Phytochemistry, Vol. 21, No. 1, pp. 55–59, 1982.
Printed in Great Britain.

0031-9422/82/010055-05 $03.00/0

VERY LONG CHAIN FATTY ACIDS: OCCURRENCE AND BIOSYNTHESIS IN MEMBRANE FRACTIONS FROM ETIOLATED MAIZE COLEOPTILES

RENÉ LESSIRE, MARIE-ANDRÉE HARTMANN-BOUILLON* and CLAUDE CASSAGNE

Institut de Biochimie Cellulaire et Neurochimie, 1 rue Camille Saint-Saëns, 33000 Bordeaux, France; * Institut de Botanique, Laborataire de Biochimie Végétale, ERA du CNRS No. 487, 28 rue Goethe, 67083 Strasbourg Cedex, France

(*Revised received* 31 *May* 1981)

Key Word Index—*Zea mays*; Gramineae; etiolated maize; coleoptile; endoplasmic reticulum; plasmalemma; composition; biosynthesis; long-chain fatty acids.

Abstract—The endoplasmic reticulum from maize coleoptiles elongates stearoyl-CoA more effectively than the plasmalemma-enriched fraction. The alkane and very long chain fatty acid content of the different membrane fractions have been determined. The plasma membrane contains larger amounts of very long aliphatic chains than the endoplasmic reticulum. In maize coleoptile, the endoplasmic reticulum fraction seems to be the major site of the biosynthesis of the very long chain fatty acids and the plasma membrane the site of their final intracellular insertion.

INTRODUCTION

Saturated very long chain fatty acids (VLCFA) in the C_{20}–C_{30} range are normal components of the wax layer of higher plants [1, 2]. Numerous studies, both *in vivo* and *in vitro*, have been devoted to their biosynthesis [3, 4]. From the studies with developing leaves [5, 6] and/or germinating seeds [7, 8] there is general agreement that NADPH is the preferred reductant and that malonyl-CoA is the elongating agent [5, 6]. The nature of the primer of the elongase remains, however, controversial: in *Allium porrum* epidermal cells stearoyl-CoA is a better substrate than stearoyl-ACP or free stearic acid [9] but in microsomes from developing jojoba seeds, stearoyl-CoA and stearoyl-ACP are both used by the elongase [10].

In leaves of higher plants, the epidermis seems to be the main site of biosynthesis of VLCFA but the precise site in the epidermis cell is still in debate. From electron microscopic examinations, it was assumed that in *Libertia elegans* the synthesis is extracellular [11], but a subcellular fractionation of leek epidermal cells revealed that the main site of stearoyl-CoA elongation is probably the endoplasmic reticulum [9].

An analysis of the subcellular fractions of leek enriched either in endoplasmic reticulum or plasmalemma showed that both contained VLCFA and alkanes [12] but that the amount in the plasmalemma-enriched fraction was some 3 times higher than the endoplasmic reticulum fraction. Very similar results were obtained with purified membrane fractions from yeast [13]. Thus the very long chain aliphatic components could be normal constituents of the plasmalemma, and not only characteristic wax components. This possibility has now been investigated in purified membrane fractions from maize coleoptiles.

In this paper, we describe the analysis of the VLCFA and alkanes in the endoplasmic reticulum- and plasmalemma-enriched fractions from maize coleoptiles, as well as a study of stearoyl-CoA elongation by these membrane fractions.

Table 1. Biosynthesis of saturated VLCFA by membrane fractions of etiolated maize coleoptiles

Membrane fraction	^{14}C-Labelled saturated VLCFA		
	Protein (nmol/mg per hr)	Total activity (nmol per hr)	Odd/even
Endoplasmic reticulum	12.1 ± 0.8*	8.47 ± 0.5	0.48 ± 0.06
Plasmalemma	4.3 ± 0.9	2.9 ± 0.6	0.84 ± 0.07

The reaction mixture contained in 1 ml of Tris–HCl 0.1 M, pH 7.5, buffer: 0.25 μmol malonyl-CoA, 0.5 μmol NADPH, 2 μmol ATP 1 μmol $MgCl_2$, 0.2 μmol [1-^{14}C]stearoyl-CoA (2 Ci/mol) and 0.14 mg of resuspended membrane proteins.

