Biosynthesis of Fatty Acids by a Soluble Extract From Developing Soybean Cotyledons[1]

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Abstract. **Fractionation of developing soybean cotyledons into cellular components demonstrates that most of the activity necessary to incorporate acetate-1-^{14}C into lipid remains in the supernatant from a 198,000*g* spin for 1 hr. The system studied is dependent upon ATP, CoA, and CO_2. Concentrations of ATP greater than 4×10^{-3} M are inhibitory, while 1×10^{-4} M CoA is needed for optimal activity. Avidin inhibition of acetate incorporation into lipid could be reversed by biotin. Studies indicated that NADPH is a better source of reducing power than NADH. The system studied is inhibited by *p*-chloromercuribenzoic acid and this inhibition can be reversed by an excess of GSH. The system studied shows maximum activity in tris buffer at pH 8.6 or in glycine buffer, pH 9.4.**

The distribution of acetate into the various fatty acids is greatly influenced by the temperature of incubation. Cooler incubation temperatures favor the distribution of acetate into the more unsaturated fatty acids.

Commercially grown soybeans weigh in the range of 140 to 200 mg dry weight per bean at maturity. This dry weight consists of approximately 40 % protein, 20 % lipid, and 40 % carbohydrates and crude fiber. Starch is normally not present in soybeans at maturity (10). The time from flowering to maturity is normally about 60 days (3). The deposition of storage protein and lipid begins somewhere from 25 to 30 days after flowering and continues to maturity (19). At 32 to 40 days after flowering, the percentage of lipid per dry weight of bean has already reached a value of about 20 %. Also at this time, the composition of fatty acids in the lipid fraction has reached a constant value. The lipid fraction of mature soybeans consists of 10 to 12 % palmitic acid, 3 to 5 % stearic acid, about 26 % oleic acid, 48 to 52 % linoleic acid, and 5 to 8 % linolenic acid.

These investigations were undertaken in hopes of obtaining a better understanding of the mechanisms involved in the formation of lipids during the development of the soybean seed. A preliminary report has been presented (18).

Materials and Methods

Materials. CoA, acetyl CoA, ATP, NAD, NADP, and NADPH were purchased from P&L Biochemicals, Inc.[2] Glucose-6-P, *p*-chloromercuribenzoic acid (*p*CMB), and GSH were purchased from Sigma Chemical Company. Avidin and D-biotin were purchased from Nutritional Biochemical Corporation. Acetate-1-^{14}C and acetyl-1-^{14}C CoA were purchased from New England Nuclear Corporation.

Preparation of Enzyme. Developing soybeans [*Glycine max* (L.) Merr., var. Harosoy 63] were picked 35 to 45 days after flowering as needed. Plants were grown in gravel culture (2) under a 14 hr photoperiod in the greenhouse. The soybeans were removed from the pods and the seed coats, plumule, hypocotyl, and radicle were removed from the cotyledons. The cotyledons were placed in distilled water at room temperature until grinding. About 10 g of soybean cotyledons were ground with 20 ml of 0.5 M sucrose + 0.01 M MES [2-(*N*-morpholino)ethanesulfonic acid], pH 6.1, at room temperature. The slurry was passed through 8 layers of cheesecloth and the homogenate was considered the crude enzyme. The crude enzyme was spun at 21,600*g* for 20 min at 10°. The supernatant was then spun at 198,000*g* for 60 min at 10°. Protein was determined by the biuret method.

Assay Method. The reaction was carried out in a 25 ml Erlenmeyer flask at the temperature and for the time specified. The reaction was stopped with 15 % ethanolic KOH followed by saponification for 3 to 4 hr at 50°. After saponification, the

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samples were acidified with 6 N HCl and the fatty acids were extracted in a partition of water and chloroform. The chloroform layer containing the fatty acids was taken to dryness under a stream of nitrogen in a water bath at 50°. The samples were then taken up with 10 ml of chloroform, and an aliquot was taken to dryness under a stream of nitrogen and counted in a Packard liquid scintillation spectrometer. All counts were corrected for quenching.

