total radioactivity in the lipids of the 150 000 *g* pellet and of the membrane fractions A, B, C and D after 120 min pulse followed by varying chase times

Chase time (min)	Total label in lipids (cpm × 10^{-5}/g of seedlings)				
	150 000 *g* pellet	A	B	C	D
0	10	2.5	1.2	0.9	5.1
30	9.6	1.7	1.4	1	5.3
60	10.9	0.9	1.8	1.3	5.9
120	10.2	0.7	1.1	1.4	5.8
240	10.6	0.8	1.6	1.5	6

Pulse chase experiments and the membrane subfractionation were performed as described in the Experimental. The lipids were extracted as previously reported [6] and their radioactivity determined in a liquid scintillation counter.

The analysis of the fatty acid methyl esters obtained from each membrane fraction shows that the decrease of the radioactivity in the membrane fraction A was due, for a great part, to the decrease of labeled C_{16} and C_{18} fatty acids (Table 5). After a 240 min chase, the radioactivity in C_{16} fatty acids of the membrane fraction A decreased by about 50% and that of C_{18} fatty acids decreased by about 70%. In contrast, after a 60 min chase, the amount of labeled C_{16} fatty acids increased in fractions B and C and then decreased to a level close to the initial value (120 min pulse). The amount of labeled C_{16} fatty acids in the fraction D increased by about 40% after a 60 min chase and then remained constant until 240 min. No significant change in the amount of labeled C_{18} faty acids was observed in the fractions B, C and D. The distribution of the radioactive VLCFA between the four membrane fractions is also shown in Table 5. The amount of labeled VLCFA associated with the membrane fraction A decreased as a function of the chase time and, after a 120 min chase, these fatty acids disappeared completely from this fraction leaving only labeled C_{16} and C_{18} fatty acids.

A behaviour similar to that observed for the VLCFA in fraction A was also observed for the C_{20} fatty acids in all the membrane fractions. During the same period, the amount of labeled C_{22} fatty acid increased in membrane fractions B and D, while that of labeled C_{24} fatty acid increased in fractions B, C and D. The most important increase in labeled C_{22} and C_{24} fatty acids was observed in membrane fraction D, where a two-fold increase in the amounts of labeled C_{22} and C_{24} fatty acids was observed after a chase of 30 min and 60 min respectively.

Figure 2 shows the radioactivity in the VLCFA per mg of proteins in each membrane fraction as a function of the chase time. After the 120 min labeling period, the highest label of the VLCFA per mg of protein was associated with the membrane fraction A (due to the small quantity of proteins in this fraction). It then decreased rapidly during the chase in fraction A, whereas it increased at different

Table 5. Fatty acid methyl esters analysis of the various membrane fractions as a function of the chase time following a 120 min labeling period (results are expressed in cpm × 10^{-3} per g of seedling)

Membrane fractions	Acid chain length	Labeling period (120 min)	Chase time (min)			
			30	60	120	240
Fraction A	16	77	23	41	38	30
	18	85	88	28	15	22
	20	2.3	0.5	0	0	0
	22	6	2.7	1.6	0	0
	24	6.3	3.3	1.9	0	0
Fraction B	16	46	49	72	54	46
	18	31	38	35	31	29
	20	1.5	1.1	1.2	—	0
	22	5.8	7.5	9.7	—	9.6
	24	8.1	6.9	12.2	—	12.6
Fraction C	16	38	41	62	59	51
	18	23	21	29	26	27
	20	1.2	0	1.1	1	0
	22	8.4	6.3	7.5	7.6	8.3
	24	6.4	6.8	8.9	5.8	13.1
Fraction D	16	190	180	264	251	269
	18	120	94	114	137	131
	20	6.5	4.3	2.2	1.2	1.3
	22	15.6	30	31.3	27.5	23
	24	16.2	27.7	34	29.3	33.6

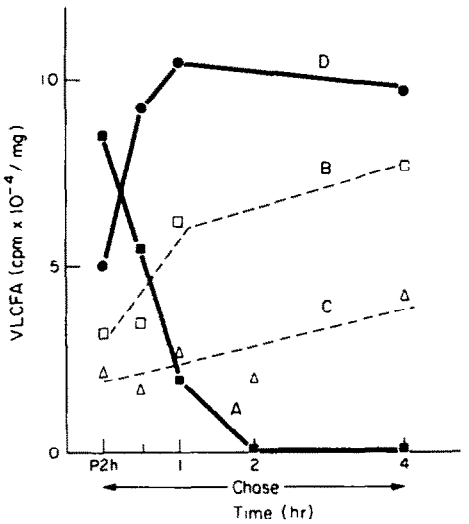

Fig. 2. Radioactivity in the VLCFA per mg of proteins of each membrane fraction, A, B, C and D after a 120 min pulse as a function of the chase time. Same experimental conditions as in Table 5.

rates in the other membrane fractions. The increase of the radioactivity of the VLCFA per mg of proteins was very rapid during the first hr of chase for the membrane fractions B and D. In contrast, the increase was slower for the membrane fraction C, but, after the maximal chase period (240 min), the label of the VLCFA per mg of proteins in the membrane fractions B, C and D was two times higher than after the initial 120 min labeling period. It should be noticed that, during the increase of labeled VLCFA in the heavier membranes possibly resulting from intermembrane transfer events, the VLCFA continue to be synthesized (Table 2), so this increase must in fact be considered as the result of the two phenomena (i.e. synthesis and transfer).

