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Efficient Production of Biosynthetically Labeled Fatty Acids[1]

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Abstract

By short-term exposure of a photosynthesizing oilseed plant at seed-setting stage to high levels of $C^{14}O_2$, radioactivity is efficiently incorporated into glycerides yielding randomly labeled fatty acids of high specific activity.

Introduction

BIOSYNTHETIC METHODS for labeling fatty acids in seeds of higher plants have involved their culture from seedling stage to maturity in the presence of a radioactive isotope (3). This procedure assures randomness of labeling; however, there are two disadvantages: a) fatty acids have very low specific activities, necessarily limited by the lethal levels of radiation, and b) providing a plant with a continuous supply of an isotope during the growth period increases production costs of the desired fatty acids by labeling the undesired leaf and stem portions of the plant.

An efficient procedure has been developed to produce high specific activity, randomly labeled fatty acids by short-term exposure of a nearly mature plant to levels of radioactivity that would be lethal in the continuous culture method.

Experimental

Production of Labeled Fatty Acids. Several species of oilseed plants were employed for biosynthetic labeling at various times, including flax, soybean and safflower. The use of a perilla plant *(Perilla frutescens)* is described here because its fatty acid composition is favorable for the isolation of labeled linolenate (6). The plant was grown in a pot, buried in the ground with the top level with the surface, for five months to the seed-setting stage under otherwise ordinary field conditions. It was then exposed to radioactive carbon dioxide of high specific activity in a closed system (Fig. 1) as described by Burris et al. (2), but with modifications. The pressure in the system was reduced by 2 cm to prevent leaks to the outside; the pressure increased only slightly during the exposure of the plant.

$C^{14}O_2$ (0.5 mc in 0.34 cc gas) was generated into the glass exposure vessel (volume was 36 liters) by the reaction of 3 mg Ba $C^{14}O_3$, specific activity of 32.8 mc/mmole, with 3 ml 30% perchloric acid. Photosynthetic uptake of CO_2 was stimulated by irradiating with twenty-six 20-w fluorescent tubes cylindrically arranged about the vessel. To counteract the heating effects of the lights, tap water was sprayed on the top and sides of the vessel. Absorption of $C^{14}O_2$ by the plant was monitored by a continuous-flow ion chamber in a recycling pumping system. A 2-hr period of illumination was required for absorption of 90% of radioactivity; illumination was continued for an additional 2 hr. Figure 2 is the ion current recording showing the generation and loss of $C^{14}O_2$. During a 12-hr dark period the plant, maintained in the exposure chamber with the pumping system off, respired carbon dioxide. As shown in the lower curve of Fig. 2, the radioactivity respired amounted to 30% of the original dosage and required an additional 3.5-hr of illumination for reabsorption. Before returning the plant to natural light conditions, the chamber was flushed with air and the plant was enclosed in a battery jar with a loose fitting lid to ensure recovery of any leaves which might drop off.

After allowing 12 days for maturing, the seeds (1.06 g) were harvested, and the lipids were extracted by crushing them in a micro-Waring Blendor with 20 ml diethyl ether. The ether solution was decanted, filtered, dried over anhydrous Na_2SO_4, and filtered again. Two additional 20-ml ether extractions were made with the volumes added together. Ether was removed *in vacuo* on a rotary pump. The wt of lipids recovered was 371 mg, corresponding to a 35.1% yield; the meal weighed 615 mg.

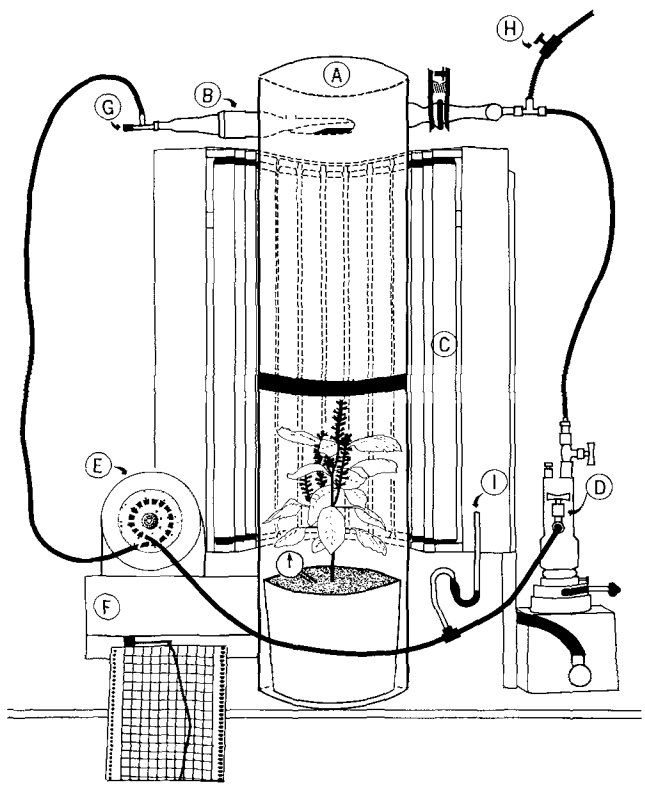

FIG. 1. Gas exposure system: A, sealed exposure vessel; B, $C^{14}O_2$ generator; C, fluorescent tubes: D, continuous-flow ion chamber; E, recycling pump; F, recorder; G, rubber injection septum; H, vacuum line; I, open-end manometer.