Analysis of Fatty Acids. Methyl esters of the fatty acids were prepared by using 5 % H_2SO_4 in methanol. Recovery of counts of fatty acids and those in methyl esters was above 90 %. Fatty acid methyl esters were separated by gas-liquid chromatography by using a Micro Tek 2000-R gas chromatograph fitted with a flame ionization detector. The column (6 ft by one-fourth in) was packed with 10 % diethylene glycol succinate on 80/100 Diatoport S. Column temperature was 180° with a flow rate of 100 ml/min of helium. The signal from the ionization detector was recorded on a Brown recorder.

Housed between the ionization detector and the column was a gas stream splitter which divided the gas stream coming from the column in a ratio of 50/1 in favor of collection. Fatty acid methyl esters were collected in 2 mm × 200 mm glass tubes as they emerged from the splitter. The glass tubes were washed directly into scintillation vials with scintillation fluid and counted. The technique routinely gave 85 % or greater recovery of injected counts.

Results and Discussion

Localization. When acetate is used as the substrate over 85 % of the fatty acid synthesizing activity is found in the soluble protein fraction (table I).

The main site of fatty acid synthesis in the developing safflower is a particulate fraction (13).

Table I. *Incorporation of Acetate-1-^{14}C Into Lipid by Various Cellular Fractions*

The reaction mixture contained in a volume of 2.5 ml the following in μmoles: tris 100, pH 8.6; CoA 1.3 (0.1 mg); ATP 10; GSH 2; $MnCl_2$ 0.5; $KHCO_3$ 30; NADPH 0.25, and acetate-1-^{14}C 2 (2 μc). The ppt were suspended in 2.5 ml of grinding media and 1 ml was used for an enzyme source while 1 ml of the other fractions was used for an enzyme source. The reaction mixture was incubated for 2 hr with shaking at 37°.

Fraction	Acetate incorporation	
	mμmoles	*mμmoles/mg protein*
Crude enzyme	76.1	1.90
21,600*g* ppt 20 min	12.1	0.17
21,600*g* supernatant 20 min	71.6	2.86
198,000*g* ppt 60 min	0.5	0.02
198,000*g* supernatant 60 min	66.9	3.18

The avocado possesses both a soluble and particulate system (25) depending on the substrate used. The soluble system from avocado can only utilize malonic acid or malonyl-CoA as its substrate while the particulate system from avocado can utilize acetate, acetyl-CoA, or malonyl-CoA. The system from developing castor beans (24) requires both a particulate fraction and a supernatant fraction for the incorporation of acetate into ricinoleic acid. Therefore, it appears that the developing soybean is unique in that the major site of fatty acid synthesis using acetate as the substrate is found in the soluble protein.

The supernatant from the 21,600*g* spin for 20 min was used as an enzyme source for the remaining experiments reported. This fraction normally contains about 25 to 30 mg of protein per ml. The supernatant from the 21,600*g* spin for 20 min was quite unstable and fresh enzyme was prepared as needed. Initial attempts have been made to purify the enzymes and although they have been unsuccessful, the work is continuing. Work with malonyl-CoA as the substrate has been started and will be reported shortly.

Effect of pH. The fatty acid synthesizing system has 2 peaks of maximum activity (fig 1). With

