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Elongation Pathway for α-Linolenic Acid Synthesis in Spinach Leaves

A REEXAMINATION[1]

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ABSTRACT

Leaf slices from spinach exhibited considerable variation in their incorporation of [^{14}C]bicarbonate and [1-^{14}C]acetate into fatty acids. Reductive ozonolysis studies indicated that all the ^{14}C-labeled fatty acids were synthesized *de novo*; no *in vivo* evidence was found for the previously proposed elongation of hexadecatrienoate to α-linolenate.

Although the location and pathway of synthesis of long chain saturated and monosaturated fatty acids in plant cells have been well characterized (7, 10, 12, 15), the nature of the final steps of the biosynthesis of polyunsaturated fatty acids has remained controversial. The original proposed pathway for their biosynthesis involved sequential desaturation of oleate → linoleate → α-linolenate (4). Many investigators have demonstrated that isolated chloroplasts synthesized large quantities of oleate but did not convert this acid to α-linolenate that makes up the bulk of the endogenous fatty acids in chloroplast lipids (15).

Using *Spinacia oleracea* leaf discs and disrupted chloroplast preparations, this laboratory observed that the labeling pattern of the α-linolenate formed from [1-^{14}C]acetate was inconsistent with its synthesis from oleate (5, 6, 8, 9). Stromal enzymes from disrupted spinach chloroplasts readily elongated $\Delta^{7,10,13}$ hexadecatrienoic acid to α-linolenic acid (5, 6). With spinach leaf tissue, carboxyl-labeled fatty acids were converted into α-linolenic acid only if their chain length was 12 carbon atoms or less (10). It was therefore postulated that a specific elongation system existed for the conversion of medium chain ($<C_{12}$) trienoic fatty acids to α-linolenic acid in the leaf cell. In the light of subsequent *in vivo* studies with *Cucumis sativus* (11), *Zea mays* (Hawke JC, PK Stumpf, unpublished results) and *Spinacia oleracea*, we have reappraised the contribution of the putative elongation pathway to α-linolenic acid biosynthesis. Our evidence now suggests that the principal mechanism for α-linolenic acid production involves a sequential desaturation of C_{18} fatty acids and is in agreement with the earlier results of Harris and James (4).

MATERIALS AND METHODS

Plant Material. Seedlings of *Spinacia oleracea* L. were grown hydroponically in a nutrient medium as described in (10). The plants were kept in foil-wrapped 3-liter jars under constant aeration and illuminated by a bank of G.E. FI5T8D 15 w fluorescent strips supplemented by 100-w tungsten bulbs. Leaves were selected for incubation after 6 to 8 weeks. The leaves were excised and floated in distilled H_2O for 1 h in the light prior to incubation with lipid precursors. Alternatively, the seedlings were kept in a Vermiculite medium after germination and grown for 6 to 8 weeks. The seedlings were watered every other day with half-strength hydroponic nutrient medium. Store-bought spinach was obtained from a local market and kept in a cold cabinet at 4 C until required. Both Vermiculite-grown and store-bought leaves were also floated on water for a 1 h period of illumination before incubation.

Reagents and Substrate. ^{14}C-Bicarbonate (40–60 Ci/mol) and [1-^{14}C]acetate (40–60 Ci/mol) were purchased from New England Nuclear. All solvents were reagent grade.

Incubation of Tissue. Leaves were deribbed and sliced into strips (1 mm × 2 cm). Each treatment consisted of 0.5 to 1 g tissue incubated with 10 μCi [1-^{14}C]acetate (0.2 μmol) or 100 μCi [^{14}C]bicarbonate (2 μmol) (8) in 2 ml of a medium containing 50 μmol K-phosphate buffer at pH 7.9. Incubations were performed in an illuminated water bath at 20 C and were halted after 4 h by extraction into hexane-isopropyl alcohol 3:2 (v/v) (11).

Lipid Extraction and Analysis. Total lipid mixtures were extracted in hexane-isopropyl alcohol, derivatized with diazomethane and methyl esters separated by GLC as previously described (12).