* Mean ± s.d. (4 expts).

RESULTS

Biosynthesis of VLCFA by membrane fractions

Endoplasmic reticulum and plasmalemma fractions incubated with [1-^{14}C]stearoyl-CoA formed 8% or 20% of hydroxy and/or polyunsaturated fatty acids. No monoenoic and dienoic acids were detected. This result was quite different from when stearoyl-ACP was used as substrate [9]. The endoplasmic reticulum fraction synthesized about 12 nmol saturated VLCFA/mg protein per hr (Table 1) and the plasmalemma fraction only 4.5 nmol/mg per hr. The endoplasmic fraction activity was about 3 times higher than that of the plasmalemma fraction. These results are very similar to those reported for membrane fractions from *A. porrum* epidermal cells [9].

The distribution of the radioactivity in saturated fatty acids is given in Table 2. Eicosanoic acid and docosanoic acid were the major fatty acids synthesized in the endoplasmic reticulum fraction. In the plasmalemma fraction, eicosanoic acid was also the major acid formed from stearoyl-CoA. Its rate of formation (1 nmol/mg protein per hr), however, was five times lower than in the endoplasmic reticulum fraction. Except for C_{28} synthesis, the even-numbered VLCFA were predominant in the endosplasmic reticulum whereas in the plasmalemma fractions the level of biosynthesis of odd- and even-numbered VLCFA was very similar. The ratio of the radioactivity in odd-numbered VLCFA/even-numbered VLCFA was 0.84 for the plasmalemma and 0.48 for the endoplasmic reticulum fraction. This fact, previously reported in yeast and in *A. porrum* epidermal cells [13, 9], suggested the presence in the plasmalemma membrane of an α-oxidation system for the formation from even-numbered VLCFA of odd-numbered VLCFA.

These results showed that in etiolated maize coleoptiles, the endoplasmic reticulum was more active in synthesizing saturated VLCFA than was the plasmalemma and could be the site of biosynthesis of saturated VLCFA.

Analysis of membrane fractions

The fatty acid and alkane content of membrane fractions is given in Table 3. The total amount of fatty acids including medium, long and very long chains in the two fractions were not very different. The total alkane content of the plasmalemma fraction (18.2 μg/mg protein) was 3 times higher than that of the endoplasmic reticulum fraction (5.5 μg/mg protein).

The purification and identification of alkanes by GC/MS has been reported previously [14]. The analysis of alkanes of membrane fractions of etiolated maize coleoptile showed the presence of all even- and odd-numbered alkanes in the C_{22}–C_{31} range (Table 4). Triacontane squalene was the most important alkane, and accounted for more than 36% of the alkanes in the plasmalemma fraction. The alkane distribution was very similar in the two membrane fractions with the C_{27}, C_{28}, C_{29} and C_{30} alkanes representing 67% and 74.9% of the total alkanes in the endoplasmic reticulum and plasmalemma membranes respectively. These distributions were markedly different from those of waxes, and particularly leek waxes, for in waxes odd-numbered alkanes are the major components [2, 14]. This was taken as an indication that the alkane content of the membrane fraction was not due to contamination occurring during

Table 3. VLCFA and alkane contents of membrane fractions of etiolated maize coleoptiles

	Endoplasmic reticulum	Plasmalemma
Total fatty mass (μg/mg proteins)	406.0 ± 21.2*	475.0 ± 16.9
VLCFA (μg/mg proteins)	20.0 + 4.6	59.0 ± 4.0
VLCFA (%)	4.9 ± 0.7	12.4 ± 2.0
Unsaturated fatty acids (μg/mg proteins)	223 ± 15.3	216 ± 21.4
Unsaturated fatty acids (%)	54.9 ± 1.6	45.5 ± 4.6
Saturated fatty acids (μg/mg proteins)	183 ± 13.2	259 ± 27.4
Unsaturated fatty acids / Saturated fatty acids	1.2 ± 0.1	0.8 ± 0.2
Alkanes (μg/mg proteins)	5.5 ± 0.7	18.2 ± 2.2

* Mean ± s.d. (six expts).

