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ELONGATION OF ACYL-CoAs BY MICROSOMES FROM ETIOLATED LEEK SEEDLINGS

RENÉ LESSIRE, HÉLÈNE JUGUELIN, PATRICK MOREAU and CLAUDE CASSAGNE

Institut de Biochimie Cellulaire et Neurochimie du CNRS, 1 rue Camille Saint-Saëns, 33077 Bordeaux Cedex, France

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Key Word Index—*Allium porrum*; Liliaceae; leek; microsomes; fatty acid biosynthesis; acyl-CoA elongation.

Abstract—Long chain fatty acid synthesis was studied using etiolated leek seedling microsomes. In the presence of ATP, [2-^{14}C]malonyl-CoA was incorporated into fatty acids of C_{16}–C_{26}. The omission of ATP, even in the presence of acetyl-CoA, led to a complete loss of activity, which was restored by addition of exogeneous acyl-CoAs. Comparison of acyl-CoA (C_{12}–C_{24}) elongation showed that stearoyl-CoA, in the presence of [2-^{14}C]malonyl-CoA, was the more efficient precursor leading to the formation of fatty acids having a chain length of C_{20}–C_{26}. [1-^{14}C]C_{16}-CoA and [1-^{14}C]C_{18}-CoA were elongated in the presence of malonyl-CoA, without degradation of the acyl chain. The time-course and the malonyl-CoA concentration curves showed that [1-^{14}C]C_{18}-CoA was a better primer than [1-^{14}C]C_{16}-CoA. Acyl-CoA elongation was also studied over the concentration range 4.5–45 μM [1-^{14}C]C_{18}-CoA. Comparison of the radioactivity incorporated into the fatty acids formed using [2-^{14}C]malonyl-CoA in the presence of C_{18}-CoA, on the one hand, and [1-^{14}C]C_{18}-CoA in the presence of malonyl-CoA, on the other, demonstrated clearly that the acyl chain of the acyl-CoA was elongated by malonyl-CoA.

INTRODUCTION

Long chain fatty acids (LFA) have been considered to be characteristic wax components of higher plants [1, 2]. However, it seems that this oversimplified view has to be reconsidered, because LFA have been demonstrated in plant plasma membrane [3, 4] raising the question of the metabolic interrelations between plant plasmalemma and the wax layer. Supporting the presence of LFA in membrane lipids, recent experiments [5] have demonstrated that etiolated leek seedlings synthesize radioactive, saturated LFA which are incorporated into polar lipids including the major phospholipids as well as the galactolipids. Interestingly, the purified acyl-CoA fraction contained C_{16}, C_{22} and C_{24} labelled acyl-moieties, the label found in C_{22} and C_{24} representing 13% and 24% of the total radioactivity, respectively [5]. This fact suggested that, *in vivo*, the leek elongase(s) could accept long chain acyl-CoAs as substrates and also that the LFA could be released as acyl-CoAs and further transferred to polar lipids. In this paper, we present evidence that, *in vitro*, long chain acyl-CoAs and unknown, endogeneous precursors may be elongated by microsomes from etiolated leek seedlings.

RESULTS

Etiolated leek seedling microsomes synthesize saturated fatty acids (C_{16}–C_{26}) from [2-^{14}C]malonyl-CoA, in the presence of NADPH, NADH, ATP and magnesium chloride. The microsomal pellet, containing *ca* 90% of the total activity (unpublished results), was used as enzyme source.

LFA biosynthesis from various substrates

Different substrates were tested for LFA biosynthesis (Table 1). [1-^{14}C]Acetyl-CoA (10 μM) was poorly incorporated into fatty acids. [2-^{14}C]Malonyl-CoA used without a primer led to a high fatty acid synthesis, provided that ATP was added to the reaction mixture. The omission of ATP (1 mM) resulted in a complete loss of activity and the addition of acetyl-CoA (100 μM) led to a slight decrease of the [2-^{14}C]malonyl-CoA incorporation into fatty acids. These results, in good agreement with those obtained recently [6], confirm the presence of endogeneous substrates in the particulate fraction which can act as primers only in the presence of ATP.