[1] Presented at the AOCS Meeting in Atlanta, 1963.
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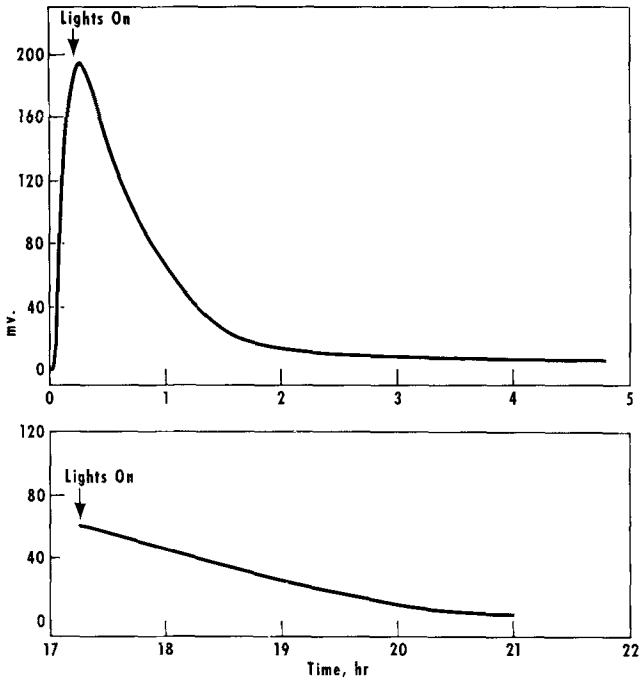

FIG. 2. Ionization current monitoring of radioactivity in chamber during exposure of perilla plant.

Analysis of Lipids. Ether-soluble lipids were transesterified by the sodium methoxide-methanol procedure (9). The methyl esters recovered weighed 314 mg. Their radioactivity was assayed in a liquid scintillation spectrometer [solvent: toluene containing 4 g, 2,5-diphenyloxazole plus 30 mg, 1,4-bis-2-(5 phenyloxazole)-benzene/liter]. The esters were also analyzed by gas-liquid chromatography (GLC) on a 6 ft, 20% DEGS on 60/80 mesh Chromosorb W column at 200C with a helium gas flow of 30 cc/min.

A partially vulcanized rubber column with 88% aqueous-acetone as the moving phase (7) was used to fractionate the esters, which yielded 134 mg 99% pure methyl linolenate as determined by GLC. In addition, six other fractions containing a mixture of the remaining fatty esters were recovered. The seven fractions were radioassayed and individually injected into a GLC-radioactivity system previously developed at the Northern Laboratory (4).

The fraction collected over the oleate-palmitate peak was diluted with an equal wt of pure methyl

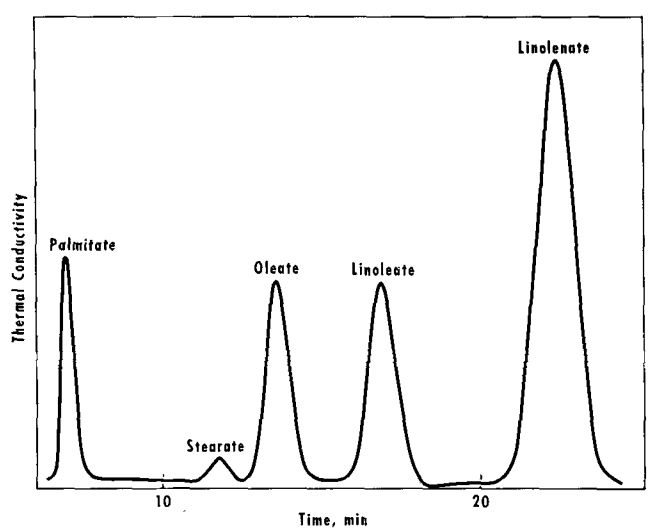

FIG. 3. Gas chromatogram of C^{14}-labeled methyl esters from a 6 ft x ¼ in. column packed with 20% DEGS on Chromosorb W and operated at 200C, 30 cc helium/min.

