Desaturation of fatty acids in seeds of higher plants

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ABSTRACT Photosynthesizing flax, soybean, and safflower plants were exposed to $^{14}CO_2$ at seed-setting stage for a 1 hr period. Seed was sampled periodically thereafter and the lipids were extracted. A triglyceride-rich fraction was methanolyzed; the resultant methyl esters were analyzed by gas–liquid chromatography and assayed for radioactivity.

Of the C_{18} unsaturated acids, oleic was the first to acquire radioactivity, which subsequently and successively appeared in linoleic and linolenic acids. The shapes of the radioactivity–time curves provide evidence that consecutive desaturation reactions occur in the seeds of these higher plants.

KEY WORDS desaturation · oleic acid · linoleic acid · triglycerides · seeds · flax · soybean · safflower · biosynthesis · linolenic acid · $^{14}CO_2$

THE PATHWAYS of fatty acid biosynthesis from low molecular weight precursors in tissue slices, homogenates, and excised parts of plants are receiving increasing attention, particularly with the use of radioactive tracers. Simmons and Quackenbush (1) obtained indications that oleic acid might be the first unsaturated C_{18} fatty acid formed by immersing slices of green soybean cotyledons in labeled acetate. Using cotyledon slices of castor beans, Coppens (2) reported that the labeled acetate precursor found its way primarily into C_{18} fatty acids, but that the addition of glucose resulted in C_{14} and C_{16} acids being most active. Newcomb and Stumpf showed (3) that labeled acetate is the precursor of fatty acids in peanut cotyledons and they and their coworkers also studied the formation of saturated and unsaturated C_{18} acids by avocado particles (4, 5). Gibble and Kurtz (6) observed the synthesis of long-chain fatty acids through multiple condensation of acetate by floating flax flowers on an acetate-1-^{14}C solution. Eberhardt and Kates (7) and also James (8), working with excised leaves of runner bean and castor plants, respectively, found that acetate-^{14}C is incorporated into long-chain fatty acids.

In recent work James concluded that oleic acid is the precursor of linoleic acid in leaves, which is in agreement with the pathway found by Yuan and Bloch for yeast (9); but James observed only a slow conversion of linoleic to linolenic acid (8, 10). Isolated chloroplast suspensions from lettuce, although capable of carrying out syntheses of long-chain fatty acids, did not desaturate oleic to linoleic to linolenic acid, according to Stumpf and James (11). McMahon and Stumpf (12) have now reported a cell-free system from safflower seeds which converts oleoyl CoA to linoleic acid.

Changes in composition of unsaturated fatty acids during maturation has most recently been studied by McKillican and Sims (13) who also reviewed the earlier literature; however, no study of the kinetics of biosynthesis in seeds of the intact plant using ^{14}C has yet been described (14).

In experiments described here, photosynthesizing soybean, safflower, and flax plants at the seed-setting stage were exposed to $^{14}CO_2$ and the fatty acid composition and radioactivity of the fatty acids of the seed glycerides measured at various intervals of time. The experiments provide kinetic evidence that the desaturation reactions, oleate to linoleate to linolenate, occur readily in the seeds of higher plants.

EXPERIMENTAL PROCEDURE

Growth of Plants

Flax, soybeans, and safflower were planted in 8-inch pots and grown indoors during the winter. Daylight

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illumination was fortified and extended by a 16 hr/day exposure from fluorescent lamps as described in a later section. These plants arrived at the seed-setting stage after approximately 3 months and had normal growth characteristics.

Exposure to $^{14}CO_2$

The exposure chamber used for soybeans and flax has been previously described (15). For safflower a somewhat different exposure system was used. The stem and lower leaves of the plant were inserted in 3-inch diameter glass tubing. Bottom and top ends of the tube were closed with drilled and slit rubber stoppers, and the stem was sealed in the stopper hole with Apiezon high vacuum plastic. Because the safflower plant protruded above the upper stopper, seed clusters were readily available for sampling. Ion-chamber monitoring of the radioactivity in gas phase was also provided.