In the absence of ATP, that is in experimental conditions under which no endogeneous substrates are elongated, exogeneous acyl-CoAs may be used as primers in the presence of [2-^{14}C]malonyl-CoA. The amount of malonyl-CoA incorporation depends on the chain length of the acyl moiety of the exogeneous acyl-CoA, with a maximum for C_{18}-CoA. In this case, the malonyl-CoA incorporation is significantly higher than that observed with endogeneous substrate(s).

The analysis of the label distribution among the fatty acids is given in Table 1. With [2-^{14}C]malonyl-CoA + ATP, the major products were C_{18}, C_{20} and C_{22} fatty acids. The addition of acetyl-CoA did not change the label distribution pattern. On the other hand, when using exogeneous long chain acyl-CoAs, without ATP, the label distribution was modified. The acyl moiety of the various acyl-CoAs was incorporated as a whole, so that C_{12}-CoA, C_{14}-CoA, C_{18}-CoA and C_{20}-CoA led chiefly to C_{16}, C_{18}, C_{20} and C_{22} labelled fatty acids, respectively. The C_{16}-CoA led mostly to the formation of C_{20} and C_{22} fatty

Table 1. Fatty acid biosynthesis from various substrates

Conditions	Total ^{14}C incorporation (nmol/mg·hr)	^{14}C-labelled fatty acid composition (%) C_{14}	C_{16}	C_{18}	C_{20}	C_{22}	C_{24}	C_{26}	LFA formation (C_{20}–C_{26}) (nmol/mg·hr)
[1-^{14}C]Acetyl-CoA + malonyl-CoA	0.32	nd	—	—	—	—	—	—	—
[1-^{14}C]Acetyl-CoA + malonyl-CoA + ATP	0.50	nd	—	—	—	—	—	—	—
Acetyl-CoA + [2-^{14}C]malonyl-CoA	0.39	nd	—	—	—	—	—	—	—
Acetyl-CoA + [2-^{14}C]malonyl-CoA + ATP	2.80	—	—	21.2	58.4	20.4	—	—	2.2
[2-^{14}C]Malonyl-CoA	0.38	nd	—	—	—	—	—	—	—
[2-^{14}C]Malonyl-CoA + ATP	5.21	—	—	13.3	47.9	28.2	10.6	—	4.5
C_{12}-CoA + [2-^{14}C]malonyl-CoA	2.60	41.5	22.5	7.4	9.5	13.4	5.7	—	0.7
C_{14}-CoA + [2-^{14}C]malonyl-CoA	4.90	—	37.1	6.0	8.7	25.1	16.2	6.9	2.8
C_{16}-CoA + [2-^{14}C]malonyl-CoA	5.92	—	—	19.1	31.2	28.6	15.3	5.8	4.8
C_{18}-CoA + [2-^{14}C]malonyl-CoA	9.77	—	—	3.1	43.4	32.6	15.8	5.1	9.5
C_{20}-CoA + [2-^{14}C]malonyl-CoA	5.01	—	—	—	3.8	60.0	28.5	7.7	5.5
C_{22}-CoA + [2-^{14}C]malonyl-CoA	0.39	nd	—	—	—	—	—	—	—
C_{24}-CoA + [2-^{14}C]malonyl-CoA	0.40	nd	—	—	—	—	—	—	—

Activity was determined as described in the Experimental. The concentrations used were: [1-^{14}C]acetyl-CoA (46 Ci/mol), 10.3 μM; acetyl-CoA, 106 μM; [2-^{14}C]malonyl-CoA (58 Ci/mol), 17 μM; malonyl-CoA (17 μM), each acyl-CoA (9 μM) and ATP, 1 mM, respectively. nd, Not detected. The incubation time was 1 hr at 30°.

acids, indicating that the labelled C_{18} acyl moiety formed from C_{16}-CoA was very rapidly converted into C_{20} and then to C_{22}. From C_{18}-CoA, no label was detected in C_{16} and only 3.1 % in C_{18} fatty acids. Icosanoic acid contained more than 43 % of the total label, suggesting an elongation by [2-^{14}C]malonyl-CoA. The presence of label in C_{22} and C_{24} is probably not due to the elongation of endogeneous substrate, since the experiments were carried out without ATP and suggest that the neosynthesized C_{20} was further elongated, giving rise to C_{22} which, in turn, was elongated to C_{24}.

