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The Sequence of Formation of Fatty Acids in Developing Soybean Seeds[1]

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STUDIES of the fatty acid synthesis in higher plants have been conducted along two general lines. One approach has been to study the conversion of carbohydrates (1, 2) and other substrates (3) to fatty acids in seeds during maturation. A second general group of studies have been conducted on the changes in degree of unsaturation and the fatty acid composition of the oil during the oil deposition period (4, 5). None of these studies have given conclusive evidence on the interconversion of the C_{18} acids in plants as has been shown to occur between oleic and stearic acid in animals (6).

This paper reports results obtained from a study of the synthesis of the individual fatty acids by soybeans on excised stems which were supplied with sucrose labeled with C^{14}. The fatty acids were separated chromatographically as their 2,4-dinitrobenzenesulfenyl chloride derivatives (7).

Experimental

Methods. Cuttings, three nodes in length, with leaves intact and bearing 8 to 10 bean pods were cut from the main stems of Lincoln soybean plants which had bloomed 35 to 40 days prior to cutting. The cut end of each stem was dipped 1/8 to 1/4 in. into melted paraffin immediately after cutting, and then the basal 2 in. of the stem was sterilized by immersion in a 5% solution of calcium hypochlorite for 5 minutes. After washing thoroughly with sterile water, a fresh cut was made under water about 1 cm. above the basal end of the stem, and the cutting was then placed in a 10-ml. Erlenmeyer flask fitted with a cotton plug and containing sterile Nitsch (8) medium (solution of sucrose and essential inorganic salts, autoclaved 15 min. at 15 p.s.i.). The flask with its cutting was then placed in a desiccator fitted with suitable connections and traps. Dry air ($CaCl_2$ tube) was passed through the desiccator and exhausted through two sodium hydroxide traps and one barium hydroxide trap. The desiccators were placed in a dark room at 26-30°C. except when otherwise stated.

Radioactive carbon was supplied to the cuttings in the form of uniformly labeled sucrose,[3] which was substituted for ordinary sucrose at different activity levels in the medium. When cuttings were transferred to the non-radioactive medium, they were washed thoroughly with sterile water and a new cut was made about 1 cm. from the end of the stem.

TABLE I

Oil Changes in Soybean Cuttings on Sucrose Medium[a]

Sample	Av. wt. per bean	Av. oil per bean	Av. wt. of fatty acids per bean			
			Saturated	Oleic	Linoleic	Linolenic
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Expt. 1 (26-30°C.)						
0–Day	106	21.8	2.0	8.2	9.1	1.3
2–Day	117	24.8	2.5	7.9	8.7	2.7
3–Day	113	23.6	2.5	7.7	8.0	2.1
5–Day	140	29.0	1.6	10.7	12.5	2.7
7–Day	145	30.0	3.0	10.5	12.3	2.1
Expt. 2 (Radioactive)						
2–Day	146	29.4	3.0	4.8	15.7	2.4
4–Day[b]	152	30.4	3.0	5.8	17.2	3.3
12–Day	167	36.8	2.7	8.1	19.3	3.4
18–Day	156	33.0	2.7	7.8	18.2	2.5
Expt. 2 (Control)						
2–Day	156	30.6	3.5	5.6	15.9	....
4–Day	152	30.3	2.3	7.9	17.3	2.4
12–Day	148	31.6	3.6	7.8	17.9	2.7
18–Day	140	28.8	3.0	8.1	16.0	2.5
Expt. 3 (28°C.)						
0–Hr.	127	23.5	2.3	5.4	12.0	2.6
6–Hr.	132	22.2	2.0	4.1	10.3	2.6
8–Hr.	128	21.8	2.1	4.3	10.6	2.3
12–Hr.	131	22.6	1.9	4.0	10.7	2.6
15–Hr.	139	24.6	2.0	4.3	12.0	2.8
21–Hr	110	18.6	1.8	4.0	9.3	2.1

[a]Isolation method of analysis.
[b]From second to fourth day, cuttings held at 36°C. without sucrose, thereafter at 18°C. with sucrose.

Samples of 6-12 pods were collected at each sampling. The pods were placed in boiling water for 3 minutes to aid in the removal of the beans. The beans were cut into pieces about 1 mm. in diameter and dried in a vacuum oven at 60°C. for 24 hrs. The dried bean samples were pulverized in a mortar and extracted for 4 hrs. with hot redistilled hexane. The residue was washed with hot solvent, and the solvent was then removed from the combined extract and washings under a stream of nitrogen. The oil samples were dried in a vacuum oven for 2 hrs. at 50°C.