Sampling

At various time intervals following the 1 hr exposure to radioactive CO_2, a sample of seeds of uniform maturity was selected and removed from the plant. For flax, six capsules served as the sample; for soybeans, two green immature beans; and for safflower, the contents of one head which amounted to approximately 10 seeds.

Extraction, Methanolysis, Liquid Partition, and Assay

Seed samples removed at specified intervals were ground and extracted in a glass tissue grinder with 20 ml of absolute methanol. The extract was filtered and then dried by passing it through a column of sodium sulfate. After the addition of 0.5 ml of sodium methylate solution (0.4% Na in methanol), the extract was heated to reflux temperature and 10 ml of the methanol was distilled off. Glacial acetic acid (0.5 ml) was then added to inactivate the catalyst and 10 ml each of water and petroleum ether were added in a separatory funnel. The lower alcoholic layer was drawn off in a 25 ml volumetric flask and made up to volume with methyl alcohol. For radioassay, 0.5 ml of this solution, 6 ml of methanol, and 8 ml of scintillation solvent (4 g/liter of 2,5-diphenyloxazole in toluene) were placed in duplicate scintillation vials. The hexane layer, after being washed with two 10-ml portions of water, was made to 25 ml and assayed for radioactivity by placing 1 ml of the methyl ester solution in 15 ml of scintillation solvent.

Methyl esters in the hexane-soluble fractions come largely from the triglycerides. Free fatty acids react with the sodium methoxide to form soaps; therefore, they would not be methylated under the conditions of methanolysis.

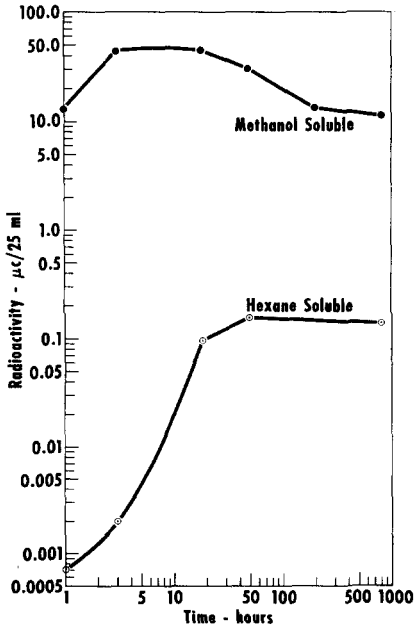

FIG. 1. Changes in radioactivity of methanol- and hexane-soluble fractions of linseed oil.

Gas Chromatography–Radioactivity Analysis

The samples from the hexane layers, after evaporation and weighing, were analyzed by a gas chromatograph equipped with a 6 ft column of 20% diethylene glycol succinate polyester on Chromosorb W (60–80 mesh) at 190°C and a thermal conductivity detector. Radioactive effluents were condensed in the scintillation solvent according to the automatic technique previously described (16). The percentage composition figures for methyl esters were calculated by planimetric integration of the area under the thermal conductivity peaks; quantitative results with National Heart Institute Fatty Acids Standards E and D agreed with the stated composition with a relative error of less than 9% for major components and less than 11% for minor components. To obtain relative specific activities, the percentage radioactivities at each time interval were divided by the percentage methyl ester composition as determined by thermal conductivity.