DISCUSSION

Only an *in vivo* investigation will provide the demonstration of the existence of the intermembrane transfer events of VLCFA, from their site of synthesis (ER) to the plasmalemma [5], that have been proposed from the results of *in vitro* experiments. Consequently, the material used for this study must satisfy the following conditions: the *in vivo* biosynthesis of lipids and fatty acids (including VLCFA) must be high and reproducible, the material must allow the preparation of subcellular membrane fractions with a sufficiently high VLCFA label and, finally, one must dispose of a methodology which allows the study of intermembrane transfer events of these molecules.

It has been shown that 7-day-old etiolated leek seedlings provided *in vivo* with [1-^{14}C]acetate, allow high and reproducible biosynthesis of lipids [6]. The technique employed in this study for the subcellular membrane fractionation yields reproducible proportions of the different membrane fractions (Fig. 1) and the level of the VLCFA label (Table 5) recovered in these fractions (after a 120 min labeling period) is sufficiently high.

'Pulse chase' experiments with a 120 min labeling

period, followed by various chase times, were used to study eventual intermembrane transfer events of VLCFA. The results shown in Tables 1 and 4 clearly demonstrate the efficiency of the chase, which allowed the demonstration of the existence of intermembrane transfer events of VLCFA *in vivo* (Table 5 and Fig. 2). The subfractionation of the microsomal pellet led to the resolution of four membrane fractions and, in particular, a light fraction (A), from which C_{16} and C_{18} fatty acids and VLCFA were transferred to heavier membrane fractions during the chase.

The fact that some label remains associated with the membrane fraction A after the maximal chase period (Table 5) indicates that some lipids containing C_{16} and C_{18} fatty acids, probably constitutive of this membrane, were not involved in the intermembrane transfer. The increase of labeled C_{22} and C_{24} fatty acids in the heavier membrane fractions, which probably results from the transfer of these fatty acids from the fraction A (Table 5, Fig. 2) and the biosynthesis of new labeled VLCFA (Table 2), raises the question concerning the order in which the transfer events take place between the various membranes.

The results reported here show that the methodology used is appropriate for the study of the *in vivo* intermembrane transfer of lipids in higher plants. However, they do not definitely establish that very long chain fatty acids are transferred from endoplasmic reticulum to the plasmalemma. Some uncertainties have to be resolved in order to make the demonstration more convincing: the various fractions A, B, C and D have to be further examined by means of positive and also negative markers according to Quail [7], in order to characterize the bands and their purity. The latter point is of crucial importance in the case of the fraction D; β-glucan synthetase II has been demonstrated only in this band, indicating that plasmalemma is confined to this fraction. But this does not mean that the fraction D is made only of membrane vesicles originating from the plasma membrane: other marker enzymes indicate that the fraction D is relatively heterogeneous. For this reason, the purification of the plasmalemma from the fraction D, or directly from the microsomal pellet has been undertaken using phase partition in an aqueous two polymer phase system [8].

EXPERIMENTAL

Plant material. Leek seeds, stored overnight at 4°, were sterilized with sodium hypochlorite in the presence of Triton X 100 for 5 min and then washed with H_2O. They were then grown for 7 days in the dark, at 25°, on a growth medium consisting of: 5 g agar-agar, 900 ml H_2O and 100 ml of a nutritive soln containing 7.5 g/l KCl, 6 g/l $NaNO_3$, 2.5 g/l $MgSO_4$, 0.95 g/l $CaCl_2$ and 1.25 g/l NaH_2PO_4.

Substrates and reagents. [1-^{14}C]Acetate (52.5 Ci/mol) was from Amersham. All other chemical products were purchased from Merck or Sigma.

In vivo *pulse-chase experiments.* One hundred 7-day-old etiolated leek seedlings were incubated for 30, 60 and 120 min with [1-^{14}C]acetate (52.5 Ci/mol) as reported [6]. They were then washed with H_2O and incubated with unlabeled 0.2 M NaOAc (0.5 ml for 20 seedlings) for 30, 60, 120 or 240 min.

Membrane subfractionation. The seedlings were homogenized in a 0.1 M Tris–HCl buffer, pH 7.5. The homogenate was centrifuged for 5 min at 1000 *g*. The supernatant so obtained was centrifuged for 15 min at 12000 *g* and the resulting supernatant was spun for 60 min at 150000 *g*. The microsomal pellet was loaded onto a linear sucrose gradient (0.35–1.7 M) which was centrifuged for 14 hr at 130000 *g*.

Succinate dehydrogenase was tested according to ref. [9]. NADPH cytochrome *c* reductase was assayed using the method of ref. [10]. Mg^{2+} ATPase activity, stimulated by Na^+ and K^+, was measured according to ref. [11]. Glucan synthetase II was assayed according to ref. [12]. Carotenoids were extracted from the various pellets (1000, 12000 and 150000 *g*) by Me_2CO–H_2O (4:1). The spectra were determined in hexane between 400 and 500 nm using a Philips SP8-100 spectrophotometer.

Lipid extraction and fatty acid analysis. The lipids of the seedlings (after wax lipid extraction by $CHCl_3$ for 20 sec) or of the membrane fractions were extracted by $CHCl_3$–MeOH (2:1), as previously reported [6] and aliquots were taken for radioactivity measurement in a liquid scintillation counter. The fatty acid methyl esters were prepared by direct transesterification in the presence of BF_3–MeOH (1:9) and purified as described [6]. The radio-GLC of methyl esters was performed using a Packard 429 chromatograph fitted with a 10% SE 30 column (1/8″ × 1 m) on WHP (90–10 mesh size). The temp. programming was from 180° to 280° at 4°/min. The effluent gases were continuously monitored for radioactivity in a Packard 894 unit connected to a Spectra Physics SP 4100 integrator.

Proteins were estimated by Bradford's method [13], using bovine serum albumin as standard.

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