[1-^{14}C]*Palmitoyl-CoA and* [1-^{14}C]*stearoyl-CoA elongation*

To test further the elongation of the exogeneous primers, labelled acyl-CoAs were used in the presence of unlabelled malonyl-CoA and all the cofactors as described in the Experimental. In this case, any eventual elongation of endogeneous substrates would not be detected.

Time-course experiment. In the presence of malonyl-CoA (167 μM), both [1-^{14}C]C_{16}-CoA and [1-^{14}C]C_{18}-CoA led to the formation of LFA. In these experiments the GC–RC was critical because whether or not the radioactive methyl esters with the longest chains were detectable depended on the radioactivity injected. Routinely, the whole methyl esters were subjected to GC–RC. Under these conditions, C_{20}, C_{22}, C_{24} and sometimes C_{26} acids were detected when [1-^{14}C]C_{18}-CoA was the primer and only C_{18} and C_{20} acids when [1-^{14}C]C_{16}-CoA was the primer. The time-course curve showed that, in the experimental conditions used, the elongation of [1-^{14}C]C_{18}-CoA was *ca* five times more effective than that of [1-^{14}C]C_{16}-CoA (Fig. 1). The conversion of C_{18}-CoA into LFA increased almost linearly for *ca* 30 min and then reached a plateau. This plateau could be explained by the fact that 80 % of C_{18}-CoA was either hydrolysed or elongated and/or had its acyl moiety transferred to lipids after 40 min (unpublished data). The analysis, by GC–RC, of the synthesized fatty acids showed that, whatever the acyl-CoA used as primer,

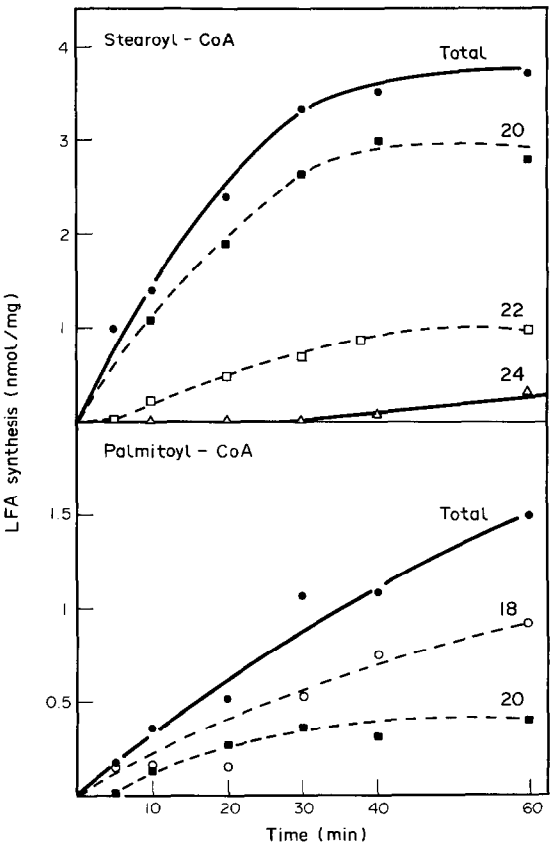

Fig. 1. Time-course curve of [1-^{14}C]C_{18}-CoA and [1-^{14}C]C_{16}-CoA elongation. The incubation medium contained 9 μM [1-^{14}C]acyl-CoA, 167 μM malonyl-CoA and 36 μg proteins and cofactors as indicated in the Experimental. Total fatty acid synthesis is represented by the curve designated as 'total'. The formation of individual fatty acid is indicated by the numbered curves corresponding to the chain length of each fatty acid.