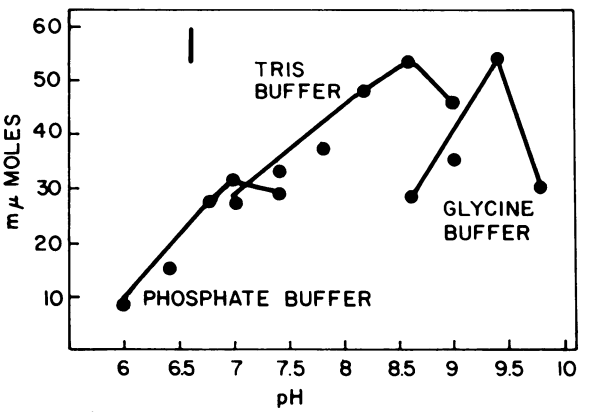


FIG. 1. Effect of pH on the incorporation of acetate-1-^{14}C into lipid by the 21,600*g* supernatant fraction. The reaction mixture contained in a volume of 2.5 ml the following in μmoles: various buffers 100; CoA 0.1 (0.1 mg); ATP 10; GSH 2; $MnCl_2$ 0.5; NADPH 0.25; $KHCO_3$ 0.25 acetate-1-^{14}C 2 (2 μc), and 1 ml enzyme. The reaction mixture was incubated for 1 hr with shaking at 37°.

tris buffer there is a peak of maximum activity at pH 8.6 while in glycine buffer the peak of maximum activity is at pH 9.4. Phosphate buffer exhibited a peak of activity at pH 7.0, though this activity was only 40 % of that obtained with either tris buffer at pH 8.6 or glycine buffer at pH 9.4. Tris buffer has been reported to inhibit the avian liver system (22) while the particulate system from avocado mesocarp has maximum activity in tris buffer at pH 8.0 (15). A soluble enzyme system prepared from avocado mitochondrial particles is inhibited in tris buffer at pH 8.0 and has optimal activity in phosphate buffer at pH 7.1 (1).

Table II. *Cofactor Requirements for Acetate-1-^{14}C Incorporation by the 21,600g Supernatant Fraction*

The reaction mixture contained in a volume of 2.5 ml the following in μmoles: tris 100, pH 8.6; CoA 0.3 (0.25 mg); ATP 10; GSH 2; $MnCl_2$ 0.5; $KHCO_3$ 30; NADPH 0.25; acetate-1-^{14}C 3 (0.5 μc), and 1 ml enzyme. The reaction mixture was incubated for 1 hr with shaking at 37°.

Reaction mixture	Acetate Incorporated	Decrease
	mμmoles	%
Complete	206.2	0
—CoA	7.7	96
—ATP	0	100
—CoA and ATP	0	100
—GSH	184.3	11
—$MnCl_2$	144.3	31
—NADPH	160.4	23
—$KHCO_3$	167.9	18
+Enzyme and acetate-1-^{14}C	0	100

Time. The incorporation of acetate into fatty acids is linear up to 90 min at 37° under the conditions shown in figure 2. A reaction time of 60 min was chosen for the remaining experiments reported.

Cofactors. Table II shows the effect on the fatty acid synthesizing system caused by omitting different cofactors and substrates from the reaction mixture.

The cofactor requirements of the enzyme system from developing soybean cotyledons does not differ substantially from other fatty acid systems which synthesize fatty acids *via* the malonyl-CoA pathway (1, 4, 11, 16, 17, 20, 21, 23).

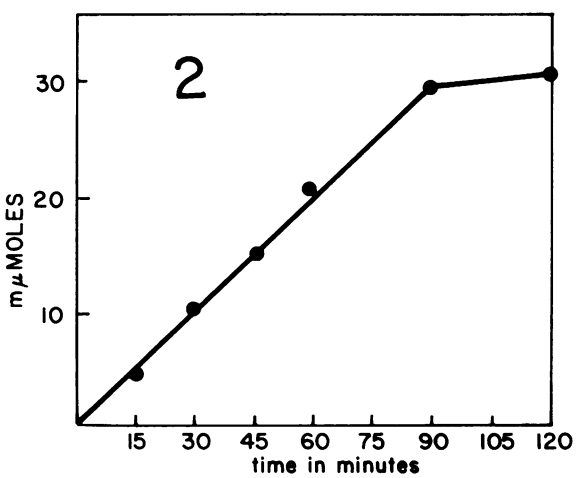


FIG. 2. Effect of time on the incorporation of acetate-1-^{14}C into lipid by the 21,600*g* supernatant fraction. Reaction mixture contained in a volume of 2.5 ml the following in μmoles: tris 100, pH 8.6; CoA 0.1 (0.1 mg); GSH 2; $MnCl_2$ 0.5; NADPH 0.25; $KHCO_3$ 30; acetate-1-^{14}C 2 (2 μc); and 1 ml enzyme. The reaction mixture was incubated for the times indicated with shaking at 37°.

