CHAIN ELONGATION OF FATTY ACIDS BY CELL-FREE EXTRACTS OF EPIDERMIS FROM PEA LEAVES (PISUM SATIVUM)

P. E. Kolattukudy and J. S. Buckner
Department of Agricultural Chemistry
Washington State University
Pullman, Washington 99163

Received November 29, 1971

Summary: A 27,000 x g supernatant from extracts of epidermis of pea leaves (Pisum sativum) incorporated acetate-1-^{14}C into C_{18} to C_{26} fatty acids in the presence of ATP, CoA, NADPH (but not NADH), HCO_3^- and Mn^{+2}. Avidin inhibition of this reaction and incorporation of malonyl-2-^{14}C-CoA in the absence of HCO_3^- showed that malonyl-CoA was the elongating agent. Palmitoyl-CoA did not stimulate the reaction nor was stearic acid-1-^{14}C elongated. Micro-Schmidt degradation of the individual acids showed that the synthesis of the very long acids was exclusively by elongation of preformed endogenous chains. Subcellular fractionation indicated that the elongating activity was in the microsomal fraction.

Long chain hydrocarbons are widely distributed in nature (1,2). Experimental evidence thus far obtained with plants indicate that alkanes are synthesized from fatty acids by an elongation-decarboxylation mechanism. However, a condensation between two biochemically dissimilar fatty acid derivatives followed by decarboxylation can not be ruled out as a mechanism for alkane biosynthesis (3). According to the elongation-decarboxylation hypothesis a preformed fatty acid is elongated by C_2 units on an enzyme complex until the chain length of the acid reaches C_{30} or C_{32}, and then it is decarboxylated releasing the alkane. Some elongated products are released from the complex giving rise to C_{20}-C_{30} fatty acids. The major site of alkane synthesis in higher plants is the epidermal layer of cells (4,5). In this communication we demonstrate that a cell-free extract prepared from the epidermis catalyzes elongation of preformed fatty chains.

Scientific Paper 3781, Project 2001, College of Agriculture, Washington State University, Pullman, Washington 99163. This work was supported in part by U.S.P.H.S. Grant GM18278.

EXPERIMENTAL

The epidermal layer of cells of young pea leaves was peeled into a grinding buffer consisting of 50 mM tris, 250 mM sucrose, and 5 mM dithioerythritol, pH 7.2. The tissue was ground with a Ten-Broeck homogenizer and the homogenate cetrifuged at 27,000 x g for 30 minutes. The supernatant was used as the enzyme source in most experiments. The microsomal fraction was obtained by centrifugation at 100,000 x g for 90 minutes. All these operations were done below 4°C.

The reaction mixture contained 8.2 μmoles ATP, 0.6 μmoles CoA, 1.0 μmole NADPH, 30 μmoles glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase, 3.0 μmoles $MnSO_4$, 10 μmoles $NaHCO_3$, 0.17 μmole sodium acetate-1-^{14}C (59 mCi/mmole) and epidermal extract containing 4 to 6 mg protein in a total volume of 3 ml grinding buffer. Similar experiments were run with 0.07 μmole malonyl-2-^{14}C-CoA (20.6 mCi/mmole) and 0.1 μmole stearic acid-1-^{14}C (48.4 mCi/mmole) as substrates. The reaction mixture was incubated at 30°C for 2 hrs in a gyratory water bath shaker. At the end of the incubation period 12 ml of 10% KOH in ethanol was added and the reaction mixture refluxed for 2 hrs under N_2. Fatty acids were extracted with chloroform after acidification and purified by thin-layer chromatography with hexane:ether:formic acid (40:10:1) as the solvent system. Fatty acids were esterified with 14% BF_3 in CH_3OH.

Chain length distribution of the methyl esters of the labeled fatty acids was determined by radio gas-liquid chromatography with a combination of a Perkin-Elmer 800 gas chromatograph attached to a Barber-Coleman radioactivity monitor. For degradation purposes, individual fatty acids were separately isolated by gas chromatography followed by hydrolysis and thin-layer chromatography. Micro-Schmidt degradation was done according to the method of Brady et al. (6). Protein concentration was determined by the method of Lowry et al. (7). Radioactivity was measured with a Packard Tri-Carb liquid scintillation

spectrometer. Counting was done with 74% efficiency and a standard deviation less than 3%.