The specific activity of the crude oil sample was determined by counting replicate 0.1-mg. samples in a continuous flow counter[4] until a total count of at least 10,000 was obtained. Individual fatty acids from a 100-mg. sample were separated chromatographically as their 2,4-dinitrobenzenesulfenyl chloride derivatives (1). The derivatives were dissolved in 100 ml. of benzene, and the specific activities of the individual fatty acids were determined by plating 1 ml. of this solution on an aluminum dish (8.5 sq. cm.) and counting the sample until a total count of 10,000 was reached.

Three experiments were performed, each with five or six soybean cuttings, on media containing different

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[4]Q-gas counter, Model 046a, Nuclear Instruments and Chemical Company, Chicago, Ill.

levels of radioactive sucrose. In Experiment 1 six cuttings were placed in a medium containing 20 microcuries of activity per flask for 48 hours, then in inactive sucrose medium for five days. In Experiment 2 six cuttings were placed in a medium containing 6 microcuries per flask for 48 hours, then in mineral salts-water without sucrose at 36°C. for 2 days, and finally in inactive sucrose medium at 18°C. for 14 days. As a control parallel experiments were made with cuttings in media containing ordinary sucrose. In Experiment 3 five cuttings were placed in a medium containing 3 microcuries of activity per flask at 28°C. for 21 hours. Samples were then taken for analysis at intervals as shown in the tables and graphs.

Results and Discussion

Analyses of the beans showed that the cuttings continued to develop and produce oil during 7 days (Experiment 1) on the 20 microcurie sucrose medium (Table I). Substantial increases were observed in bean size, oil content, and amounts of each of the fatty acids, and these increases were nearly equal to those observed earlier with intact soybean plants (5).

Relatively large amounts of radioactivity were also found in the oil and in the individual fatty acids after two days on the sucrose medium (Figure 1). The oleic acid showed the highest activity (13,000 CPM/mg.) and the linolenic the lowest (2,500 CPM/mg.). These differences suggested either that oleic acid was being formed from sucrose at an accelerated rate or that oleic acid was partly a transient intermediate

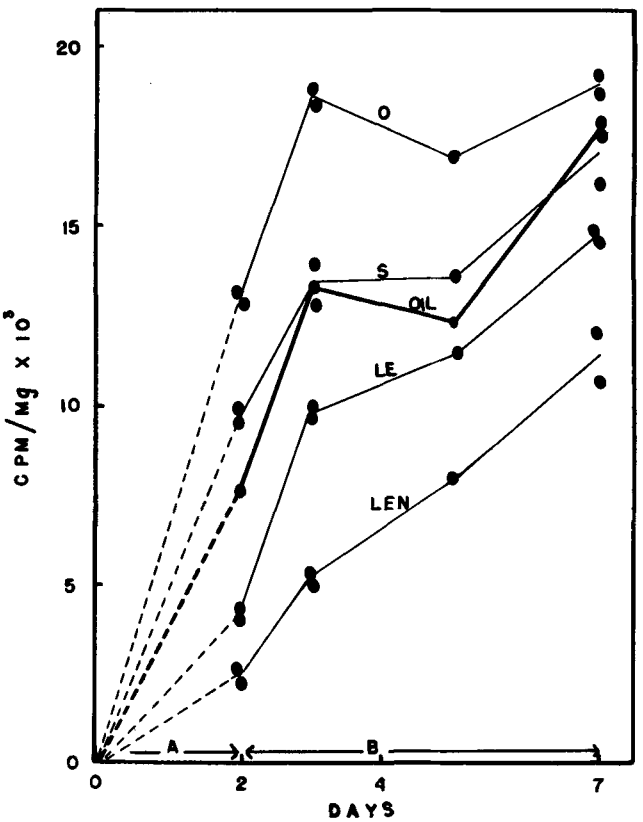

FIG. 1. Changes in specific activity with continuous sucrose feeding at ordinary temperature, 26-30°C. (O, oleic acid; S, saturated acids; LE, linoleic acid; LEN, linolenic acid; A, cuttings in radioactive sucrose medium, 20 microcuries/flask; B, cuttings in ordinary sucrose medium. All cuttings kept in dark.)

