IN VIVO BIOSYNTHESIS OF α-LINOLENIC ACID IN PLANTS[1]

C. Gamini Kannangara, Bruce S. Jacobson and P. K. Stumpf

Department of Biochemistry and Biophysics
University of California
Davis, California 95616

Received April 6, 1973

SUMMARY

[1-^{14}C]acetate was readily incorporated into unsaturated fatty acids by leaf slices of spinach, barley and whole cells of Chlorella pyrenoidosa and Candida bogoriensis. In these systems the [^{14}C] label in newly synthesized oleate and linoleate was approximately equally distributed in the C_{1-9} and the C_{10-18} fragments obtained by reductive ozonolysis of these acids, whereas in α-linolenic acid over 90% of the total [^{14}C] was localized in the C_{1-9} fragment. While [1-^{14}C]oleic acid was converted by whole cells of Chlorella to [1-^{14}C]linoleic and [1-^{14}C]linolenic acids, [U-^{14}C]oleic acid yielded [U-^{14}C]linoleic acid but α-linolenic acid was labeled only in the carboxyl terminal carbon atoms. When spinach leaf slices were supplied with carboxyl labeled octanoic, decanoic, dodecanoic, tetradecanoic and octadecanoic acids, only the first three acids were converted to α-linolenic acids while the last two acids were ineffective. Thus we suggest that (a) linoleic acid is not the precursor of α-linolenic acid and (b) 12:3(3,6,9) is the earliest permissible trienoic acid which is then elongated to α-linolenic acid.

INTRODUCTION

It has been repeatedly proposed that biosynthesis of α-linolenic acid in all plants and algae takes place by a mechanism involving sequential desaturation of stearic acid via oleic and linoleic acids (1-5). Recently we suggested the presence of an enzyme system capable of elongating 16:3(7,10,13) to 18:3 (9,12,15) in a disrupted spinach chloroplast preparation (6). In the light of this observation it became necessary to reinvestigate the pathway for the synthesis of α-linolenic acid in vivo in plant tissues. In this communication we shall report in vivo evidence for the presence of a pathway in which the precursor of α-linolenic acid is not linoleic acid but a shorter chain trienoic acid which is elongated to α-linolenic acid by a pathway separate from the

[1]This work was supported by NSF Grant GB-19733X and NIH Grant GM-19213-01.

stearic → oleic → linoleic pathway. Furthermore, the evidence interestingly implies that the three non-conjugated cis double bonds of α-linolenic acid are formed at a 12 carbon chain precursor level which is then elongated to α-linolenic acid. The data suggest that this pathway operates in green algae, yeast and higher plant leaf tissues.

MATERIALS AND METHODS

Spinach was purchased from the local market. Seedlings of barley (Hordeum vulgare L. var. Svalof's Bonus) were grown in a large Buchner funnel with a continuous spray of water in 1000 ft-c white light. The primary leaves of 5 day old seedlings were used for this investigation. Chlorella pyrenoidosa was grown under photoautrophic conditions according to Hutner et al. (7), in a liquid medium devoid of acetate with CO_2 as the sole carbon source. Candida bogoriensis was grown in liquid culture as described by Deinema (8).

Substrates: [^{14}C]sodium bicarbonate (59 μCi/μmole), [1-^{14}C]acetate (62 μCi/μmole), [1-^{14}C]octanoic (17 μCi/μmole), [1-^{14}C]decanoic (14.3 μCi/μmole), [1-^{14}C]dodecanoic (31.2 μCi/μmole), [1-^{14}C]tetradecanoic (15.4 μCi/μmole), [1-^{14}C]hexadecanoic (55 μCi/μmole) were obtained from Amersham-Searle; [1-^{14}C]oleic (51.2 μCi/μmole) was purchased from Schwarz-Mann and [U-^{14}C] oleic was prepared by $^{14}CO_2$ incorporation into spinach leaf tissue, isolated and purified by $AgNO_3$-TLC and gas liquid chromatographic techniques. The approximate specific activity was 3-5 μCi/μmole.

Incubations: The incorporation of [^{14}C] fatty acids and [^{14}C]bicarbonate into lipids of leaf slices was carried out according to Hawke and Stumpf (9) while [1-^{14}C]acetate incorporation into lipids by leaf slices was performed according to Appelqvist et al. (10). Labeled acetate and oleate incorporations into Chlorella lipids were performed essentially according to Harris and James (3).