Table III. *Effect of Pyridine Nucleotides on Acetate-1-^{14}C Incorporation Into Lipid by the 21,600g Supernatant Fraction*

The reaction mixture and incubation same as table II except for source of reduced pyridine nucleotides listed below in μmoles.

Source of reduced pyridine nucleotide	Acetate incorporated
	mμmoles
NADPH 0.25	97.7
None	54.7
NADP 0.5 and glucose-6-P 0.5	98.0
NADH 0.25	74.0
NADPH 0.25 and NADH 0.25	104.6

Substrate. The effect of increasing the concentration of acetate on the fatty acid synthesizing activity is shown in figure 3. The results show that the fatty acid synthesizing system becomes saturated at about 2.5 μmoles or 1×10^{-3} M.

Coenzyme A. Figure 4 demonstrates the dependence of the fatty acid synthesizing system from developing soybean cotyledons on CoA. The system shows a linear response to the amount of CoA added up to the concentration of 1×10^{-4} M. No inhibition of acetate incorporation occurred up to a concentration of 1.5×10^{-4} M, and therefore a concentration of 1.2×10^{-4} M was used routinely in the assay. These results are similar to those reported for the avocado system (1).

ATP. The fatty acid synthesizing system is dependent on ATP as shown in figure 5. A concentration of 4×10^{-3} M is needed for optimal activity while optimal incorporation with the avocado system is at 6×10^{-3} M (1).

Reducing Power. The NADPH dependence is shown in figure 6. Optimal activity is achieved at a concentration of 1.04×10^{-4} M NADPH and a concentration of 1×10^{-4} M was used routinely in the assay. Table III demonstrates that the system can generate its own reducing power since equal

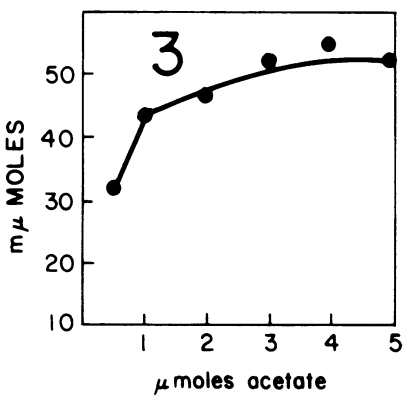
Table IV. *Effect of Avidin on the Incorporation of Acetate-1-^{14}C Into Lipid by the 21,600g Supernatant Fraction*

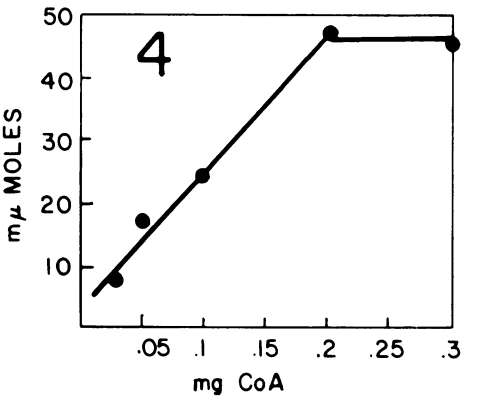
The reaction mixture and incubation same as table II. Avidin preincubated with enzyme for 5 min at room temperature.