Methyl esters were separated on the basis of chain unsaturation by TLC on activated Silica Gel H plates containing 5% $AgNO_3$ which were developed in hexane-diethyl ether, 80:20 (v/v). Location of the separated methyl esters was with reference to corresponding unlabeled standards containing saturated, mono-, di-, and trienoic methyl esters. Separations were also checked by means of a Packard model 7201 radiochromatogram scanner. To determine their fatty acid labeling patterns, separated methyl esters were then analyzed by reductive ozonolysis according to the method of Stein and Nicolaides (14). The C_9 aldehyde ester fragments from reductive ozonolysis of C_{18} unsaturated fatty acid methyl esters were separated on a 10% DEGS-PA GLC column from the C_{10}–C_{18} fragments containing C_9 aldehyde (from oleate), C_6 aldehydes, and C_3 dialdehydes (from linoleate) and C_3 aldehydes and C_3 dialdehydes (from α-linolenate). Due to the high volatility of the C_{10}–C_{18} fragments, and in particular malonyl dialdehyde, it was necessary to prevent solvent evaporation before injection onto the GLC column. Samples not stored on ice prior to injection were found to be deficient in label in the C_{10}–C_{18} fragments presumably mainly because of loss of malonyl dialdehyde by volatilization. These precautions were also observed in the earlier investigation (5, 6).

RESULTS AND DISCUSSION

Jacobsen *et al.* (6) reported a considerable variability in the capacity of disrupted spinach chloroplast preparations to synthe-

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size α-[^{14}C]linolenic acid from [1-^{14}C]acetate. The variability was reduced if highly concentrated chloroplast preparations were "aged" by incubation for several hours at 2 C. We observed marked variations in the capacity of spinach leaf slices to incorporate both [^{14}C]bicarbonate and [1-^{14}C]acetate into fatty acids. Store-bought leaves showed 1.5- to 4-fold higher rates of fatty acid biosynthesis from the labeled precursors than did hydroponically grown leaves. This differential capacity for fatty acid synthesis increased during the storage at 2 C of store-bought leaves; the hydroponic leaves were always used fresh from the plants. Later experiments showed some variation between different batches of store-bought leaves and between hydroponically grown and Vermiculite-grown leaves, with the latter exhibiting a higher capacity for synthesis of polyunsaturated fatty acid.

This variation in over-all fatty acid biosynthesis was also reflected in the labeling patterns of the fatty acids. The store-bought leaves were always able to synthesize 2 to 17% more polyunsaturated fatty acid than the hydroponically grown leaves. This effect was magnified if longer incubation times were employed. However, both types of spinach leaf synthesized the same range of fatty acids from both [^{14}C]bicarbonate and [1-^{14}C]acetate. Storage at 2 C of the store-bought leaves had little effect on the proportions of the labeled fatty acids synthesized, in spite of the higher over-all rates of fatty acid synthesis. In all cases during time course studies, ^{14}C label appeared in the C_{18} fatty acids in a sequential manner suggestive of a progressive desaturation from oleate → linoleate → α-linolenate.

Table I demonstrates that in all cases, regardless of tissue age, origin, or fatty acid precursor, all of the C_{18} fatty acids were uniformly labeled. At no time was a preponderance of counts observed in the C_1–C_9 reductive ozonolysis fragments that would suggest a chain elongation sequence. In particular, α-linolenic acid was invariably labeled in a similar manner to the other C_{18} fatty acids, in contrast to the earlier findings (5, 6).

We also observed that conditions such as darkness or anaerobiosis inhibited the formation of oleic and linoleic acid and thereby also affected α-linolenic acid synthesis. It has already been demonstrated that spinach leaves fed [1-^{14}C]oleate and [1-^{14}C]linoleate will produce [^{14}C]linolenate (3), although the authors did not check the products by reductive ozonolysis and so did not convincingly demonstrate a direct synthesis of [^{14}C]linolenate from the labeled substrates. As early as 1965, however, the direct conversion of [1-^{14}C]oleate to [1-^{14}C]linoleate and [1-^{14}C]linolenate was observed in both *Ricinus gibsonii* and *Lactuca sativa* leaf preparations (4) and in 1974 *Pisum sativum* leaves were shown to have a similar activity (16). More recently, we have demonstrated the desaturation of exogenous [1-^{14}C]oleate and [1-^{14}C]linoleate to [1-^{14}C]linolenate in greening cucumber cotyledons (11) and in developing maize leaves (Hawke JC, PK Stumpf, unpublished results). In both cases, α-linolenic acid retained the carboxyl-labeling position of the substrate fatty acids.

