THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 236, No. 10, October 1961
Printed in U.S.A

Fat Metabolism in Higher Plants

XV. ENZYMIC SYNTHESIS OF FATTY ACIDS BY AN EXTRACT OF AVOCADO MESOCARP

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(Received for publication, March 24, 1961)

Cell-free systems that catalyze the condensation of acetylcoenzyme A to form long chain fatty acids have been prepared from chicken liver by Wakil *et al.* (1), from yeast by Lynen (2), and from rat liver and brain by Brady, Bradley, and Trams (3, 4). In 1957, Stumpf and Barber (5) described a mitochondrial system isolated from avocado mesocarp that incorporated C^{14}-acetate into long chain fatty acids, mostly as triglycerides. In 1958, Squires *et al.* (6) prepared a water-soluble system from these particles that readily formed long chain fatty acids, provided CO_2 was added, and in 1959, Stumpf *et al.* (7) demonstrated sensitivity of this system to avidin.

This paper will describe the preparation and properties of a water-soluble enzyme system from mitochondrial particles of the avocado mesocarp. This system catalyzes the incorporation of acetylcoenzyme A and malonylcoenzyme A to long chain fatty acids.

EXPERIMENTAL PROCEDURE

Preparation of Acetone Powder—The Fuerte variety of avocado (*Persea americana*) used throughout this study was purchased locally. The McArthur variety can also be used as a source of enzyme. The fruit, ripened 2 to 3 days at room temperature, was peeled and deseeded, and the mesocarp was sliced into thin pieces. Approximately 550 g of the mesocarp were homogenized with an equal volume of 0.25 M reagent grade sucrose at the number 1 position (8000 r.p.m.) of the Waring Blendor (1 gallon, Model CB-3) for 10 seconds and the number 2 position (10,000 r.p.m.) for an additional 5 seconds. One-third of the original volume of sucrose used was again added and the homogenization repeated, to produce a smooth thin paste. This paste was centrifuged either in a Lourdes model L-R or Servall RC-2 centrifuge for 10 minutes at 600 to 1000 $\times$ *g*. Three layers were obtained: (*a*) the sediment; (*b*) a slightly turbid middle fraction; and (*c*) an upper layer composed of lipids. The middle layer was siphoned off and saved, the other two layers were resuspended with 0.25 M sucrose and recentrifuged, and the middle layer was again saved. The sediment and the top lipid fraction were discarded. The slightly turbid middle layer was then centrifuged at 15,000 $\times$ *g* for 30 minutes. The resulting sediment was suspended in a minimal volume of 0.25 M sucrose, and an acetone powder was prepared. The yield was approximately 1 g of acetone powder of particles from 1000 g of mesocarp. The fatty acid-synthesizing system was prepared by suspending 100 mg of the particle acetone powder in 0.85 ml of 0.2 M PO_4 buffer at pH 7.1. The suspension was stirred for about 1 minute and then centrifuged at 27,000 to 30,000 $\times$ *g* for 10 minutes. Whereas the dry acetone powder is stable for at least 6 months at 0°, the clear supernatant loses activity rather rapidly when frozen overnight, *i.e.* a 75% loss.

Methods—The protein concentration was determined by a biuret reaction (8). Paper chromatography of the fatty acids was carried out on silicon-treated paper according to the method of Mangold, Lamp, and Schlenk (9). The technique of Buchanan (10), with acetic acid-formic acid-H_2O_2 (9:1:1), was utilized for the separation of unsaturated and saturated fatty acids by paper chromatography on silicon-treated paper.

Dyer's monophasic procedure (11) was used for extraction of lipid from the reaction mixture. The lipid samples were counted on a Packard liquid scintillation spectrometer, with a toluene-phosphor mixture. Chromatographic strips were scanned with a Nuclear-Chicago Actigraph II strip counter. When necessary, the areas under the peaks were measured by planimetery. Hydrolysis of thiol esters were carried out for 15 minutes at 50° at approximately pH 11. The phospholipid and glycerides were hydrolyzed for 3 hours in 2 or 3 ml of 0.3 N ethanolic KOH and the fatty acids isolated by Dyer's procedure. The neutral lipids were separated from phospholipids by silicic acid chromatography (12). Chloroform was the eluting solvent for neutral lipid, whereas 5% chloroform in methanol was used to elute phospholipids. The procedure of Barron and Hanahan (13) was used for total fractionation of the neutral lipid with a 2.5-g silicic acid column. The fatty acids were separated from triglycerides by the procedure of Borgström (12) by washing with 0.05 N NaOH in 55% ethanol.