RESULTS

Changes in the radioactivity of methanol- and hexane-soluble fractions of linseed with time are shown in Fig. 1. After 1 hour's exposure to $^{14}CO_2$, the methanol-soluble fraction reached a maximum radioactivity after about 5 hr and then decreased. The hexane-soluble layer, which consists of the methyl esters derived from the glycerides, showed only slight radioactivity after 1 hr, and increased to a constant value. This behavior is similar to that described by Zilversmit (17) for the model case in which the specific activity of the precursor decreases exponen-

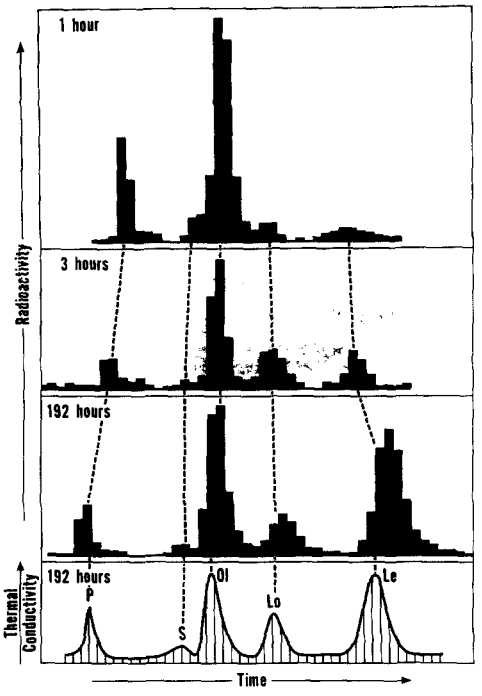

FIG. 2. Mass (by thermal conductivity detector) and radioactivity analyses for the gas chromatographic separation of 1-, 3-, and 192-hr samples of linseed esters. *P*, palmitic, *S*, stearic, *Ol*, oleic, *Lo*, linoleic, and *Le*, linolenic.

tially with time. Curves comparable to these, but for soybeans, have also been obtained.

Gas chromatography–radioactivity data for linseed extracts are presented in Fig. 2. The mass elution curve for the 192 hr sample is at the bottom of the figure. Since the analysis for other sampling periods did not change, the curves are not shown. The bar graph at the top of the figure illustrates the radioactivity analysis of fractions collected from the 3 hr sample at 30-sec intervals. The major peaks for radioactivity correspond to methyl palmitate and to methyl oleate, with only slight amounts of radioactivity in the methyl linoleate and methyl linolenate. The radioactivity data for 3 and 192 hr in Fig. 2 support the concept that the radioactivity of the linoleate fraction is increasing at the expense of the oleate radioactivity and that it "spills over" subsequently into the linolenate fraction. Further, it is also apparent that at 192 hr the distribution of radioactivity is approximately that of the mass curve. Data for stearate have been calculated but are not presented; they are subject to large error because of their small proportion and the inadequate resolution of the gas chromatographic peak.

Data in Fig. 3 further support the indications given in Fig. 2. The percentage of radioactivity in methyl oleate decreased from an initially high value to approximately that in the mass analysis. Linoleic acid goes through a maximum before approaching its equilibrium value, whereas linolenic acid radioactivity increased with time to achieve it.

The relative specific activity data of Fig. 4, calculated from those of Fig. 3 by dividing the radioactivity percentage by the weight percentage, show that all fatty acid methyl esters approach a steady-state specific activity level of 1; oleate and palmitate approach this equilibrium from high values; linolenate rises from zero to a limiting value; and linoleate increases from zero more rapidly and goes through a slight maximum at 3 hr before approaching 1.

Soybean data, because of the lower content of linoleic acid in the oil, are less dramatic than data for flax. However, from the gas chromatography–radioactivity curves in Fig. 5, it is apparent that most of the radioactivity again appears in oleate and palmitate at an early stage and that the radioactivity curves eventually assume the general shape of the corresponding mass curves.

