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IN VIVO INCORPORATION OF ACETATE INTO THE ACYL MOIETIES OF POLAR LIPIDS FROM ETIOLATED LEEK SEEDLINGS

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Abstract—Seven-day-old leek seedlings actively synthesize lipids *in vivo* from [1-^{14}C]acetate, both in the light and in the dark In the dark, phospholipid synthesis is more effective than galactolipid synthesis Whatever the time of acetate incorporation by the etiolated seedlings, very long chain fatty acids having from 20 to 26 carbon atoms are found in all the polar lipids, including the acyl-CoAs All of the labelled very long chain fatty acids incorporated into the polar lipids are saturated On the other hand, the labelled C_{18}-fatty acids are unsaturated in phospholipids and galactolipids and almost no label is found in the saturated or unsaturated C_{18}-fatty acids of the acyl-CoAs

INTRODUCTION

It has been shown in this laboratory that an endoplasmic reticulum-enriched membrane fraction from leek epidermis is able to synthesize very long chain fatty acids from stearoyl-CoA *in vitro* [1–3] The C_{20}–C_{30}-fatty acids are most probably released from the membrane elongase(s) as acyl-CoAs [4], and subsequently transferred to polar lipids, chiefly to PC* [5] These data obtained from *in vitro* experiments may be related to previous analyses which indicated that VLCFAs are especially abundant in the plasma membrane of leek epidermis [1] and of maize coleoptile [6], suggesting that these membrane fatty acids could be esterified to phospholipids The aim of the work described in this paper was to study whether the transfer of VLCFAs to phospholipids observed *in vitro* under artefactual conditions is also operative *in vivo* The *in vivo* biosynthesis of the C_{20}–C_{30}-fatty acids is well documented [7, 8] and they have been mainly observed in the wax layer Only a few reports [9–11] have shown their possible occurrence in polar lipids

The phospholipids from leek epidermis provided with [1-^{14}C]acetate contained some labelled VLCFAs [5], but large variations in the incorporation of label excluded the use of epidermis for kinetic studies In order to overcome this source of variation, leek seedlings were grown in the laboratory and their ability to synthesize lipids was studied The leek seedlings synthesizing lipids most actively and reproducibly were used and the nature of the labelled fatty acids of the various polar lipids after acetate incorporation was investigated

RESULTS

Study of the synthetic capacity

[1-^{14}C]Acetate incorporation into lipids by seedlings aged from 2 to 28 days was measured in the light or in the dark (Fig 1) During the first few days, lipid synthesis was lower in the dark than in the light, but for seedlings aged from 5 to 8 days, it attained the same level under both sets of experimental conditions Then, in the dark, the synthetic capacity decreased to 10^5 cpm incorporated into lipids/g of seedlings, whereas in the light, after 20 days, the

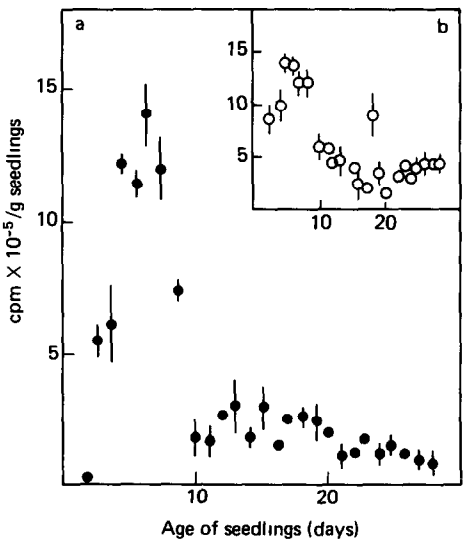

Fig 1 Capacity of leek seedlings for synthesis of internal lipids For each experiment, ten seedlings were incubated with 5 μCi [1-^{14}C]acetate (51 μCi/μmol) in the dark (a) or in the light (b) Mean value of three experiments ± s d