the most labelled and the most rapidly formed fatty acid resulted from the addition of only one molecule of malonyl-CoA, to give C_{18} and C_{20} from [1-^{14}C]C_{16}-CoA and [1-^{14}C]C_{18}-CoA, respectively. The longer acids, C_{20}, C_{22} and C_{24} appeared after a lag of 5 min for C_{20} and C_{22} and 20 min for C_{24}. The absence of radioactive acids shorter than C_{16} or C_{18} showed that the acyl chains of C_{16}-CoA and C_{18}-CoA were incorporated without degradation, thus supporting the elongation process hypothesis.

Malonyl-CoA concentration curve. Figure 2 shows that whatever the acyl-CoA used as primer, LFA synthesis was dependent upon the malonyl-CoA concentration. In this experiment also, C_{18}-CoA was a better substrate than C_{16}-CoA and, at the highest concentration of malonyl-CoA (167 μM), *ca* 22% of the [1-^{14}C]C_{18}-CoA was converted into LFA, but only 6% of [1-^{14}C]C_{16}-CoA was elongated to C_{18} and C_{20} acids under the same conditions. Under these conditions also, the analysis of fatty acids by GC/RC showed a sequential formation of LFA: at the lowest malonyl-CoA concentration, C_{18} and C_{20} were formed from [1-^{14}C]C_{16}-CoA and [1-^{14}C]C_{18}-CoA, respectively, then the next acids C_{20} and C_{22} and, finally, C_{24} appeared. This picture was typical of an elongation process, the results again clearly indicating that C_{18}-CoA is a better primer than C_{16}-CoA.

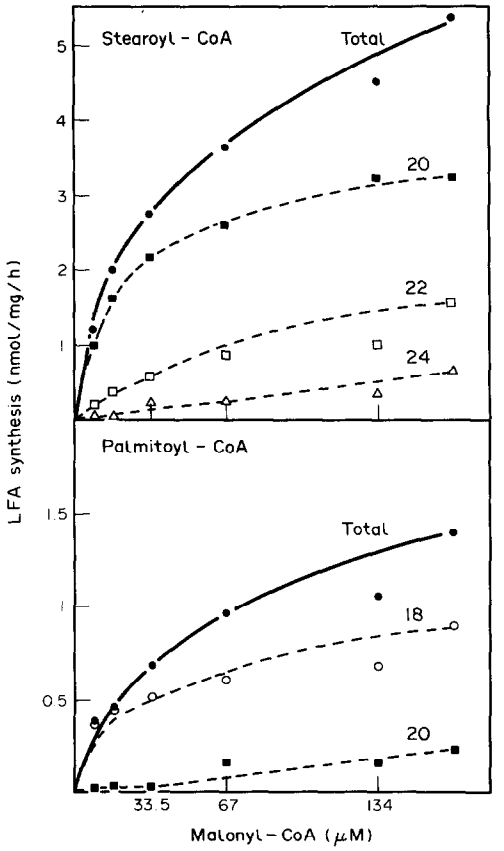

Fig. 2. Malonyl-CoA concentration curve for [1-^{14}C]C_{18}-CoA and [1-^{14}C]C_{16}-CoA elongation. Same conditions as in Fig. 1.

Stearoyl-CoA elongation

[1-^{14}C]*Stearoyl-CoA concentration curve.* LFA synthesis was assayed for different concentrations of [1-^{14}C]C_{18}-CoA, using a malonyl-CoA concentration of 167 μM. Figure 3 shows that LFA formation was dependent upon the primer concentration and increased linearly as the C_{18}-CoA concentration increased from 4.5 to 27 μM. The maximal synthesis was observed for a C_{18}-CoA concentration of 36 μM. For each assay, only *ca* 100 000 cpm of the total radioactivity of the methyl ester fractions were subjected to GC–RC, so the detection of C_{24} and C_{26} acids was not possible with these conditions. The fatty acid label distribution showed that, whatever the concentration of C_{18}-CoA, C_{20} acid was always the most labelled product. The results also indicated that the ratio of the malonyl-CoA and C_{18}-CoA concentrations may be an important regulation factor because, when this ratio was high (between 10 and 20, corresponding to the lowest C_{18}-CoA concentrations) the elongation of the primer, expressed in percentage of conversion, was more efficient.