Acetyl-1-C^{14} was synthesized from acetic anhydride by the method of Simon and Shemin (14). Cold malonyl-CoA was synthesized by the procedure of Vagelos (15) with diketene. Malonyl-1,3-C^{14}-CoA was prepared by the method of Brady (16). Avidin was obtained from Nutritional Biochemical Corporation, Cleveland, and had 2.5 units per mg.

RESULTS

General Properties—In the avocado, the enzyme complex responsible for the synthesis of fatty acid is localized in the large particulate fraction usually associated with mitochondria. These particles sediment at 15,000 $\times$ *g*, have Krebs cycle activity,

* Postdoctoral Trainee of the National Institutes of Health training grant program (G-119).
† Supported in part by a grant from the National Science Foundation G-4582.

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TABLE I
Incorporation of acetate-1-C^{14} into lipids by different cellular fractions

Reaction mixtures contained either 100 mμmoles of acetate-1-C^{14} (110,000 c.p.m.) or 188 mμmoles of acetyl-1-C^{14}-CoA (86,000 c.p.m.) in 1 ml.

"Microsomes" were prepared by centrifuging 250 ml of supernatant, which had been submitted to centrifugation at 15,000 × g, for 92,000 × g for 1 hour. The particles were suspended in 1 ml of 0.25 M sucrose, and 0.2 ml was used in each experiment.

Supernatant enzymes were obtained by precipitating proteins from 5 ml of the supernatant (from the 92,000 × g centrifugation) by adjusting to 80% saturation with respect to $(NH_4)_2SO_4$. The precipitate was dissolved in 1 ml of 0.9% NaCl. Protein, 1.6 mg, was used in each experiment. Except when noted, reaction conditions were described in Fig. 1.

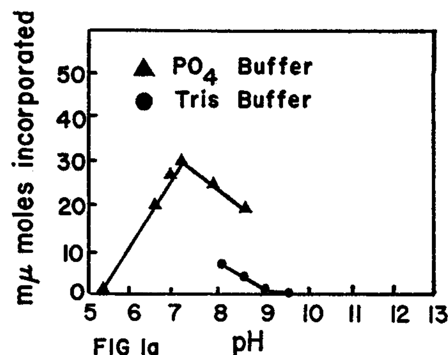
Substrate	Enzyme source	Substrate incorporated
		mμmoles
Acetate	Particle acetone powder	7.2
Acetate	Particle acetone powder + supernatant	6.1
Acetate	Supernatant	0.4
Acetyl-CoA	Particle acetone powder	120
Acetyl-CoA	Particle acetone powder + supernatant	54
Acetyl-CoA	Supernatant	0.9
Acetyl-CoA	Particle acetone powder + "microsomes"	16.2
Acetyl-CoA	"Microsomes"	0.5

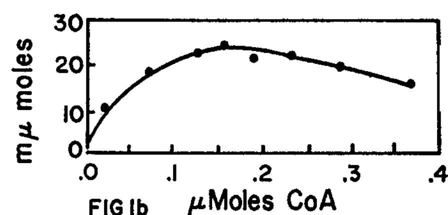
oxidize fatty acids[1] and carry out oxidative phosphorylation (17). The small particles that are centrifuged at 100,000 × g and the supernatant fraction derived from this centrifugation have essentially no lipid synthetic activity and in fact inhibit the incorporation of acetate (see Table I).