*Abbreviations VLCFAs, very long chain fatty acids, PA, phosphatidic acid, PC, phosphatidylcholine, PE, phosphatidylethanolamine, PG, phosphatidylglycerol, MGDG, monogalactosyldiglyceride, DGDG, digalactosyldiglyceride, ACP, acyl carrier protein

synthetic capacity reached a plateau around 4 $\times 10^5$ cpm/g of seedlings The highest incorporation of acetate into lipids was repeatedly observed in 5- to 8-day-old seedlings (Fig 1) As VLCFA biosynthesis in leek seems to involve chiefly the endomembranous system and the plasmalemma [12], the subsequent studies were performed with etiolated seedlings

The incorporation of acetate into lipids was next studied as a function of time (Fig 2) The incorporation of acetate into lipids in the 6- and 7-day-old seedlings increased with time more rapidly than in the 14-day-old seedlings Effectively, as early as 2 hr the level of acetate incorporation into lipids reached 10^6 cpm/g of seedlings in 6- and 7-day-old seedlings, whereas the incorporation did not exceed 3×10^5 cpm/g of seedlings in 14-day-old seedlings, whatever the length of the incubation period Thus, in good agreement with the study of the synthetic capacity in the dark, Fig 2 clearly shows the efficiency of 6- and 7-day-old etiolated seedlings for lipid biosynthesis

The galactolipids/phospholipids ratios were always found to be smaller than 1, which was in good agreement with the results obtained by Tremolières and Mazliak [13] for etiolated seedlings from *Trifolium repens*

In the 7-day-old etiolated seedlings, this ratio did not exceed 0 25, while it was between 0 5 and 0 9 for 6-day-old etiolated seedlings

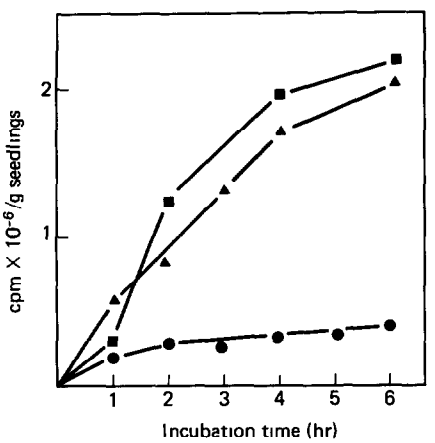

Fig 2 Internal lipid synthesis as a function of time Six-day-old (▲, 20 seedlings, 0 22 g ± 0 02), 7-day-old (■, 5 × 20 seedlings, 0 30 g +0 03) or 14-day-old (●, 20 seedlings, 0 85 g ±0 04) etiolated seedlings incubated in the dark with 10 μCi [1-^{14}C]acetate (51 μCi/μmol)

In order to study the synthesis of the lipids of the endomembranous system and of the plasmalemma (i e chiefly phospholipids and not galactolipids), 7-day-old seedlings rather than 6-day-old seedlings were used for further experiments

Lipid synthesis by 7-day-old etiolated seedlings

The detailed analysis of the lipids synthesized is presented in Table 1 After a 2 hr incubation period, the labelling of neutral lipids represented 65% of the total radioactivity and then decreased with time to 50% PC and PE synthesis reached, respectively, 17% (after 4 hr of incubation) 16 5% (after 6 hr of incubation) of the total lipid biosynthesis, whereas the synthesis of PG represented only 7% It must be noted here that on TLC PA migrated to almost the same level as PG, which must be considered as PG + PA Gardiner *et al* [14] have shown the possibility of two different biosynthetic pathways for PC and PG, the latter taking place in the chloroplasts So the weak labelling found in PG could be explained by the use of dark-grown and not light-grown seedlings

The labelling of MGDG and DGDG did not exceed 3 2 and 5 5% of the total lipid label, respectively The galactolipids are concentrated in, if not confined to, the chloroplastic membranes [15–20] and may amount to *ca* 90% of the total lipids of these membranes [21] Their synthesis, which could be localized in the envelope of chloroplasts [22], is more important in the chloroplasts than in the etioplasts [23] In good agreement with this, the synthesis of MGDG and DGDG by 7-day-old etiolated seedlings did not exceed 8 7% of the total lipid synthesis