Methods: Lipid extraction, methyl transesterification, gas-liquid and thin layer chromatography and scintillation counting were conducted as previously described (11). Chemical α-oxidation and reductive ozonolysis of fatty

acids were performed according to Harris et al. (4), and Stein and Nicolaidis (12) respectively.

RESULTS AND DISCUSSION

Over fifty percent of the total fatty acids produced by higher plants and green algae consists of α-linolenic acid. The biosynthesis of this important fatty acid has been assumed to be by a desaturation of linoleic acid at the C_{15-16} position based on experiments published by a number of workers (1-5). No direct evidence has as yet been provided on an enzyme level defining a linoleic desaturase. When we observed (11) with intact spinach chloroplasts that cyanide completely inhibited the incorporation of [^{14}C]acetate into linoleic acid with no inhibition of α-linolenic acid formation, we initiated a further investigation to resolve this apparent paradox. Very recently we (6) submitted evidence which clearly indicated that α-linolenate was not being formed from linoleic acid but rather from 16:3(7,10,13). The in vivo experiments described here fully support this conclusion and indicate a pathway for α-linolenate synthesis in a variety of photosynthetic and heterotrophic systems completely different from the sequential desaturation pathway proposed earlier (3,5).

Table I summarizes the ozonolysis data of [^{14}C]oleic, [^{14}C]linoleic and [^{14}C]linolenic acids synthesized from [1-^{14}C]acetate by spinach and barley leaf slices. The results clearly indicated that while the degradation pattern of [^{14}C]oleic and [^{14}C]linoleic acids were essentially identical, the pattern for [^{14}C]linolenic acid was drastically different. In contrast, $^{14}CO_2$ incorporation patterns into these three C_{18} fatty acids differed from the [^{14}C] acetate incorporation data. This suggests that the systems responsible for the in vivo synthesis of the proposed short chain trienoic acid precursor were employing [^{14}C]C_2 pools which differed from the C_2 pools utilized for de novo C_{16} and C_{18} fatty acid synthesis.

Results with intact cells of Chlorella pyrenoidosa and Candida bogoriensis are summarized in Table II and demonstrate the same characteristically different labeling patterns of 18:1, 18:2 and 18:3 fatty acids.

Table I. Products of reductive ozonolysis of the unsaturated fatty acids synthesized from [1-^{14}C]acetate and NaH$^{14}CO_3$ by leaf slices of spinach and barley. Each reaction mixture contained 1.2 g wet weight of 1 mm^2 leaf slices, 2.0 ml of water, 50 µmoles $KHCO_3$, 50 µmoles potassium phosphate buffer, pH 7.9 and either 10 µCi (0.15 µmoles) of [1-^{14}C]acetate or 100 µCi (0.21 µmoles) of NaH$^{14}CO_3$. The incubations were carried out for 4 hours at 15° and 1000 ft-c.

Substrate	Fatty acid analyzed	Total cpm isolated	Radioactivity in C_1-C_9 fragment %	Radioactivity in C_{10}-C_{18} fragment %
Spinach				
[1-^{14}C]acetate	18:3	53,818	100	0
	18:2	348,744	51.1	48.9
	18:1	1,153,872	50.1	49.9
NaH$^{14}CO_3$	18:3	99,966	76.0	24.0
	18:2	2,021,875	58.5	41.5
	18:1	4,247,321	47.7	52.3
Barley				
[1-^{14}C]acetate	18:3	127,331	95.2	4.8
	18:2	1,340,799	59.6	40.0
	18:1	2,699,911	51.9	48.1
NaH$^{14}CO_3$	18:3	180,365	52.9	47.1
	18:2	2,008,959	52.2	47.8
	18:1	3,259,687	46.7	53.3

Results depicted in Table III confirm earlier observations by Harris and James (3) that [1-^{14}C]oleic acid has its [^{14}C] label transferred to [^{14}C] linolenic acid and it also indicates some breakdown of the C_{18} substrate to C_{16} products presumably by β-oxidation and resynthesis. However, as outlined in Table IV, the reductive ozonolysis data clearly show that α-linolenic acid cannot be derived from [U-^{14}C]oleate acid but is formed rather by the removal of a [^{14}C]C_2 unit and its transfer to an endogeneous acceptor, presumably 16:3(7,10,13) to form [^{14}C]α-linolenate. Chemical α-oxidation of [^{14}C]stearic acid obtained by reduction of the [^{14}C]α-linolenate supported the data of reductive ozonolysis.