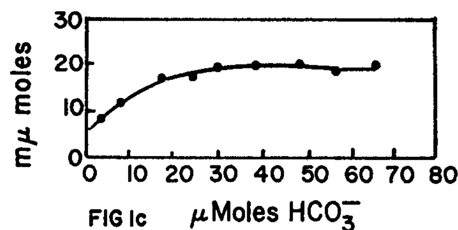
The fatty acids synthesized by the soluble extract of the acetone powders were consistently found in triglycerides and phospholipids and not as free fatty acids. However, when this extract is centrifuged at 140,000 × g for 90 minutes, a small pellet is obtained that has no capacity to form fatty acids from C^{14}-acetate but does rapidly incorporate stearate or palmitate into triglycerides in the presence of ATP and CoA. Although the supernatant fraction retains all the capacity to synthesize palmitate and stearate from acetate, these acids are now free and not esterified with glycerol or CoA (Table II). Although Lynen (18) reports that the fatty acid-synthesizing system isolated from yeast is centrifuged down quantitatively at 140,000 × g for 75 minutes, in the present case, the synthesizing system remains in the supernatant fraction.

Closer investigations indicate that the pellet, obtained by prolonged centrifugation of particle acetone powder extracts, is derived from "microsomal" particles. With a fresh supernatant fraction freed from mitochondrial particles by centrifugation at 12,000 × g for 30 minutes, a "microsomal" pellet is obtained after centrifugation for 140,000 × g for 90 minutes which contains active systems for the synthesis of triglycerides and phospholipids. Presumably, in the preparation of our standard mitochondrial fraction, a considerable number of "microsomal" particles contaminate the fraction and account for the synthesis of glycerol lipids in fresh mitochondrial preparations and in extracts of acetone powders of these preparations. All the work to be reported here is with nonultracentrifuged extracts of particle acetone powder that catalyzes long chain fatty acid esterification into triglyceride.

[1] Dr. James Nance, personal communication.







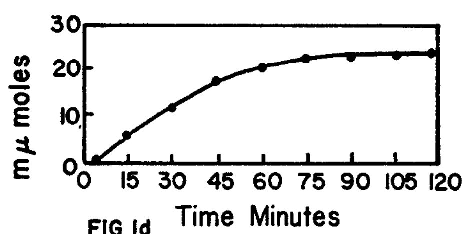


FIG. 1. *a*, effect of pH on acetate-1-C^{14} incorporation; *b*, effect of CoA; *c*, effect of HCO_3^-; *d*, effect of time. The usual reaction mixture contained: ATP, 6 μmoles; CoA, 0.133 μmoles; TPN, 0.067 μmole; Mn^{++}, 1.25 μmoles; glucose-6-P, 1 μmole, added HCO_3^-, 30 μmoles; acetate-1-C^{14}, 0.10 μmole; protein, 6 mg; and potassium phosphate buffer, pH 7.1 (including buffer enzyme extract), 0.1 mmole. The total volume of the reaction mixture was 1 ml, and the reaction time was 1½ hours.

As shown in Fig. 1*a*, the enzyme system[2] has optimal activity at pH 7.1 in 0.2 M phosphate buffer. Tris buffer at 0.2 M and pH 8.0 seemed to be inhibitory. Optimal incorporation of acetate occurred at an ATP concentration of approximately 6

[2] Extract of particle acetone powder centrifuged at 25,000 × g for 10 minutes.

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TABLE II

Incorporation of fatty acid precursors by high speed supernatant of particle acetone powder

Reaction mixtures contained 0.1 μmole of acetate, or 0.22 μmole of acetyl CoA, or 0.12 μmole of malonyl-CoA. Calculation of incorporation of malonyl-1,3-C^{14} CoA included loss of 1 equivalent of CO_2. The supernatant and particles were obtained by centrifuging the particle acetone powder extract for 90 minutes at 140,000 × *g*. The unwashed particles were suspended in two-thirds the original volume of the extract. Except when noted the reaction conditions were the same as described in Fig. 1.

Substrate	Enzyme	Cofactor omitted	Substrate incorporated	
			mμmoles	%
Acetate-1-C^{14}	Supernatant		37	37
Acetyl-1-C^{14}-CoA	Supernatant	CoA	78	35
Acetyl-1-C^{14}-CoA	Supernatant	ATP, CO_2	1	1
Malonyl-1,3-C^{14}-CoA	Supernatant	CoA, ATP	41	34
Acetate-1-C^{14}	Particles		0.2	0.2
Acetyl-1-C^{14}-CoA	Particles	CoA	2.6	1.2
Malonyl-1,3-C^{14}-CoA	Particles	ATP, CoA	1.2	1.0

TABLE III

Effect of cofactors and avidin on incorporation of acetyl-CoA and malonyl-CoA

Reaction mixtures contained 188 mμmoles of acetyl-1-C^{14}-CoA (86,000 c.p.m.) and 310 mμmoles of malonyl-1,3-C^{14}-CoA (12,280 c.p.m.). Calculation of malonyl-1,3-C^{14} incorporation includes loss of 1 equivalent of CO_2. Avidin, 250 μg, was used in each case. Except where indicated, the reaction conditions were the same as described in Fig. 1.