Table 2. Radio-GLC analysis of saturated VLCFA synthesized by membrane fractions of etiolated maize coleoptiles

Fatty acids	Plasmalemma (nmol/mg protein per hr)	Endoplasmic reticulum (nmol/mg protein per hr)
C_{20}	1.03	5.35
C_{21}	0.81	1.59
C_{22}	0.45	2.50
C_{23}	0.26	0.68
C_{24}	0.31	0.68
C_{25}	0.32	0.11
C_{26}	0.19	0.45
C_{27}	0.20	0.34
C_{28}	0.24	0.06
C_{29}	0.29	0.22
C_{30}	0.19	

the preparation, but that alkanes are normal constituents of membranes, a suggestion which is confirmed by the analysis of yeast plasmalemma [13].

The VLCFA were identified by comparison of their R_ts on GLC with standards and by GC/MS. The acids present were 20:0, 20:1, 22:0, 23:0, 24:0 and 26:0. Table 3 shows that the endoplasmic reticulum fraction contained about three times less VLCFA than the plasmalemma: 20 μg/mg protein compared with 59 μg/mg protein. The distribution of fatty acids is reported in Table 5 and shows that palmitic, stearic, oleic and linoleic acids were the most abundant in the two fractions, and accounted for 90.3% and 84.3% of the fatty acids in the endoplasmic reticulum and plasmalemma fractions respectively. Oleic acid was more abundant in the plasmalemma than in the endoplasmic reticulum; the reverse was true for linoleic acid. The plasmalemma fraction contained 2.8% 16:1 and the complete series of even- and odd-numbered VLCFA. The even-numbered VLCFA were more abundant than the odd-numbered VLCFA. In the endoplasmic reticulum fraction, the only fatty acids detected were 20:0, 23:0 and 24:0. Another difference between endoplasmic reticulum and plasmalemma fractions, appeared on examination of the quantity of unsaturated acids in each fraction (Table 3). Of the fatty acids of the plasmalemma, 45.5% were unsaturated as compared to 54.9% in the endoplasmic reticulum fraction. This decrease in the ratio of unsaturated to saturated acids in the plasmalemma was in good agreement with previous analyses of the fatty acids of the plant plasmalemma [15]. This difference was due, for a great part, to a higher percentage of 18:2 in the endoplasmic reticulum in maize coleoptile membranes (34.0% in endoplasmic reticulum as compared with 9.2% in plasmalemma fraction).

Plasmalemma was enriched in very long chain saturated aliphatic molecules when compared with endoplasmic reticulum membrane.

Table 4. Alkane composition of membrane fractions of etiolated maize coleoptiles

Alkanes	Endoplasmic reticulum (%)	Plasmalemma (%)
C_{22}	Traces	—
C_{23}	4.3	2.0
C_{24}	4.4	2.1
C_{25}	6.4	3.3
C_{26}	10.4	7.9
C_{27}	15.2	9.6
C_{28}	18.3	15.0
C_{29}	14.9	13.8
C_{30}		
C_{30} + squalene	18.6	36.5
C_{31}	7.6	9.9

* Mean values of six expts.