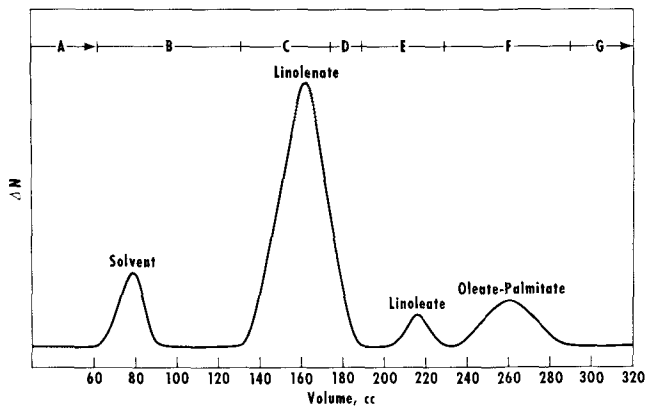

FIG. 4. Liquid-liquid partition chromatogram of C^{14}-labeled methyl esters from partially vulcanized rubber column, 88% aqueous-acetone moving phase. Samples separated into seven fractions, A through G.

oleate to provide sufficient sample wt. This was saponified, converted to the acids, and then oxidized with permanganate-periodate solution (8). The methyl esters of the cleavage products were recovered, assayed and gas chromatographed with the effluent esters also being assayed.

Analysis of Meal and Plants. Seed hulks and shells (615 mg), recovered after extraction of the lipids, and the leaves and stem of the plant (3.42 g) were analyzed, after grinding, for radioactive carbon. Combustion of 20–80 mg samples of these materials was performed in an oxygen flask with IR ignition (1). After combustion, the flask was cooled to room temp, and 10 ml hyamine hydroxide solution (10) was then injected through a rubber septum in a side arm. The flask was shaken vigorously and then allowed to stand for one-half hr to ensure complete absorption of $C^{14}O_2$. Aliquots of these solutions were assayed in a liquid scintillation spectrometer.

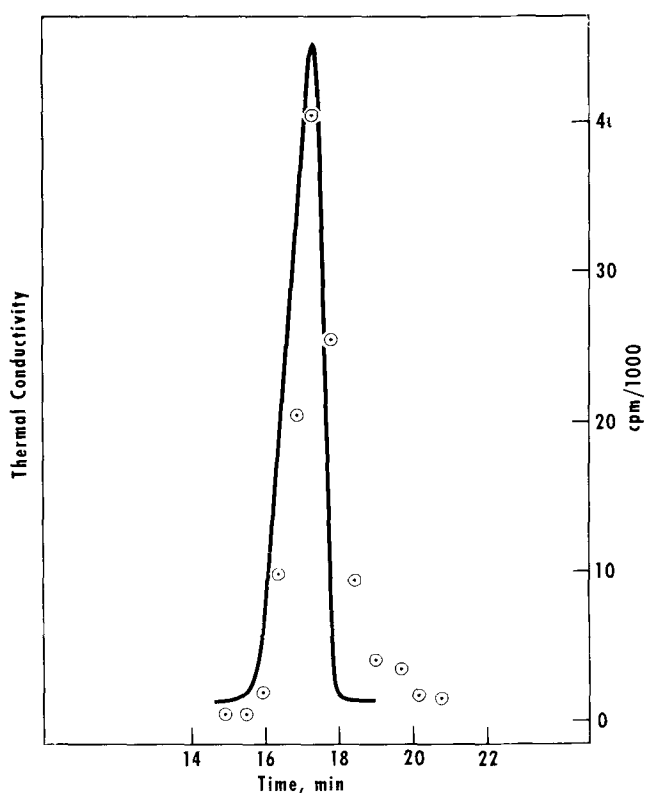

FIG. 5. Gas chromatogram of Fraction C from 6-ft column, with automatic collection of effluent esters in scintillation solvent. Scintillation assay = ⊙.

TABLE I
Composition and Radioactivity of C^{14}-Labeled Methyl Esters and Fractions Separated by Reverse-Phase Partition Chromatography

Fraction	Total recovered wt, %	Fatty acid composition, %					Specific activity μc/mg	Radioactive content μc
		Steric	Palmitic	Oleic	Linoleic	Linolenic		
C^{14}-Labeled esters		1.7	9.3	15.2	19.1	54.7	0.59	186.8
A	4.4	....	....	....	....	....	0.01	0.1
B	8.4	....	....	....	....	....	0.05	1.3
C	44.2	....	....	....	....	100.0	0.64	85.5
D	10.5	....	....	....	8.7	91.3	0.53	16.9
E	17.2	....	....	....	78.1	21.9	0.56	29.2
F	11.9	....	36.6	63.4	Trace	....	0.60	21.7
G	3.4	....	....	....	....	....	0.60	6.3

Results and Discussion

The C^{14}-labeled methyl esters contained 187 μc and had a specific activity of 0.6 μc/mg. The percentage composition of the methyl esters, obtained by integrating the areas under the thermal conductivity curve (Fig. 3), shows in Table I.