Time-course of [2-^{14}C]*malonyl-CoA incorporation.* LFA synthesis was studied as a function of the incubation time, using [2-^{14}C]malonyl-CoA as the labelled substrate, in the presence of 9 μM C_{18}-CoA. Figure 4 shows that, under these conditions, the radioactivity in the LFA increases rapidly and linearly for *ca* 30 min. As expected, this curve was similar to the one obtained in the reciprocal experiment, where [1-^{14}C]C_{18}-CoA was the labelled substrate (Fig. 1). In this experiment also, the longer fatty acids were synthesized less rapidly than the shorter ones. For technical reasons inherent to GC–RC, the use of [2-^{14}C]malonyl-CoA allows the detection of labelled LFA up to C_{28}. These are undetectable when using [1-^{14}C]C_{18}-CoA.

Comparison of LFA synthesis using [1-^{14}C]*stearoyl-CoA and* [2-^{14}C]*malonyl-CoA.* In order to investigate

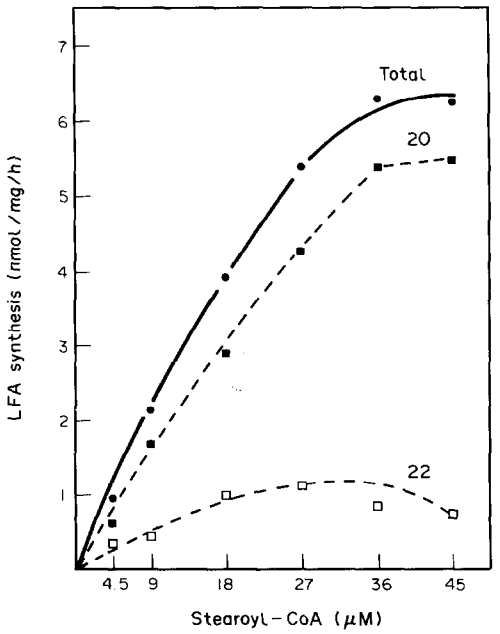

Fig. 3. [1-^{14}C]C_{18}-CoA concentration curve for LFA formation. Microsomal proteins (60 μg) were incubated in the presence of various [1-^{14}C]C_{18}-CoA concentrations for 1 hr at 30°.

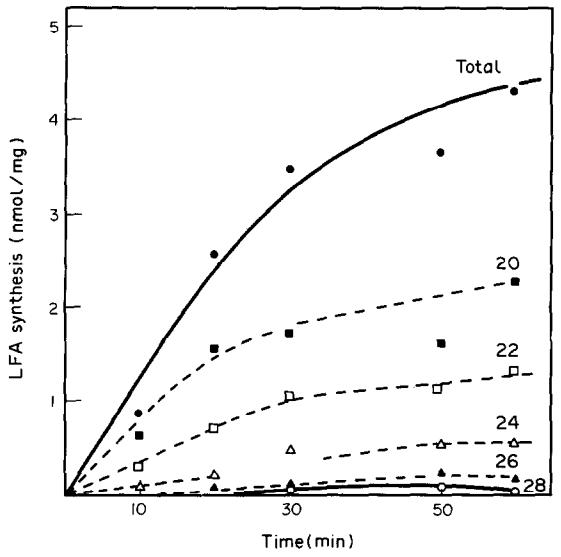

Fig. 4. Time-course curve of [2-^{14}C]malonyl-CoA incorporation into LFA. Proteins (60 μg) were incubated in the presence of 17 μM [2-^{14}C]malonyl-CoA, 9 μM stearoyl-CoA and cofactors as indicated in the Experimental.