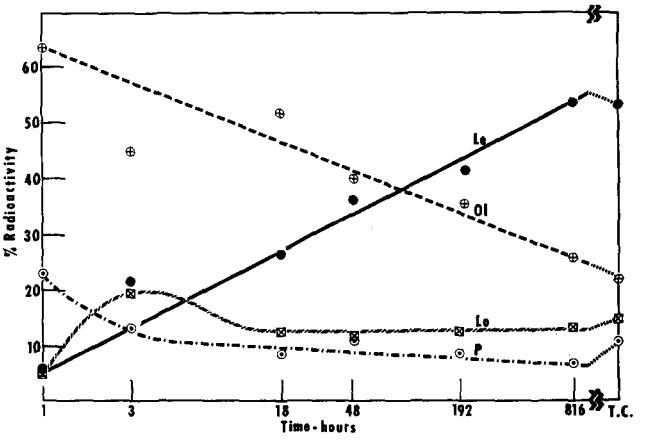

FIG. 3. Radioactivity of methyl esters (derived from triglycerides) of linseed oil. Final points represent the fatty acid composition determined by the thermal conductivity detector (*T.C.*). Abbreviations as in Fig. 2.

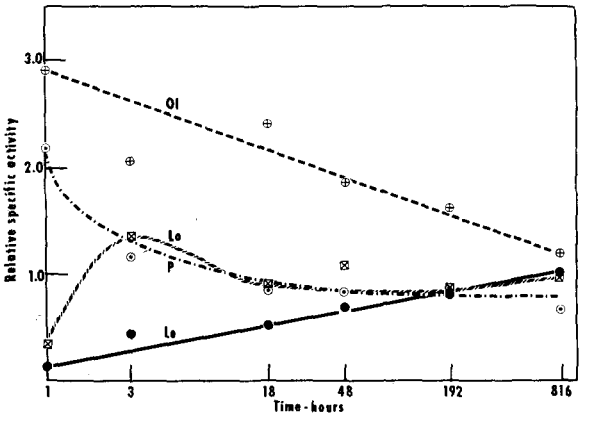

FIG. 4. Relative specific activity of methyl esters derived from glycerides of linseed oil at various times after exposure of plant to $^{14}CO_2$. Abbreviations as in Fig. 2.

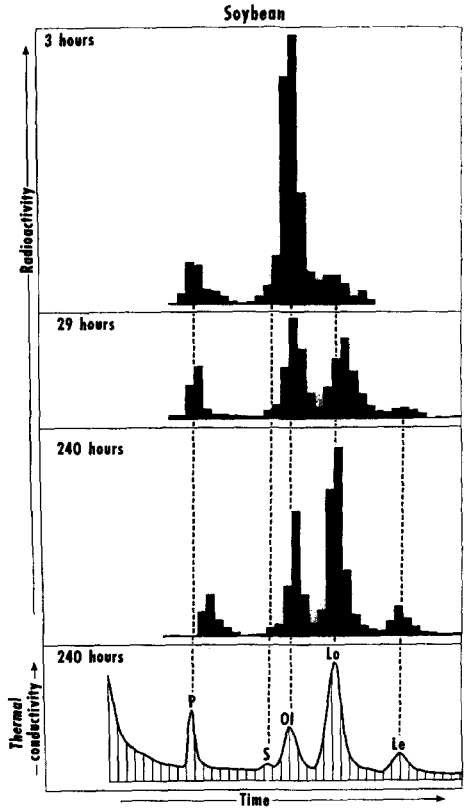

FIG. 5. Mass (by thermal conductivity detector) and radioactivity analyses for the gas chromatographic separation of 3-, 29-, and 240-hr samples of soybean esters. Abbreviations as in Fig. 2.

The specific activity for oleate decreases from a ratio of 4 to a ratio of 1, whereas the activities for linoleate and linolenate increase from 0 to 1 in 240 hr.

Data on the *safflower* are not as complete as for soybean and flaxseed. The gas chromatography–radioactivity curves for two samples at 1 and 24 hr are recorded in Fig. 6. It is apparent that, in safflower, palmitate and oleate initially have high percentages of radioactivity and then decrease with time, that linoleate activity increases, and that at 24 hr the radioactivity curve approaches the thermal conductivity curve in shape and therefore in composition.