In these experiments, some label remained at the origin after multiple development two-dimensional TLC This spot was weakly labelled during the first 3 hr, but an increase of its radioactivity content was observed after longer incubation periods As newly synthesized long chain acyl-CoAs remain at the origin under these experimental conditions, the spot was examined for their presence After analysis of all the other lipids, the spot remaining at the origin was developed with butanol–acetic acid–water to separate CoA esters, malonate and acyl-ACP (see Experimental) Malonyl-CoA and malonate were only weakly labelled, most of the radioactivity being associated with the long chain acyl-CoA fraction No label was associated with the acyl-ACP

The total yield of acyl-CoAs was not great in comparison to other fractions after 4 hr of incubation, long chain acyl-CoAs accounted for 1 8% of the total lipid label and 2 1% after 6 hr This experiment demonstrated

Table 1 Lipid synthesis by 7-day-old etiolated seedlings

Incubation time (hr)	Incorporation of the radioactivity into lipids (cpm × 10^{-3}/g seedlings) Acyl-CoAs	PC	PE	PG	DGDG	MGDG	NL	Total
1	—	36 5	8 5	6 5	5	2	173	266
2	16	126	69	64	42	20	527	880
4	36 5	343	286 5	146 5	113	66 5	1000	2065
6	48 5	246 5	386 5	160	60	63	1165	2330

For each incubation time, 5 × 20 seedlings were incubated in darkness as described in Fig 2 NL, neutral lipids

the presence of labelled long chain acyl-CoAs synthesized *in vivo*

Fatty acid analysis of the various lipids

The seedlings were incubated with [1-^{14}C]acetate for 2, 4 and 6 hr The lipids were extracted, purified by multiple development two-dimensional TLC and the fatty acid methyl esters prepared and purified as described in the Experimental

The distribution of label within the acyl moieties from acyl-CoA was determined after 4 hr of incubation (Fig 3) More than 90% of the total label of the acyl-CoAs was found in C_{16} (54%), C_{22} (13%) and C_{24} (24%) On the other hand, the labelling of acyl chains with 18, 20 and 26 carbon atoms represented only 3, 2 and 3%, respectively, of the total radioactivity of the acyl-CoAs No label was associated with C_{14} So, after 4 hr of acetate incorporation, two types of long chain acyl-CoA were synthesized *in vivo* the first was palmitoyl-CoA and the second was C_{22}-CoA and C_{24}-CoA The presence of very long chain acyl-CoAs (chiefly saturated and unsaturated C_{20}-acyl-CoAs) had already been shown [24] The results reported in this study constitute the first demonstration of the *in vivo* biosynthesis not only of C_{20}-acyl-CoAs but also of C_{22}- and C_{24}-acyl-CoAs

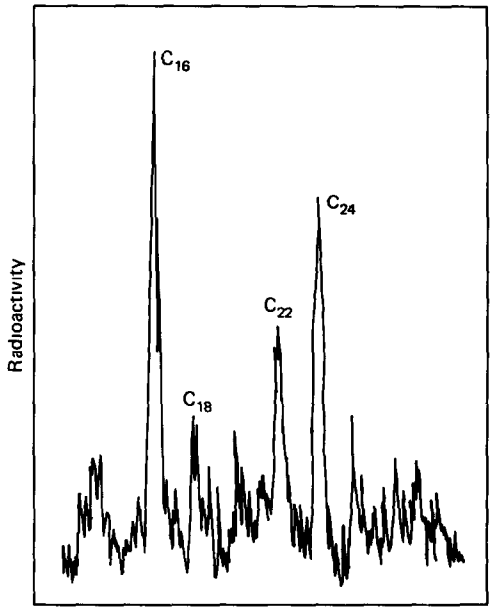

Fig 3 *In vivo* [1-^{14}C]acetate incorporation into the acyl moieties of acyl-CoA by 7-day-old etiolated seedlings 5 x 20 seedlings were incubated in the dark for 4 hr as described in Fig 2 Acyl-CoAs were purified as described in the Experimental and analysed by radiochromatography (attenuation 0 3 K)

The distribution of label between the various fatty acyl moieties of phospholipids and DGDG is shown in Table 2 All acyl chains having from 16 to 26 carbon atoms were found in PC, PE, PG and DGDG Radiochromatographic analysis showed that the labelled C_{16}, C_{22}, C_{24} and C_{26} were saturated whereas the labelled C_{18} was always unsaturated