Table 5. Fatty acid composition of membrane fractions of etiolated maize coleoptiles

Acids	Endoplasmic reticulum (%)	Plasmalemma (%)
16:0	35.4	40.6
16:1	—	2.8
17:0	0.3	1.8
18:0	6.3	7.1
18:1	14.6	24.6
18:2	34.0	9.2
20:0	2.6	2.8
20:1	—	2.8
NI†	—	1.8
22:0	2.8	1.6
23:0	1.4	1.2
NI	1.0	—
24:0	1.7	2.8
25:0	—	0.3
26:0	—	0.8
27:0	—	traces

* Mean values of six expts.

† NI, not identified.

DISCUSSION

The measurements of saturated VLCFA biosynthesis by subcellular fractions from *A. porrum* epidermal cells showed previously that crude mitochondrial fraction and the soluble supernatant were devoid of that activity. Some synthesis occurred in the plasmalemma-enriched fraction but the main site was located in the endoplasmic reticulum. The results with yeast were basically the same, although the synthesis observed in the pure plasmalemma reached 50% of that found in the endoplasmic-reticulum-enriched fraction [13]. The results reported here using well-defined plant membrane fractions are in fairly close agreement and seem to indicate that the endoplasmic reticulum is the main intracellular site of VLCFA biosynthesis. Recently, the study of the biosynthesis of VLCFA and alkanes by barley protoplasts indicated that the digestion enzymes inhibited the synthesis. This was taken as an argument that the plasmalemma and/or the outermost cell-wall could be involved in the wax synthesis [16]. As in *A. porrum* epidermal cells and yeast, in maize coleoptile membranes no alkane biosynthesis was observed using stearoyl-CoA as substrate. Stearoyl-CoA was elongated to saturated VLCFA uniquely and, in good agreement with the 'CoA track–ACP track' hypothesis proposed by Shine *et al.* [17], no radioactivity was detected in monoenoic and/or dienoic acids.

The fatty acid analysis of plasmalemma membrane of etiolated maize coleoptile gave the characteristic pattern reported for plant plasma membranes. The C_{16} and C_{18} family acids were preponderant and the degree of saturation of the fatty acids was higher than in the endoplasmic reticulum membrane. In addition, it was demonstrated that plasmalemma accumulated preferentially very long chain aliphatic components. This fact was previously reported for yeast [13], *A. porrum* cell epidermis plasmalemma [9] and numerous mammalian membranes [18–22] including the highly specialized myelin membrane [23, 24]. The presence of the very long chain aliphatic molecules in such different tissues

indicates that these components may be normal constituents of the plasmalemma membrane. This characteristic will be definitively established by the analysis of alkanes and fatty acids of numerous higher plants and mammalian plasmalemma membranes. The composition of VLCFA of the plasmalemma membrane is very different to those known for the wax layer and excludes the possibility of some contamination during membrane preparation. This result suggested that in the epidermal cell two different systems could be involved in the biosynthesis of very long chain fatty acids: one leading to the formation of the wax fatty acids; and one responsible for the synthesis of membrane VLCFA. Alternatively, the same system could synthesize the very long aliphatic chains for both the plasma membrane and wax layer. The analysis of the fate of the VLCFA formed in the endoplasmic reticulum, chiefly their insertion into polar lipids of plasma membranes or excretion to the wax layer is under way.

EXPERIMENTAL

Chemicals. [1-^{14}C] Stearoyl-CoA (52 Ci/mol) was purchased from New England Nuclear (Dreieich RFA). ATP, malonyl-CoA and NADPH were supplied from Sigma Chemicals (St. Louis, U.S.A.).

Plant material. Seedling of *Z. mays* (cv INRA 258) were grown for 6 days at 26° in the dark. The harvesting of coleoptiles and all subsequent procedures were performed in the light.