In order to determine at what chain length the trienoic double bond

Table II. Products of ozonolysis of the unsaturated fatty acids synthesized by Chlorella pyrenoidosa and Candida bogoriensis from [1-^{14}C]acetate. Chlorella pyrenoidosa was grown in an acetate free medium under photoautotrophic conditions; 250 mg wet weight of cells were incubated with 1.0 ml of 0.2 M phosphate buffer, pH 7.4 containing 10 µCi of [1-^{14}C]acetate and 200 µmoles of $KHCO_3$ for 2 hours at 15° and 1000 ft-c. Yeast cells (250 mg wet weight) were incubated in 1.0 ml of culture medium instead of phosphate buffer for 2 hours at 15°.

Substrate	Fatty acid	Radioactivity in % C_1-C_9 fragment	C_{10}-C_{18} fragment	Position of the first double bond
Chlorella	18:3	70.9	29.1	9
	18:2	52.0	48.0	9
	18:1	50.8	49.2	9
	16:3	70.8	29.2	7
Candida	18:3	96.0	4.0	9
	18:2	53.1	46.9	9
	18:1	51.4	48.6	9

Table III. Incorporation of [1-^{14}C] and [U-^{14}C]oleate into intact Chlorella cells. Chlorella pyrenoidosa was grown in an acetate free medium under photoautotrophic conditions, 250 mg wet weight of cells were harvested and incubated with 1.0 ml of 0.2 M phosphate buffer, pH 7.4 containing approximately 1.0 µmole of [^{14}C]oleate (3-5 µCi). Incubations were for 48 hours at 25° and 1000 ft-c under aerobic conditions.

Substrate	$^{14}CO_2$ evolved nmoles	Radioactivity in newly formed fatty acids % 16:0	16:1	18:2	18:3
[U-^{14}C]oleate	9.1	6.3	6.2	75.0	12.5
[1-^{14}C]oleate	80.5	1.9	1.1	85.8	11.2

system was introduced into the hydrocarbon chain, carboxyl labeled hexanoic, octanoic, decanoic, dodecanoic, tetradecanoic and hexadecanoic acids were incubated with spinach tissue slices for 4 hours and the products were isolated, purified and degraded (Table V). Of interest, the most effective substrate for α-linolenic acid synthesis was dodecanoic acid with decanoic, octanoic and hexanoic acids less effective in that order. Tetradecanoic and hexadecanoic acids were ineffective as precursors. Since reductive ozonolysis of the C_{18} unsaturated fatty acids formed from these substrates showed

Table IV. Products of reductive ozonolysis of the C_{18} unsaturated fatty acids synthesized by intact Chlorella cells from [^{14}C]oleic acids.

Substrates	Fatty acid	Radioactivity in C_1-C_9 fragments %	Radioactivity in C_{10}-C_{18} fragments %
[U-^{14}C]oleate	18:3	100	0
	18:2	48.1	51.9
	18:1*	48.6	51.4
[1-^{14}C]oleate	18:3	92.6	7.4
	18:2	94.0	6.0
	18:1*	93.6	6.4

*Reductive ozonolysis of original substrate.

little significant β-oxidative degradation and resynthesis into the C_{18} acids, these substrates were directly elongated and modified to form the C_{18} unsaturated fatty acids. In addition, small amounts of [^{14}C] 12:3(3,6,9) were detected from [1-^{14}C] 12:0 as well as small amounts of [^{14}C] 16:3(7,10,13) from [1-^{14}C] 10:0. Earlier work with barley leaf tissue (9) had shown that tetradecanoic, hexadecanoic and octadecanoic acids were ineffective as precursors of α-linolenic acid, while octanoic, decanoic and dodecanoic acids served as substrates.

The evidence submitted in this and a previous communication (6), allows us now to propose the following pathways for the synthesis of the C_{18} unsaturated fatty acids:

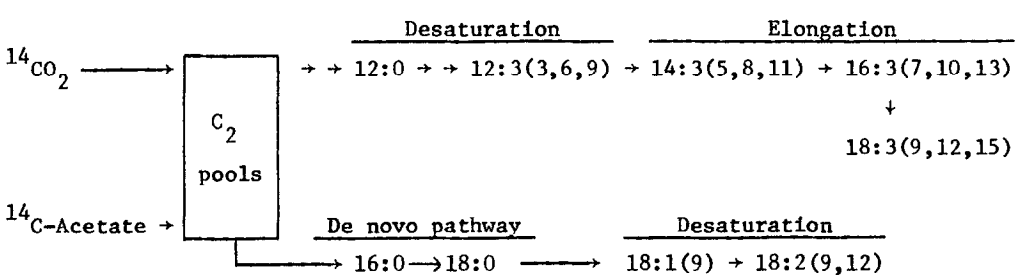

Table V. The incorporation of [1-^{14}C] fatty acids into leaf slices of spinach. Leaf pieces (1 mm^2 and 1.2 g wet weight) were incubated in 2.0 ml of water containing 250 nmoles of each substrate, 0.5 µmoles acetate, 200 µmoles Tris-HCl buffer, pH 7.9 and 30 µmoles $KHCO_3$. Incubations were performed for 4 hours at 15° and 1000 ft-c with shaking.