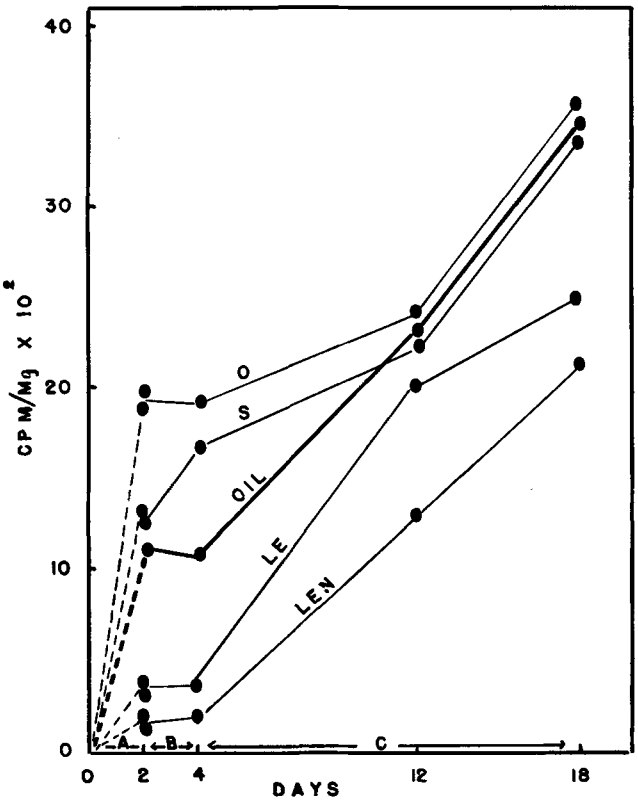

FIG. 2. Changes in specific activity following two days' starvation at high temperature (A, cuttings in radioactive sucrose medium, 6 microcuries/flask, 26-30°C.; B, cuttings in salts medium without sucrose, 36°C.; C, cuttings in ordinary sucrose medium, 18°C. All cuttings kept in dark.)

for the formation of other fatty acids, especially linoleic and linolenic. When the radioactive medium was replaced by ordinary sucrose medium, depostion of radioactive fatty acids continued during the remaining 5 days of the experiment but at different rates for the different acids. This is shown most clearly in the distribution of total radioactivity (Table II), which concerns quantities as well as specific activities of the individual acids.

TABLE II

Distribution of Radioactivity (Total CPM/Bean)

Expt. 1	At 2 days	At 7 days
Saturated	24,600	51,000
Oleic	104,000	199,500
Linoleic	34,800	182,000
Linolenic	5,200	24,100
Sum	168,600	456,600
Total oil	186,000	525,000

After the 2 days on radioactive sucrose 56% of the radioactivity of the oil was found in the oleic acid which constituted about one-third of the oil, only 18% of the radioactivity was found in the linoleic acid which also constituted about one-third of the total, thus a 3:1 ratio favoring conversion of sucrose to oleic rather than linoleic. Five days after withdrawal of the radioactive sucrose the relative distribution of radioactivity had changed sharply. While the total activity in the oleic fraction had nearly doubled, that in the linoleic fraction had increased four-fold and in the linolenic fraction seven-fold. Since in the meantime the relative percentages of the different fatty acids in the oil remained unchanged, the evidence