The *in vivo* acetate incorporation study that was performed with leek epidermis [5] had shown the presence of VLCFAs only in the neutral lipids, PE and to a lesser extent in PC, the amount of VLCFAs in PE and mainly PC not being very high By contrast, the amount of VLCFAs synthesized by the etiolated seedlings and inserted into PE represented more than 20% of the total newly synthesized fatty acids, and the amount of VLCFAs found in PC, PG and DGDG reached at least 10%

Other *in vivo* investigations had already shown that VLCFAs may be associated with polar lipids [9–11] Pollard and Stumpf [25] had shown the presence of saturated and unsaturated C_{20} and C_{22} in PC and PE, the proportion of C_{20} and C_{22} representing more than 20% of the fatty acids found in the total polar lipids In the same manner, our experiment demonstrated the presence *in vivo* of large amounts of VLCFAs with acyl chains having up to 26 carbon atoms esterified to each polar lipid Table 2 shows the evolution of each acyl chain as a function of time for PC, PE, PG and DGDG Whatever the time of acetate incorporation, the amount of C_{16} and C_{18} represented between 75 and 90% of the total radioactivity found in the fatty acid methyl esters prepared from each polar lipid

The formation of the C_{20} and C_{26} was identical for PC, PE and DGDG since the radioactivity of these two acids was higher after 4 hr of acetate incorporation and then decreased The formation over the same period of time of

Table 2 *In vivo* incorporation of [1-^{14}C]acetate into the acyl moieties of polar lipids by 7-day-old seedlings after 2, 4 or 6 hr

	Radioactivity (% total fatty acids)											
	PC			PE			PG			DGDG		
Fatty acids	2 hr	4 hr	6 hr	2 hr	4 hr	6 hr	2 hr	4 hr	6 hr	2 hr	4 hr	6 hr
16	21 2	57 2	30 1	50 6	44 5	60 8	66 1	59	40	32 6	44 8	48 9
18	66 7	15 8	53	28 7	35 5	30 1	21 6	29 9	43 1	52 1	39 6	31 7
20	2 4	16 2	3 1	3 5	7 1	1 2	t	5 3	5 4	3 3	6 6	3 3
22	4	3 2	5 8	7 5	6 1	3 4	5 8	1 2	5 3	5 5	5	5 8
24	4 6	4 5	6 3	7 8	5 1	3 4	6 6	3 4	3 1	5 2	1 7	7 5
26	1	2 9	1 3	1 7	1 6	1	—	0 7	3	1 2	2 1	2 7

The experimental conditions were the same as those described in Fig 2

C_{22}- and C_{24}-acyl chains was not comparable the C_{22} increased in PG and PC but decreased in PE and DGDG, and the C_{24} increased in PC and DGDG, decreased in PE but remained unchanged in PG It was difficult to interpret such variations and above all the behaviour of the C_{20} as a function of time

DISCUSSION

Etiolated leek seedlings allow a high and reproducible *in vivo* synthesis of phospholipids, among which those of PC and PE represent at least 70% of the total phospholipid synthesis, whereas, as expected, labelled galactolipid remains low As seen in Table 2, radioactive saturated VLCFAs are associated with phospholipids whatever the time of [1-^{14}C]acetate incorporation These results raise the question of how these fatty acids are incorporated into the phospholipids *in vivo*

It has been shown *in vitro* that the VLCFAs synthesized from stearoyl-CoA by the microsomal elongases are most probably released as acyl-CoAs [4, 26] and that PC and PE are acceptors of the acyl moieties of acyl-CoAs [5, 27] The prominent role of acyl-CoAs, rather than acyl-ACPs, in the transfer of acyl chains to phospholipids was demonstrated in avocado mesocarp microsomes [28] Accordingly, Sanchez and Harwood [29] have recently shown *in vitro* that CoA esters increase the proportion of VLCFAs and that the addition of CoA favours the incorporation of C_{20}- and C_{22}-fatty acids into the acyl lipid fraction This pathway observed *in vitro* could also be operative *in vivo* This possibility is supported by the analysis of the distribution of label between the fatty acids of the acyl-CoAs after *in vivo* acetate incorporation, which shows the occurrence of the labelled saturated C_{16}-, C_{22}- and C_{24}-fatty acids also observed in the phospholipids and DGDG Thus, *in vivo*, very long chain acyl-CoAs could be the substrates of an acyl-CoA transacylase transferring the saturated acyl moieties to phospholipids