more fully the mechanism of LFA formation, we compared the syntheses of labelled fatty acids from the two possible radioactive substrates. In both experiments, the same microsomal preparation was used and the substrates (labelled or unlabelled) were at the same concentration. The fatty acids were analysed by GC–RC after 1 hr of incubation at 30° (Fig. 5). The GC–RC trace showed the same labelled fatty acids in both experiments. The most important was C_{20} and the label decreased with the chain length. Hexacosanoic acid was well labelled using [2-^{14}C]malonyl-CoA as substrate but only traces were detected with [1-^{14}C]C_{18}-CoA. The calculations of the number of nmoles of each labelled substrate incorporated into each LFA are presented in Table 2. As shown by the radiochromatogram trace, C_{20} was the most labelled acid, but the most interesting result arose from the determination of the ratio of nmoles of [2-^{14}C]malonyl-CoA incorporated in each fatty acid to the nmoles of [1-^{14}C]C_{18}-CoA incorporated in the corresponding fatty acid. This ratio increased with the chain length and the different values were very close to those expected theoretically for an elongation process. In this hypothesis, the values should be 1, 2, 3 and 4, corresponding to the number of molecules of malonyl-CoA necessary for the formation of C_{20}, C_{22}, C_{24} and C_{26} acids, respectively, by successive elongations of C_{18}-CoA.

DISCUSSION

Microsomes from etiolated leek seedlings are able to incorporate malonyl-CoA into LFA (C_{20}–C_{26} saturated fatty acids). From labelled malonyl-CoA, the label distribution pattern is similar to that observed after *in vivo* acetate incorporation, except that the amount of C_{20} is increased in *in vitro* experiments. The fact that malonyl-CoA alone may be incorporated into fatty acids when ATP is added to the reaction mixture, raises the question of the mechanism of this synthesis. In microsomes from etiolated leek seedlings, [2-^{14}C]malonyl-CoA is not only incorporated into fatty acids, but is also decarboxylated and hydrolysed, giving rise to acetyl-CoA and malonic acid (R. Lessire, unpublished results). Thus, malonyl-CoA incorporation could reflect *de novo* synthesis by a mechanism reminiscent of the one observed, for example

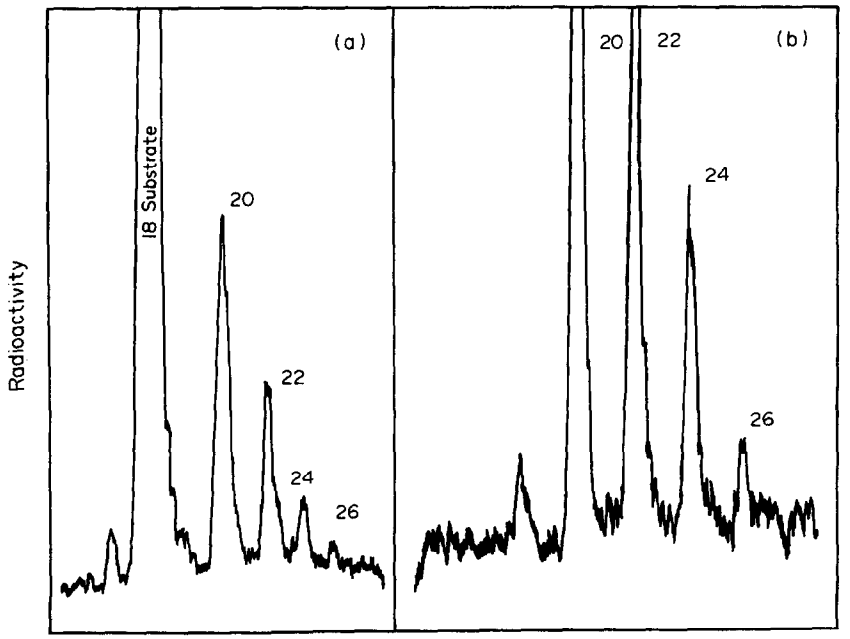

Fig. 5. GC–RC traces of labelled LFA. (a) LFA synthesized from 9 μM [1-^{14}C]C_{18} in the presence of 17 μM malonyl-CoA. (b) LFA synthesized from 9 μM C_{18}-CoA in the presence of 17 μM [2-^{14}C]malonyl-CoA.