DISCUSSION

The analysis of kinetic data from the isotope experiments described is in conformity with earlier results cited above on yeast, chloroplast suspensions, and excised plant parts but indicates further that in the intact soybean, safflower, and linseed plants, unsaturated C_{18} fatty acids are being formed by consecutive desaturation reactions beginning with oleic acid.

$$\underset{(A)}{\text{Oleic}} \xrightarrow[-H_2]{k_a} \underset{(B)}{\text{Linoleic}} \xrightarrow[-H_2]{k_b} \underset{(C)}{\text{Linolenic}}$$

This analysis supplies evidence for desaturation steps such as was sought by James in experiments on excised leaves (10) and by Stumpf and James with chloroplast suspensions (11).

Carbon dioxide is reduced photosynthetically in leaves to sugars and low molecular weight compounds, and these are subsequently translocated to seed portions of a plant. This translocation is particularly evident with safflower, where the seed clusters themselves did not come in direct contact with the radioactive CO_2. In 1 hour's time the photosynthate had migrated through the phloem cells into the maturing safflower seeds. The rapid increase in radioactivity of the methyl alcohol-soluble fractions of flax, safflower, and soybeans is in accordance with the concept that polar low-molecular weight compounds are rapidly translocated to seed tissues. The concentration of these materials then falls off with time, in the manner described mathematically by Zilversmit (17), while radioactivity in the product increases rapidly to a maximum and then falls off.

Evidence for the consecutive desaturations of oleic acid is apparent in Figs. 2, 5, and 6, since in the C_{18} acids radioactivity appears first in oleic acid, then increases in linoleic acid, and finally enters linolenic acid. The

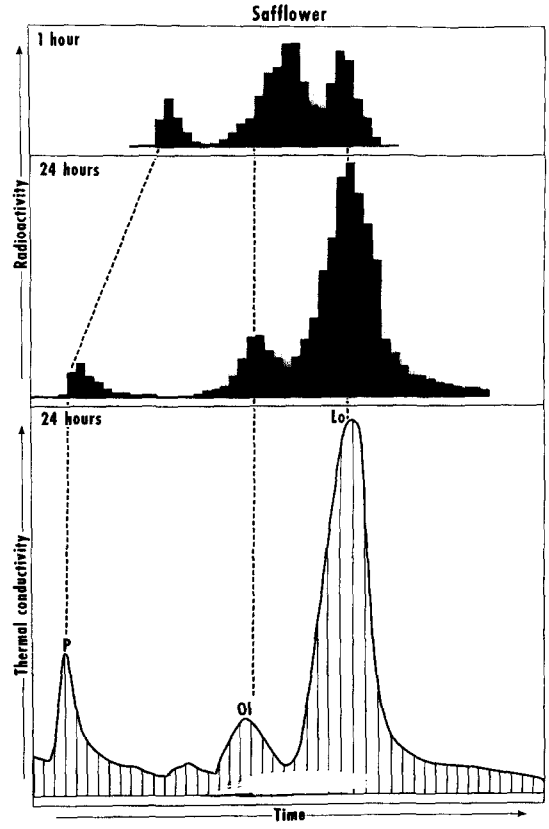

FIG. 6. Mass (by thermal conductivity detector) and radioactivity analyses for the gas chromatographic separation of 1- and 24-hr samples of safflower esters. Abbreviations as in Fig. 2.

specific activity data, such as are given in Fig. 4, are critical kinetic indices for determining generic relationships of these fatty acids. Since specific activities of oleate and palmitate decrease from high values toward the "random" value of 1, they obviously have the role of precursors, and both are perhaps formed by the branched or concurrent pathway James has suggested (10). The maximum in the curve for linoleate shows it to be an intermediate between oleate and linolenate. The steadily rising specific activity for linolenate designates it as product in the reaction series.

Flax, soybean, and safflower are representative of three orders of plants, Geraniales, Rosales, and Campanulales, respectively. The data presented here support the conclusion that dehydrogenation is a general pathway in the seeds of higher plants for the production of the C_{18} fatty acids which differ in degree of unsaturation.

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