[1-^{14}C] Substrates	$^{14}CO_2$ nmoles	[^{14}C] isolated fatty acids nmoles	% Radioactivity in 8:0	8:1	10:0	12:0	12:1	12:2	12:3	14:0	14:3	16:0	16:3	17:0	18:1	18:2	18:3
6:0	5.6	24.7	0.8	2.8	4.5	5.3	—	2.8	7.3	4.0	5.7	31.2	--	4.0	18.2	11.7	1.6
8:0	0.4	21.9	--	2.3	13.7	5.0	7.8	--	--	8.7	--	37.4	1.8	--	15.5	5.9	1.8
10:0	2.7	34.8	--	--	--	3.7	--	--	--	3.2	—	41.7	0.6	--	29.6	13.8	7.5
12:0	0.7	7.0	--	--	--	--	—	—	1.4	12.9	--	42.9	--	--	21.4	11.4	10.0
14:0	0.8	11.6	--	--	--	--	--	--	--	--	—	62.1	--	--	24.1	13.8	--
16:0	0.3	0	--	--	--	--	--	—	--	--	--	—	—	--	--	--	--

This pathway predicts (a) a series of new desaturases which function at least on the C_{12} level, the earliest permissible substrate for the introduction of a trienoic non-conjugated cis double bond system and (b) a specific elongation system which is required for the construction of the C_{18} hydrocarbon chain from the proposed dodecatrienoic acid. A recent report on the presence of a specific system which hydroxylates only the C_8, C_{10} and C_{12} acyl CoAs to form βOH acyl CoAs may very well be related to this system (13). In addition, Allen et al. (14), have reported on the presence of 0.4% of 14:3 and 10.6% of 16:3(7,10,13) in lipids of spinach lamellae. We suggest that these trienoic acids are indeed the precursors of 18:3(9,12,15). Evidence in support of this pathway with a disrupted chloroplast system will be presented in a future communication.

ACKNOWLEDGMENTS

We are indebted to Dr. Herman Phaff of the Department of Bacteriology of this campus for yeast cultures and to Dr. Jack Preiss for the Chlorella culture. The authors wish to acknowledge the critical discussions of Drs. Jan Jaworski, Ward Shine and Miss Ann Sodja. Skillful technical assistance of Mrs. Barbara Clover is greatly appreciated.

REFERENCES

1. James, A.T., Biochim. Biophys. Acta 70, 9 (1963).
2. James, A.T., "Control of lipid metabolism," Proc. Biochem. Soc. Sym. 24, 17 (1963).
3. Harris, R.V. and James, A.T., Biochim. Biophys. Acta 106, 456 (1965).
4. Harris, R.V., Harris, P., and James, A.T., Biochim. Biophys. Acta 106, 465 (1965).
5. Hitchcock, C. and Nichols, B.W., "Plant Biochemistry," Academic Press (1971).
6. Jacobson, B.S., Kannangara, C.G., and Stumpf, P.K., Biochem. Biophys. Res. Commun. (1973), in press.
7. Hutner, S.H., Provasoli, L., Schatz, A., and Haskine, C.P., Proc. Am. Phil. Soc. 94, 152 (1950).
8. Deinema, M.H., Mededelingen van de Landbouwhogeschool te Wageningen, Nederland, 61, 1 (1961).
9. Hawke, J.C. and Stumpf, P.K., J. Biol. Chem. 240, 4746 (1965).
10. Appelqvist, L.A., Boynton, J.E., Stumpf, P.K., and von Wettstein, D., J. Lipid Res. 9, 425 (1968).
11. Kannangara, C.G. and Stumpf, P.K., Arch. Biochem. Biophys. 148, 414 (1972).
12. Stein, R.A. and Nicholaides, N., J. Lipid Res. 3, 476 (1962).
13. Harwood, J.L., Sodja, A., and Stumpf, P.K., Biochem. J. 130, 1013 (1972).
14. Allen, C.F., Good, P., Davis, H.F., Chisum, P., and Fowler, S.D., J. Am. Oil Chem. Soc. 43, 223 (1966).