Table 2. Comparison of LFA synthesis using [1-^{14}C]C_{18}-CoA and [2-^{14}C]malonyl-CoA

Acids	LFA synthesis (nmol/mg·hr) Experiment A (Stearoyl-CoA + [2-^{14}C] malonyl-CoA)	Experiment B ([1-^{14}C]C_{18}-CoA + malonyl-CoA)	Ratio A/B
C_{20}	4.00 ± 0.29	3.45 ± 0.07	1.16
C_{22}	2.28 ± 0.08	1.10 ± 0.23	2.07
C_{24}	1.35 ± 0.20	0.46 ± 0.09	2.93
C_{26}	0.54 ± 0.04	0.14 ± 0.03	3.86

Microsomal proteins (21.5 μg) were incubated either in the presence of 17 μM [2-^{14}C]malonyl-CoA (58 Ci/mol), 9 μM C_{18}-CoA and all other cofactors as indicated in the Experimental, or in the presence of 17 μM malonyl-CoA, and 9 μM [1-^{14}C]C_{18}-CoA (55 Ci/mol) in the presence of other cofactors. As described in the Experimental, fatty acids were extracted and analysed by GC–RC. The ratio A/B was determined as follows: nmol [2-^{14}C]malonyl-CoA incorporated/nmol [1-^{14}C]stearoyl-CoA incorporated in each fatty acid. Mean values of two independent experiments carried out in triplicate are given.

in *Mycobacterium smegmatis* [7]. In this case, the ATP effect could be explained by a decrease of the malonyl-CoA hydrolysis and/or by a resynthesis of malonyl-CoA. In fact, the addition of an excess of acetyl-CoA to the reaction mixture did not increase the overall biosynthesis, suggesting that acetyl-CoA was not involved in this process. The use of [1-^{14}C]acetyl-CoA did not lead to any fatty acid synthesis, which ruled out any involvement of *de novo* synthesis for LFA formation. The most probable interpretation of these results would be that an endogeneous precursor(s) is present in the microsomes. The nature of the endogeneous precursor(s) and the elucidation of the ATP effect require further investigation.

On the other hand, long chain acyl-CoAs are acyl donors for the elongation process. The results presented in this paper demonstrate that C_{18}-CoA is a good primer of the microsomal elongase(s). This result is in fairly close agreement with our previous demonstration of C_{18}-CoA elongation by endoplasmic reticulum enriched membrane fractions from leek epidermis [8] and maize coleoptiles [3]. Elongation of C_{16}-CoA and C_{18}-CoA occurred and, since no degradation into shorter fatty acids was detected, it can be concluded that the acyl chain is incorporated as a whole in both cases and that C_{18}-CoA is a better substrate than C_{16}-CoA. The most important conclusion of this study may be drawn from the comparative study of the elongation of C_{18}-CoA in the presence of [2-^{14}C]malonyl-CoA (experiment a) and of [1-^{14}C]C_{18}-CoA in the presence of malonyl-CoA (experiment b) (Table 2, Fig. 5). As expected from an elongation mechanism, the C_{20} synthesis is nearly the same in experiments (a) and (b), whereas malonyl-CoA incorporation into C_{22} and C_{24} is two and three times higher, respectively, than that of C_{18}-CoA. To our knowledge, the results demonstrate for the first time that, when exogeneous acyl-CoAs are used, no elongation of endogeneous acyl donors may occur and that there is only a sequential elongation of the primer (C_{18}-CoA) by malonyl-CoA. In addition, analysis of the kinetic curves (Fig. 1) shows that C_{20} fatty acid is rapidly formed from C_{18}-CoA. The same is true for C_{18} formation from C_{16}-CoA. For the other fatty acids (C_{22}, C_{24}), there is a lag in the appearance of the label, whatever the experimental conditions. These results suggest that several elongases could exist in the microsomes from etiolated leek seedlings, so that C_{20} formed by a first elongation of C_{18}-CoA could be the substrate of a second elongase and so on. Such a hypothesis implies that C_{20} fatty acid is released as C_{20} acyl-CoA which is then further elongated, as demonstrated in this paper. The exact chemical nature of the products of elongase is extremely important and is being actively studied in our laboratory.