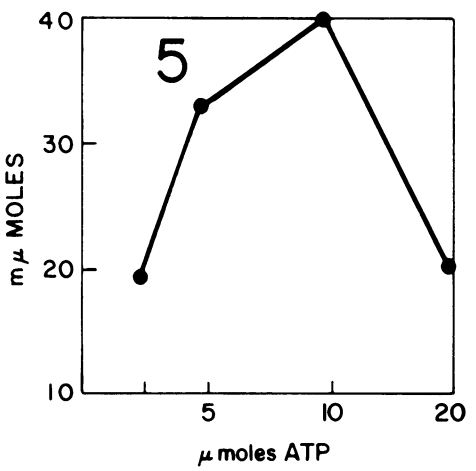
Reaction mixture	Acetate incorporated
	mμmoles
Complete	38.9
Complete + 0.25 μmole biotin	38.7
Complete + 50 μg avidin	37.1
Complete + 250 μg avidin	14.4
Complete + 0.25 μmole biotin and 50 μg avidin	38.5
Complete + 0.25 μmole biotin and 250 μg avidin	38.4

amounts of acetate can be incorporated into fatty acids when NADP and glucose-6-P are substituted for NADPH. It is interesting to note that NADH alone did increase the incorporation of acetate into fatty acids above the zero value of NADPH by 35 %. NADPH alone showed a 81 % increase above the zero value of NADPH. The use of NADH and NADPH together resulted in no additive effect. Gallard (7) recently reported that the conversion of oleic to ricinoleic requires NADH rather than NADPH. The system from developing soybean cotyledons does show a preference for NADPH over NADH.

Avidin Inhibition. Table IV shows the effect of







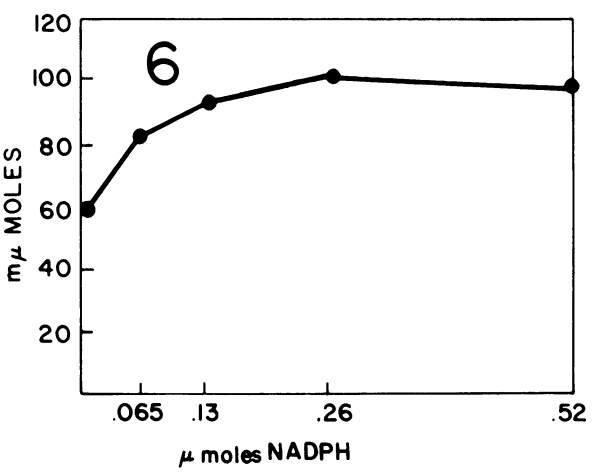


FIG. 3. Effect of varying acetate concentration on the incorporation of acetate-1-^{14}C into lipid by the 21,600g supernatant fraction. Reaction mixture contained in a volume of 2.5 ml the following in μmoles: tris 100, pH 8.6; CoA 0.3 (0.25 mg); ATP 10; GSH 2; $MnCl_2$ 0.5; NADPH 0.25; $KHCO_3$ 30; with varying amounts of K acetate as indicated with 0.5 μc acetate-1-^{14}C and 1 ml enzyme. The reaction mixture was incubated for 1 hr with shaking at 37°.

FIG. 4. Effect of varying with CoA concentration on the incorporation of acetate-1-^{14}C into lipid by the 21,600g supernatant fraction. Reaction mixture contained in a volume of 2.5 ml the following μmoles: tris 100, pH 8.6; ATP 10; GSH 2; $MnCl_2$ 0.5; NADPH 0.25; $KHCO_3$ 30; acetate-1-^{14}C 2 (0.5 μc); with varying amounts of CoA as indicated and 1 ml enzyme. The reaction mixture was incubated for 1 hr with shaking at 37°.

FIG. 5. Effect of varying the ATP concentrations on the incorporation of acetate-1-^{14}C into lipid by the 21,600g supernatant fraction. Reaction mixture contained in a volume of 2.5 ml the following in μmoles: tris 100, pH 8.6; CoA 0.3 (0.25 mg); GSH 2; $MnCl_2$ 0.5; NADPH 0.25; $KHCO_3$ 30; acetate-1-^{14}C 2 (0.5 μc); with varying amounts of ATP as indicated, and 1 ml enzyme. The reaction mixture was incubated for 1 hr with shaking at 37°.