Table I. *Products of Reductive Ozonolysis of the C_{18} Unsaturated Fatty Acids Synthesized by Spinach Leaf Slices from ^{14}C-Lipid Substrates*

	Fatty acid	Radioactivity in	
		C_1–C_9 fragments	C_{10}–C_{18} fragments
		%	
[^{14}C]Bicarbonate			
Fresh store-bought leaves	18:1	52.2	47.1
	18:2	51.1	48.9
	18:3	47.9	52.1
Hydroponically grown leaves	18:1	51.5	48.5
	18:2	60.0	40.0
	18:3	47.8	52.1
[1-^{14}C]Acetate			
Fresh store-bought leaves	18:1	52.5	47.5
	18:2	52.1	47.9
	18:3	53.2	46.8
Hydroponically grown leaves	18:1	51.9	48.1
	18:2	52.4	47.6
	18:3	49.1	50.9
Vermiculite-grown leaves	18:1	52.5	47.5
	18:2	59.1	40.9
	18:3	58.3	41.7

There is now, therefore, a considerable body of evidence from a large number of different plant species that supports the proposal of a progressive desaturation of oleate → linoleate → α-linolenate as being the primary mechanism for the generation α-linolenate *in vivo*. To date it has not been possible to isolate the linoleoyl desaturase system from any plant tissue, despite a great deal of effort. Thus, the direct *in vitro* demonstration of α-linolenate biosynthesis has yet to be made. Although linoleate desaturation is without doubt the principal way in which α-linolenate is synthesized, other minor pathways cannot be definitely excluded. Spinach leaves are unique in their relatively high hexadecatrienoic acid content, with this fatty acid forming about 10% of the total endogenous fatty acid (1). It is possible that free hexadecatrienoic acid, released by galactolipase activity in "aging" preparations or damaged leaves, may be elongated to α-linolenic acid by a nonspecific mechanism (17). In the presence of [^{14}C]bicarbonate and [1-^{14}C]acetate this would give rise to ^{14}C-labeled α-linolenate independently of oleoyl and linoleoyl desaturation. We did not observe such a reaction with slices of spinach leaves presently available and were unable to demonstrate any nonspecific (acyl carrier protein-independent) elongation reactions from C_{16} fatty acids to C_{18} fatty acids in these tissues.

However, our data demonstrate the enormous variability that occurs in different types of spinach leaf with respect to fatty acid biosynthesis. It is possible that in certain unusual circumstances the majority of the α-linolenic acid may arise from hexadecatrienoic acid and this process may be peculiar to spinach, among the higher plants. Indeed, it has been demonstrated that growing cultures of the mold *Penicillium chrysogenum* have the capacity for both linoleate desaturation and hexadecatrienoate elongation as means of generating α-linolenate, with the elongation reaction occurring preferentially under anaerobiosis when desaturation is not possible (13). Very recently Bedford *et al.* (2) described the formation of α-linolenic acid by two pathways in cells of the thermophilic alga *Cyanidium caldarium* grown at 20 C, the major one being the sequential desaturation pathway and the minor one, an elongation of preexisting hexadecatrienoic acid.

We suggest that the principal fatty acid of photosynthetic tissue, α-linolenic acid, is produced in the leaf by sequential desaturation of C_{18} fatty acids proposed first by Harris and James (4). Although *in vitro* observations by Jacobson *et al.* (6) and by Vance and Stumpf (17) supported the elongation pathway, results reported in this communication with intact spinach leaf tissues suggest that the elongation pathway, at the most, is a minor pathway.

Since procaryotic organisms do not synthesize linoleic and linolenic acids, the variations observed in the 1972 to 1973 experiments and those reported here can not be ascribed to bacterial contamination.

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