All seven fractions collected from the reversed phase chromatograph of the methyl esters, as indicated in Figure 4, were assayed and gas chromatographed. The results of the chromatographic analyses show in Table I.

The specific activities of Fractions C through G (Table I) are approx equal, indicating a substantially even distribution of radioactive carbon atoms among the fatty acids. Fractions A and B are impurities and decomposition products.

A single thermal conductivity peak was eluted in the gas chromatographic analysis of Fraction C (Fig. 5). Scintillation assay of the effluent esters indicates only one radioactive peak; this single peak coincides with the elution of the thermal conductivity peak. Thus the sample, as determined by GLC, is both chemically and radioactively pure. Fraction C accounts for 44.2% of the total wt of the recovered methyl esters. Fraction D, which contained 10.5% of the recovered wt, was also high in linolenate content. Purification of Fraction D would increase the yield of methyl linolenate to approx the percentage in the original methyl esters.

The cleavage products from the oxidation of Fraction F were analyzed with a temp-programmed gas chromatography-radioactivity instrument (4) (Fig. 6). Areas under the peaks of the thermal conductivity curve, as determined by planimetric methods, are assumed to be directly related to the wt of the individual constituents they represent. Therefore, the relative activity/carbon atoms is calculated by the following expression:

$$\frac{\text{CPM}}{1{,}000} \times \frac{\text{Mol wt}}{\text{Area}} \times \frac{1}{\text{C atoms/mole}}$$

CPM is the sum of the counts/min of the fractions collected over the specified thermal conductivity peak. The calculations for each constituent appear in Table II. The consistency of the relative activity/carbon atom indicates random labeling in the carboxyl and alkyl portions of the oleic acid molecule.

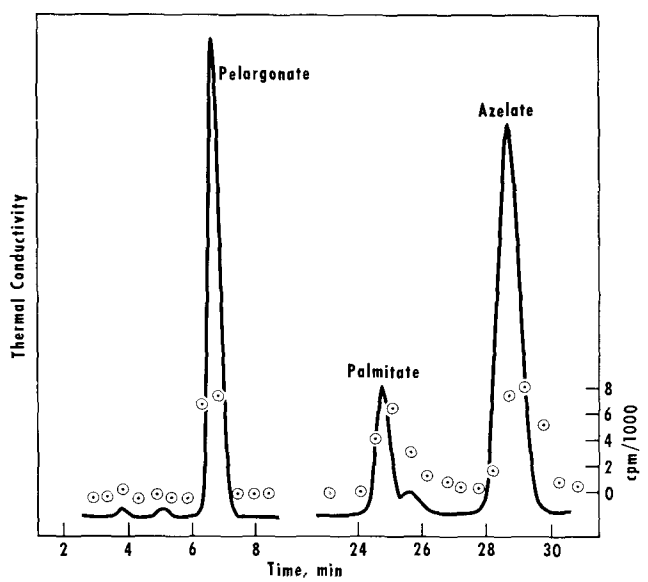

FIG. 6. Gas chromatogram of methyl esters of oxidatively cleaved acids of Fraction F, with permanganate-periodate solution, from a 6-ft column and temp programming from 140–200C.

TABLE II
Relative Activity of Products Resulting from Oxidative Cleavage of Fraction F

	Monobasic		Dibasic
	C_{16}	C_9	C_9
Activity/mole	104,500	67,700	67,500
Activity/C atom	6,540	7,520	7,500

In addition to the 187 μc of C^{14} incorporated into lipids, assays by oxygen flask combustion indicated that 82.5 μc had been incorporated into the meal and 168 μc in the leaves and stem. Of the 500 μc of radioactivity initially supplied, 87.5% was accounted for. Of this, 42.8% was deposited in fatty reserves of the seed; the remainder of the assimilated radioactivity was in the meal (18.8%) and in the leaf and stem (38.4%). In biosynthetic methods involving continuous exposure to $C^{14}O_2$, all portions of the plant are labeled approx equally; in the present method the specific activity of the methyl esters (0.6 μc/mg) is ten times as great as that of the undesired carbohydrate portions of the plant (0.06 μc/mg). Also, the specific activity of the fatty esters is ten times higher than the esters previously produced from soybeans (5) that were cultured from seedlings to maturity in the presence of $C^{14}O_2$. By exposing intact higher plants at seed-setting stage to high levels of radioactivity for short periods of time, fatty acids of relatively high specific activity are obtained and a comparatively small amt of radioactivity is lost in undesired portions of the plant.

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