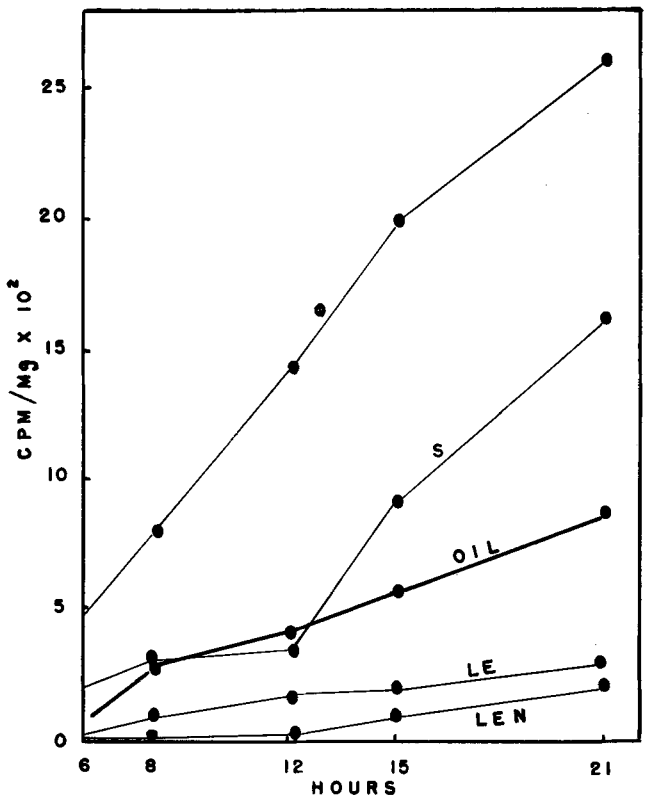

FIG. 3. Changes in specific activity during early stages of sucrose uptake. (Cuttings in radioactive sucrose medium, 3 microcuries/flask, 28°C.)

strongly favored conversion of oleic to the other acids.

The continued assimilation of radioactivity in all fatty acids after withdrawal of the cuttings from the radioactive medium indicated that a large reserve of sucrose or other radioactive metabolite had been built up prior to withdrawal. Attempts were therefore made in Experiment 2 to increase sharply the rate of respiration immediately after withdrawal of the radioactive substrate. It was hoped that this treatment, along with a lower initial level of substrate activity, would diminish the effect of the metabolic pool and permit a clearer picture of fatty acid interconversion. The results (Table I and Figure 2) showed that no substantial increases in total fatty acids occurred either during the 2-day period of starvation at 36°C. or subsequently during two weeks on ordinary sucrose at 18°C. However the oleic fraction appeared to increase and the saturated fraction appeared to decrease slightly during the 2-day starvation period. This shift in composition was substantiated in analysis by the spectrophotometric method (9).

The 2-day period of starvation at 36°C. proved ineffective as a means of depleting the radioactive from the metabolic pool of the soybean cuttings since all of the fatty acids, including oleic, continued to increase in specific activity during the subsequent 2-week period. While the experiment provided support for Experiment 1, it failed to produce the desired conditions for observing uncomplicated interconversion of fatty acids. Evident limitations in the period of viability of cuttings discouraged further work in this direction, and attention was then turned to short-term experiments to observe distribution of radioactivity during the initial stages of absorption of radioactive sucrose by soybean cuttings.

In short term experiments up to 4 hrs., oil from beans removed from the cuttings showed only traces of activity. After 6 hrs. on the radioactive medium (Experiment 3) the oil showed substantial radioactivity (Figure 3). The specific activity of the oleic acid was five-fold that of the whole oil while the activities of linoleic and linolenic were not significantly above background. Measurable activity appeared in the linoleic fraction at 8 hrs. and in the linolenic fraction at 12 hrs. At 12 hrs. two-thirds of the total radioactivity was found in the oleic acid fraction, which constituted only one-fifth of the whole oil.

The data presented in these studies clearly indicate a preferential production of oleic acid from sucrose by soybean cuttings. They indicate further that the initial preponderance of this tagged oleic acid over other tagged fatty acids is only temporary. The tagged saturated, linoleic, and linolenic acids gradually build up in concentration to approach proportionality with the tagged oleic. Although it was not found possible experimentally to establish conditions in which the specific activity of oleic acid would subsequently fall below that of the other acids as evidence of its role as a precursor, the data presented strongly support the theory of sequential conversion of oleic acid to the other fatty acids. Apparently the soybean can convert oleic to linoleic and this in turn to linolenic. Schoenheimer *et al.* (6) have demonstrated that the animal can convert stearic acid to oleic, and Clendenning (10) has reported that oil produced by algae from a tagged substrate showed its highest specific activity in the saturated fatty acid fraction. Evidently the soybean produces oleic acid prior to or along with the saturated acids.

Summary

Excised Lincoln soybean stems bearing pods and leaves were supplied with C^{14} labeled-sucrose for brief periods, and the subsequent appearance of radioactivity in the different fatty acids was observed up to 18 days. The individual fatty acids were separated as their 2,4-dinitrobenzenesulfenyl chloride derivatives and the specific activity of the different fractions were determined.

Radioactivity appeared in the fatty acids in the following order: oleic, saturated, linoleic, linolenic. Consistent presence of highest specific activity in the oleic acid fraction indicated that oleic may be converted to the other acids, at least to some extent.

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