RESULTS AND DISCUSSION

Epidermis peeled from Senecio odoris leaves had been shown to incorporate acetate-1-^{14}C into alkanes (4). Since pea (Pisum sativum) leaves synthesize hydrocarbons very rapidly (8) we peeled the epidermis from these leaves and incubated this tissue with acetate-1-^{14}C. Radioactive hydrocarbons were formed, and the fatty acids isolated from this tissue contained very long chain fatty acids (C_{20}-C_{28}). A homogenate prepared from excised epidermis also incorporated acetate-1-^{14}C into lipids. The fatty acids isolated from the labeled lipids when subjected to radio gas-liquid chromatography showed that virtually all the radioactivity was in C_{18} and longer acids with only a small amount of ^{14}C in C_{16} acid. These results suggested that the homogenate catalyzed elongation rather than de novo synthesis of fatty acids.

Fractionation of the homogenate by differential centrifugation showed that acetate incorporating activity was located mainly in the 27,000 x g supernatant. Therefore, this supernatant was used as the enzyme source for most experiments. The acetate incorporation into fatty acids was linear for 60 min and was proportional to the amount of extract used at least up to about 6 mg protein/ml (Fig. 1).

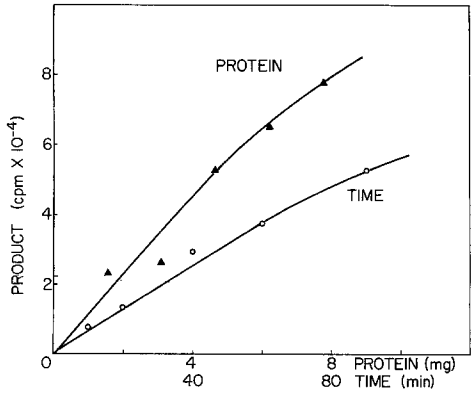

Figure 1. Time and protein concentration dependence of sodium acetate-1-^{14}C incorporation into fatty acids. Assay conditons as described in experimental.

The labeled fatty acids synthesized by the 27,000 x g supernatant from acetate-1-^{14}C were subjected to radio gas-liquid chromatography,

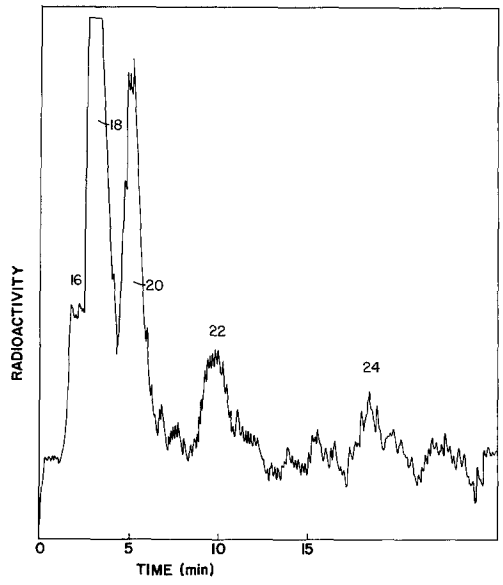

Figure 2. Radio gas-chromatogram of the labeled fatty acid methyl esters derived from acetate-1-^{14}C in the epidermal extract of pea leaves. Chromatography on 6 ft., 1/4" OD coiled stainless steel column packed with 3% OV-1 on 80-100 mesh Gas Chrome Q at 205°C and 120 ml Argon carrier gas flow.

and the results are shown in Figure 2. Only a small amount of radioactivity was found in the C_{16} acid which is usually the product of de novo synthesis. The major component was C_{18} acid followed by C_{20}, C_{22} and C_{24}. Under the present experimental conditions labeled C_{26} acid could be detected, but this component and longer acids usually did not contain significant amounts of radioactivity. The ^{14}C distribution indicates that the 27,000 x g supernatant catalyzes either elongation of preformed acids or an unusual de novo synthesis.

In order to distinguish between these two possibilities the various fatty acids were isolated by gas-liquid chromatography and then subjected to micro-Schmidt degradation. The radioactivity contained in the CO_2 released by each acid is shown in Table 1. It is obvious that all the fatty acids tested contained much more radioactivity in the carboxyl group than would be expected from de novo synthesis. In the

Table 1

Radioactivity in the carboxyl carbon of fatty acids derived from acetate-1-^{14}C in the epidermal extract of pea leaves

Fatty acid	Radioactivity in the carboxyl carbon (%)	
	Exp't. 1	Exp't. 2
C_{16}	64	84
C_{18}	85	95
C_{20}	68	69
C_{22}	--	68
C_{24}	--	74

Individual fatty acids isolated by gas chromatography were degraded by micro-Schmidt procedure (6) with a methanolic solution of hyamine hydroxide as the CO_2-trapping agent.