EXPERIMENTAL

Leek seeds stored overnight at 4°, were sterilized with NaOCl 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 the following growth medium: 5 g agar–agar, 900 ml H_2O and 100 ml of a nutritive soln containing per l. 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.

Substrates and reagents. [2-^{14}C]Malonyl-CoA (59 Ci/mol) and [1-^{14}C]C_{18}-CoA (48 Ci/mol) were from Amersham. [1-^{14}C]Acetyl-CoA (46 Ci/mol) and [1-^{14}C]C_{16}-CoA (65.8 Ci/mol) were obtained from New England Nuclear. All other chemical reagents were purchased from Sigma.

Preparation of microsomes. Etiolated leek coleoptiles (5 g) were ground in a mortar in 20 ml 0.08 M Hepes buffer, pH 7.5, containing 0.32 M sucrose and 10 mM mercaptoethanol (buffer A) and filtered through Miracloth. The residue was reground twice in the presence of 10 ml buffer A. The combined filtrates were then centrifuged at 3000 *g* for 5 min and the supernatant centrifuged at 13 000 *g* for 15 min. The resulting supernatant was centrifuged at 150 000 *g* for 1 hr. The pellet was dispersed in a glass homogenizer with 10 ml 0.08 M Hepes buffer, pH 7.5, containing 10 mM mercaptoethanol (buffer B) and recentrifuged at 150 000 *g* for 1 hr. The resulting pellet was resuspended in 5 ml buffer B and used as the enzyme source.

Assay for LFA synthesis. When using [2-^{14}C]malonyl-CoA (17 μM) as the labelled substrate, 20 μl of the microsomal

preparation (*ca* 30 μg protein) was incubated for 1 hr at 30° with 0.5 mM NADPH, 0.5 mM NADH, 1 mM ATP, 1 mM $MgCl_2$, 2 mM DTT and 0.08 M Hepes buffer, pH 7.5, in a total vol. of 0.1 ml. When acyl-CoAs were tested, ATP was omitted and the concn of each acyl-CoA was 9 μM. Routinely, the reaction was carried out for 1 hr at 30°. At the end of the incubation period, 0.1 ml 20% KOH in 90% MeOH was added to the incubation medium and then heated for 45 min at 80°. The fatty acids were extracted and Me esters prepared as described previously [6]. In expts using [1-^{14}C]C_{16}-CoA or [1-^{14}C]C_{18}-CoA as labelled substrate, the reaction mixture contained 30 μg microsomal proteins, 167 μM malonyl-CoA, 0.5 mM NADPH, 0.5 mM NADH, 1 mM $MgCl_2$ and 2 mM DTT in a 0.1 M Hepes buffer, pH 7.5, in a final vol. of 0.1 ml.

Chromatography and radioactivity measurements. GC–RC of Me esters was performed using a 10% SE 30 column (2 mm × 1 m) on WHP (90–100 mesh size) with Ar as carrier at a flow rate of 60 ml/min. In the case of expts using [2-^{14}C]malonyl-CoA, a temp. program was used from 180° to 280° at 4°/min. When [1-^{14}C]C_{16}-CoA was precursor, the temp. rise from 180° to 280° (8°/min) was preceded by a 7 min period under isothermal conditions at 180°. For expts with [1-^{14}C]C_{18}-CoA, the program was 7 min at 190° followed by an 8°/min rise up to 280°. The effluent gases were continuously monitored for radioactivity.

Proteins were estimated by the method of ref. [9] using BSA as standard.

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