Isolation of membranes. Coleoptiles were homogenized by grinding them in 0.5 M mannitol, 1 mM EDTA, 10 mM 2-mercaptoethanol and 0.1 M Tris–HCl, pH 8. Membrane fractions were isolated by differential and sucrose density gradient centrifugation as described previously [25, 26]. After centrifugation of gradients, fractions (1.2 ml) were collected using a density gradient fractionator. The enzyme marker activities and the electron microscopy examinations of the different fractions showed that in these conditions two of the isolated membrane fractions were enriched in endoplasmic reticulum and in plasmalemma respectively [26]. The gradient fractions corresponding to identified membrane fractions were pooled, pelleted by centrifugation at 100 000 *g* for 30 min in 0.1 mM Tris HCl, pH 8, containing 2.5 mM 2-mercaptoethanol and the pellet homogenized in Tris HCl, pH 7.5, containing 0.1 M BSA and 1 mM EDTA.

VLCFA synthesis. This was measured by using [1-^{14}C]stearoyl-CoA as the primer. The incubation medium contained: 1 μmol NADPH, 0.25 μmol malonyl-CoA, 0.1 μmol [1-^{14}C] stearoyl-CoA (1 Ci/mol), 1 μmol ATP, 0.5 μmol $MgCl_2$, 0.14 mg of resuspended membranes in Tris–HCl 0.1 M buffer, pH 7.5, containing 0.1 % BSA and 1 mM EDTA in a final vol. of 1 ml. The samples were incubated for 60 min at 30°. The reaction was stopped by adding 0.5 ml 5 M KOH and the reaction was saponified 1 hr at 70°. The non-saponifiable fraction was extrd as described elsewhere [5]. Fatty acids were esterified according to Metcalfe *et al.* [27] and the Me esters subjected to TLC on Ag^+–Si gel (prepared as described previously [28]) developed with C_6H_6–hexane (7:3). The radioactivity of the different classes of fatty acids was monitored after TLC by a radiochromatogram scanner and the radioactive areas extrd with $CHCl_3$–MeOH (1:2). The saturated fatty acid methyl esters were subjected to GLC on an Intersmat IGC 120 DFL gas chromatograph equipped with dual FID: injector heater 240°, detector heater 340°, 0.3 × 180 cm column packed with 10 % SE 30, carrier gas N_2 30 ml/min, temp. programme 180–310° at 4°/min. The esters were collected with a Packard 852 fraction collector in cartridges filled with anthracene and assayed for radioactivity in a liquid scintillation spectrometer. Before each assay, the purity of the labelled stearoyl-CoA was checked by radio-GLC. C_{18} represented more than 97 % of the total label and the remaining 3 % were found in the VLCFA, chiefly C_{20}. This value was considered as the background value.

Analysis of membrane fractions. Before each experiment, the solvents were freshly redistilled. The purity of hexane was checked by GLC. The glassware was carefully washed with boiling MeOH to avoid contamination. A complete blank run containing distilled H_2O and only standards was made.

The collected membrane fractions were pelleted in distilled H_2O at 100 000 *g* for 30 min. Fatty acid methyl esters were prepared as described above. The non-saponifiable fraction was layered on the top of an Al_2O_3 column and eluted with 30 ml spectrograde hexane. The eluted fraction contained alkanes. The fatty acid methyl esters were identified by GC/MS: MS conditions: ion source 270°, 70 eV, accurate voltage 3500 V; GLC conditions: injector heater 250°, glass capillary column (0.35 mm × 4 m) packed with SE 30, carrier gas 0.8 bar, temp. programme 100–250° at 2°/min. The quantitative measurements were made by the int. standard method. Octadecane (100 μg) and myristic acid (100 μg) were added before saponification. Peak areas were measured by a computing integrator.

Protein measurements. Proteins were estimated by the procedure of ref. [29]. BSA was used as the standard.

Acknowledgements—We wish to thank Dr. Salaun (Institut de Botanique, Strasbourg) and Dr. Teller (Laboratoire de Chimie Organique, Strasbourg) for help in preparing and interpreting the MS and Prof. P. Benveniste for invaluable help in preparing the experiments and discussing the results.