case of C_{18} acid most of the ^{14}C was in the carboxyl carbon showing that virtually all the labled C_{18} acid was formed by elongation of a preformed C_{16} acid. The acids longer than C_{18} could have been formed either by addition of more than one acetate unit to C_{16} chain or by the addition of one C_2 unit to preformed fatty acids of appropriate chain length. In the former case the proportion of the ^{14}C present in the carboxyl carbon should be 50%, 25% and 12% respectively in C_{20}, C_{22} and C_{24} acids, and in the latter case all the ^{14}C of each acid should be located in the carboxyl carbon. Our experimental results (Table 1) showed that the carboxyl carbon of the acids longer than C_{18} contained much less than 100% of the radioactivity of each acid suggesting that these acids were formed by the addition of more than one C_2 unit to the starting chain. However, the radioactivity in the carboxyl carbon of the C_{20}, C_{22} and C_{24} acids was higher than that expected from the

addition of two, three and four acetate units respectively to a C_{16} starter chain. These results indicate that starter chains longer than C_{16} also participated in the formation of the very long acids.

The cofactor requirements for the elongation process are summarized in Table 2.

Table 2

Cofactor requirements for chain elongation in epidermal extracts of pea leaves

Conditions	Incorporation of acetate-1-^{14}C (cpm)
All cofactors	45,000
All cofactors + NADH (1 μmole)	44,000
- ATP, CoA	7,500
- HCO_3^-	8,800
- NADPH	7,000
- NADPH + NADH (1 μmole)	10,000
All cofactors + avidin (0.5 mg)	18,000
- All cofactors	4,300
All cofactors + 12.5 mμmoles palmitoyl CoA	42,000
All cofactors + 50 mμmoles palmitoyl CoA	40,000

Reaction mixture contained 8.2 μmoles ATP, 0.6 μmole CoA, 1.0 μmole NADPH, 30 μmoles glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase, 3.0 μmoles $MnSO_4$, 10 μmoles $NaHCO_3$, 0.17 μmole sodium acetate-1-^{14}C (59 mCi/mmole) and epidermal extract containing 4 to 6 mg protein in 3 ml. Reaction mixtures incubated at 30°C for 2 hrs.

As expected, ATP and CoA are required and NADPH is the preferred reducing agent; NADH neither substituted for NADPH nor did it stimulate the activity obtained with NADPH. Bicarbonate was required for elongation and avidin inhibited the reaction showing that malonyl CoA is the elongating agent. Furthermore, malonyl-2-^{14}C-CoA was incor-

porated into very long acids in this system. In this case addition of bicarbonate stimulated the incorporation only slightly, and this stimulation can be explained on the basis of the probability that part of the incorporation of the malonyl-CoA was via acetate formed by decarboxylation. In any case, it is quite obvious that malonyl-CoA is the elongating agent. Exogenous palmitoyl-CoA did not stimulate the incorporation of acetate, and labeled stearic acid-1-^{14}C was not elongated. Therefore the starter chains for the elongation appear to be of endogenous source, and we do not know the chemical nature of these moieties.

The 27,000 x g supernatant has been further fractionated, and the elongating activity with malonyl-CoA as substrate was found to be located in the microsomal fraction. In the epidermal layer of cells chain elongation located in the microsomal fraction appears to be a major site of synthesis of fatty chains. In fact, we could detect only very little de novo synthesis in the whole homogenate. It is likely that the elongating activity described here is an integral part of wax biosynthesis which is a major function of the epidermis. A microsomal fraction which synthesized C_{20}-C_{28} acids by elongation was isolated from germinating seeds (8). This activity appeared when the germinated seed would have required wax to water-proof its aerial parts. In the present paper we demonstrate the occurrence of elongating enzyme postulated earlier on the basis of tracer studies on wax biosynthesis (10).

ACKNOWLEDGMENTS

We thank Linda Brown for technical assistance.

REFERENCES

1. Douglas, A. G. and Eglinton, G., Comparative Phytochemistry, Swain, T., Ed. (Academic Press, New York, 1965) p. 57.
2. Kolattukudy, P. E., Science 159, 498 (1968).
3. Kolattukudy, P. E., Ann. Rev. Plant Physiol. 21, 163 (1970).
4. Kolattukudy, P. E., Plant Physiol. 43, 375 (1968).
5. Cassagne, C., C. R. Acad. Sci. Ser. D. 270, 3055 (1970).
6. Brady, R. O., Bradley, R. M. and Trams, E. G., J. Biol. Chem. 235, 3093 (1960).
7. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., J. Biol. Chem. 193, 265 (1951).
8. Kolattukudy, P. E., Plant Physiol. 43, 1466 (1968).
9. Macey, M. J. K. and Stumpf, P. K., Plant Physiol. 43, 1637 (1968).
10. Kolattukudy, P. E., Biochemistry 5, 2265 (1966).