FIG. 6. Effect of varying the concentration of NADPH on the incorporation of acetate-1-^{14}C into lipid by the 21,600g supernatant fraction. Reaction mixture contained in a volume of 2.5 ml the following in μmoles: tris 100, pH 8.6; CoA 3.25 (0.25 mg); ATP 10; GSH 2; $MnCl_2$ 0.5; $KHCO_3$ 30; acetate-1-^{14}C 3 (0.5 μc); with varying amounts of NADPH as indicated, and 1 ml enzyme. The reaction mixture was incubated for 1 hr with shaking at 37°.

avidin on the incorporation of acetate into fatty acids. The higher avidin concentration inhibits the amount of acetate incorporated into fatty acids by 63 % (table IV). This inhibition can be completely reversed by additions of biotin to the reaction mixture. The fact that acetate incorporation into fatty acids is inhibited by avidin indicates the involvement of a biotin enzyme (11, 23). This suggests that the fatty acid system from developing soybean cotyledons synthesizes fatty acids from acetate *via* malonyl-CoA or *de novo*, and not by chain elongation.

Table V. *Effect of pCMB on the Incorporation of Acetyl-1-^{14}C CoA Into Lipid by the 21,600g Supernatant Fraction*

The reaction mixture and incubation same as table II except acetyl-1-^{14}C CoA 0.55 μmole (0.2 μc) was used instead of acetate-1-^{14}C. Preincubated pCMB and enzyme for 5 min at room temperature.

Reaction mixture	Acetyl CoA incorporated
	mμmoles
Complete	31.8
Complete + 2 μmoles GSH	33.5
Complete + 10^{-3} M pCMB	0
Complete + 10^{-4} M pCMB	0
Complete + 10^{-5} M pCMB	21.7
Complete + 10^{-3} M pCMB and 25 μmoles GSH	2.3
Complete + 10^{-4} M pCMB and 25 μmoles GSH	26.8
Complete + 10^{-5} M pCMB and 25 μmoles GSH	29.2

pCMB Effect. Table V illustrates the effect of *p*CMB on the fatty acid synthesizing system from soybean cotyledons. The inhibition of acetate incorporation by *p*CMB and the reversal of the inhibition by an excess of GSH is in agreement with the accepted idea of sulfhydryl groups associated with the active sites of fatty acid synthetase systems (1, 5, 11, 12).

Product of Reaction. Table VI shows the percent distribution of ^{14}C in the fatty acids isolated from reaction mixtures run at 3 temperatures. In all of the data reported so far, the experiments were carried out at 37°. At 37°, only a small percentage of the counts in the lipid fraction occur in the unsaturated fatty acids. The distribution of acetate incorporation is shifted towards more unsaturated fatty acids by cooler temperatures of incubation. The percentage of total counts found in palmitic acid does not seem to be influenced by the temperature of incubation.

Table VI. *Effect of Temperature on Incorporation of Acetate-1-^{14}C Into Fatty Acids by the 21,600g Supernatant Fraction*

Temp	% Of total ^{14}C in fatty acids				
	Palmitic	Stearic	Oleic	Linoleic	Linolenic
17°	15	51	13	6	13
27°	15	62	13	0	8
37°	15	79	5	0	0

Linolenic and linoleic acid percentages of soybean oil are inversely correlated with daily maximum temperatures during the growing season (8). Sunflower, flax, and rape produce more highly unsaturated fatty acids at cooler temperatures, but the degree of unsaturation is not affected in castor bean and safflower (6). Yeast grown at cooler temperatures has the same characteristic as sunflower, flax, and rape (9, 14).

The particulate system from avocado mesocarp (15) incorporates acetate into both saturated and unsaturated fatty acids. The incorporation of acetate by the avocado system into oleic acid was favored by higher temperatures rather than cooler temperatures. The soluble avocado system (25), which can only utilize malonic acid or malonyl-CoA as substrates, only produces saturated fatty acids.

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