REFERENCES

1. Eglinton, G. and Hamilton, R. J. (1967) *Science* **156**, 1322.
2. Tulloch, A. P. (1976) in *Chemistry and Biochemistry of Natural Waxes* (Kolattukudy, P. E., ed.) p. 236. Elsevier, Amsterdam.
3. Kolattukudy, P. E., Croteau, R. and Bückner, J. S. (1976) in *Chemistry of Natural Waxes* (Kolattukudy, P. E., ed.) p. 290. Elsevier, Amsterdam.
4. Kolattukudy, P. E. (1980) in *The Biochemistry of Plants* (Stumpf, P. K. and Conn, E. E., eds.) Vol. 4, p. 571. Academic Press, New York.
5. Cassagne C. and Lessire R. (1974) *Physiol. Veg.* **12**, 149.
6. Kolattukudy, P. E. and Bückner, J. S. (1972) *Biochem. Biophys. Res. Commun.* **46**, 801.
7. Macey, M. J. K. and Stumpf, P. K. (1968) *Plant Physiol.* **43**, 1637.
8. Bolton, P. and Harwood, J. L. (1977) *Biochem. J.* **168**, 261.
9. Cassagne, C. and Lessire, R. (1978) *Arch. Biochem. Biophys.* **191**, 146.
10. Pollard, M. P., Mac Keon, T., Grupts, L. M. and Stumpf, P. K. (1979) *Lipids* **14**, 651.
11. Sargent, C. (1976) *Ann. Botany* **40**, 355.
12. Lessire, R. and Cassagne, C. (1980) *Bot. J. Linn. Soc.* 48.
13. Blanchardie, P., Carde, J. P. and Cassagne, C. (1977) *Biol. Cell.* **30**, 127.
14. Cassagne, C. (1970) Thèse doctorat d'Etat, Bordeaux.
15. Mazliak, P. (1977) in *Lipids and Lipid Polymers in Higher Plants* (Tevini, M. and Lichtenthaler, H. K., eds.) Springer, Berlin.

16. Mikkelsen, J. (1980) *Biogenesis and Function of Plant Lipids* (Mazliak, P., Benveniste, P., Costes, C. and Douce, R., eds.) p. 285. Elsevier, Amsterdam.
17. Shine, W. E., Mancha, M. and Stumpf, P. K. (1976) *Arch. Biochem. Biophys.* **172,** 110.
18. Micklen, K. J., Abra, R. M., Knutten, S., Graham, J. M. and Parternak, C. A. (1976) *Biochem. J.* **1154,** 561.
19. Snary, E., Allen, A. K., Faulkes, R. A. and Grumpton, M. J. (1976) *Biochem. J.* **153,** 75.
20. Perkins, R. G. and Kummerow, F. A. (1976) *Biochim. Biophys. Acta* **424,** 469.
21. Gray, G. M. and Yardley, H. J. (1975) *J. Lipid Res.* **16,** 434.
22. Patton, S. and Keenan, T. W. (1975) *Biochim. Biophys. Acta* **415,** 273.
23. Darriet, D., Cassagne, C. and Bourre, J. M. (1978) *Neurosci. Letters* **8,** 77.
24. Bourre, J. M., Cassagne, C., Larrouquère-Règnier, S. and Darriet, D. (1977) *J. Neurochem.* **29,** 645.
25. Hartmann, M. A. and Benveniste, P. (1978) *Phytochemistry* **17,** 1037.
26. Hartmann-Bouillon, M. A., Benveniste, P. and Roland, J. C. (1979) *Biol. Cell.* **35,** 183.
27. Metcalfe, L. D., Schmitz, A. A. and Pelka, J. R. (1966) *Analyt. Chem.* **38,** 514.
28. Schmitt, P. and Benveniste, P. (1979) *Phytochemistry* **18,** 445.
29. Lowry, O. H., Rosebrough, W. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265.