However, the question of the transfer *in vivo* of unsaturated acyl chains to lipids remains obscure the phospholipids and DGDG contain unsaturated C_{18}-fatty acids (and almost no labelled stearic acid) whereas the acyl-CoAs are practically devoid of saturated and unsaturated labelled C_{18}-fatty acids The kinetics of the *in vivo* transfer of saturated and unsaturated acyl chains from acyl-CoAs should be considered, as well as the eventual role of the acyl-ACPs

The fact that VLCFAs synthesized *in vivo* are in part found in phospholipids is compatible with their accumulation in the plasmalemma and their biosynthesis in the endoplasmic reticulum of leek epidermal cells [2] The study of the eventual *in vivo* transfer of the VLCFAs, esterified to phospholipids or not, from their site of synthesis to the plasmalemma is under investigation

EXPERIMENTAL

Material Leek seeds stored at 4° overnight were sterilized by sodiumhypochlorite for 5 min and washed The growth medium (5 g agar-agar, 900 ml distilled H_2O and 100 ml of a nutritive soln containing 7 5 g KCl, 6 g $NaNO_3$, 2 5 g $MgSO_4$, 0 95 g $CaCl_2$ and 1 25 g NaH_2PO_4/l) was heated to 100° The vessels were sterilized for 2 hr by sodium hypochlorite Seedling culture was performed in the light or in the dark

In vivo [1-^{14}C]*acetate incorporation* Incubations with [1-^{14}C]acetate of seedlings grown in the light were carried out in the light, whereas those of seedlings grown in the dark were carried out in the dark (a) *Study of the synthetic capacity* 10 seedlings were incubated at 20° for 2 hr with [1-^{14}C]acetate (5 μCi, 51 Ci/mol) in 100 μl distilled H_2O (b) *Lipid synthesis* 20–200 seedlings were incubated at 20° for different times Each batch of 20 seedlings received 10 μCi [1-^{14}C]acetate (51 Ci/mol) in 200 μl distilled H_2O

Lipid extraction and separation After wax extraction with $CHCl_3$ for 30 sec, the lipids were extracted at 70° with $CHCl_3$–MeOH (1 1) The solvent was removed by evapn, and the lipids were dissolved in $CHCl_3$, washed and dried Aliquots were taken for radioactivity measurement in a liquid scintillation counter

Multiple development 2D-TLC separation of the different lipids was carried out on Merck 60F254 silica gel plates according to ref [30] Identification of the lipids was by comparison with standards Autoradiography of the plates was done using NS2T Kodak films The radioactive spots were then scraped off and their radioactivity was measured in 5 ml of Lumax–toluene (2 3) The spot remaining at the origin was not removed, but was subjected to development with BuOH–HOAc–H_2O (5 2 3) in order to isolate long chain acyl-CoAs The plates were analysed by radiochromatography using a Packard 7230 radiochromatography scanner The R_fs for acyl-ACP, malonyl-CoA, malonate and long chain acyl-CoAs were, respectively, 0, 0 10–0 12, 0 32–0 33 and 0 54–0 55

Fatty acid analysis (a) The fatty acid methyl esters of the various lipids separated by multiple development 2D-TLC were prepared by direct transesterification of the various spots in the presence of 2 ml 2 5% HCl–MeOH at 75° for 2 hr After adding 2 ml H_2O, the fatty acid methyl esters were extracted with 3 × 2 ml $CHCl_3$, washed and dried They were then purified by TLC on silica gel plates developed with C_6H_6 (b) The fatty acid methyl esters were analysed by radio-GLC as described earlier [2], the radioactivity of each ester being determined after trapping on anthracene cartridges Alternatively, a Nuclear Chicago selecta system 5000 apparatus was used for fatty acid methyl ester analysis The characteristics of the column in this case were SE 30 10% on Varaport 100–120 mesh (1 5 m × ⅛ in) The flow rate of the carrier gas (argon) was 31 5 ml/min

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