Substrate	Cofactor omitted	Avidin	Substrate incorporated
			mμmoles
1. Acetyl-CoA	Complete (0.26 μmole of CoA)	−	49
Acetyl-CoA	−CoA	−	122
Malonyl-CoA	Complete	−	179
Malonyl-CoA	−ATP, CoA, HCO_3^-	−	172
2. Acetyl-CoA	−CoA	−	125
Acetyl-CoA	−CoA	+	8.0
Malonyl-CoA	−ATP, CoA, HCO_3^-	−	155
Malonyl-CoA	−ATP, CoA, HCO_3^-	+	161

TABLE IV

Inhibition of acetate-1-C^{14} incorporation into fatty acids by avidin

Reaction conditions were the same as in Fig. 1, except with the addition of avidin.

Avidin	Incorporation
μg	%
0	100
20	28
50	8.2
100	6.0
150	5.7
200	4.5
300	4.4
400	3.1

μmoles per ml. Optimal concentration for TPN^+ and CoA were approximately 0.067 μmole per ml and 0.175 μmole per ml, respectively. Higher concentrations of CoA cause inhibition (Fig. 1*b*). Manganous ions at a concentration of 1.25 μmoles per ml gave optimal stimulation. A concentration of 1 μmole per ml of either glucose-6-P or isocitric acid was arbitrarily used as a substrate of TPNH generation; however, a definite requirement was never observed for these substrates. Maximal incorporation occurred with approximately 30 μmoles per ml of bicarbonate, Fig. 1*c*. With 100 mμmoles of C^{14}-acetate as the substrate, incorporation into fatty acids leveled off after 90 minutes of incubation at 31°. At higher temperatures, up to 40°, there was no effect in total incorporation or the type of fatty acids produced. The rate of incorporation of acetate-1-C^{14}, acetyl-1-C-^{14}-CoA and malonyl-1,3-C^{14}-CoA varied somewhat from one enzyme preparation to another with the total amount incorporated in 90 minutes being from 10 to 65%. However, most preparations gave an incorporation of 30 to 40% when 6 mg of protein were used.

As indicated in Table II, the incorporation of acetyl-1-C^{14}-CoA into long chain fatty acids is dependent on ATP and HCO_3^-, whereas with free acetate-1-C^{14}, CoA, ATP, and HCO_3^- are essential components. The incorporation of malonyl-1,3-C^{14}-CoA into fatty acids is independent of CoA, ATP, or HCO_3^-. Because of the presence of malonyl-CoA decarboxylase and deacylase in the system, the interdependence of acetyl-CoA and malonyl-CoA is difficult to demonstrate.

The incorporation of labeled malonyl-CoA into fatty acids is not affected by the addition of avidin, whereas incorporation of acetyl-CoA is greatly inhibited by avidin (Table III). As shown in Table IV, the addition of 100 μg of avidin caused almost the maximal inhibition of acetate incorporation with 6 mg of protein in the reaction mixture. After this point, the addition of larger quantities of avidin produced smaller increments of inhibitions. It is consistently observed that complete inhibition at higher avidin concentrations is never obtained. These results support the present hypothesis of fatty acid synthesis proposed by Wakil (1), Gibson *et al.* (18), Lynen (2), and Brady (19) in which malonyl-CoA is the prime condensing unit in fatty acid synthesis.

A series of other inhibitors were tested with the synthesizing system. Arsenite at 10^{-3} M inhibited the incorporation of acetate by 70% and at 10^{-4} M inhibited 45%. This is in accord with the results of Brady (3) and Lynen (20), which suggests the involvement of juxtaposed sulfhydryl groups at the active site for long chain fatty acid synthesis. Arsenate, cyanide, and azide at 5 × 10^{-4} M had no inhibitory effect on acetate incorporation.

Product of Reaction—Table V shows the composition of the lipids isolated from the reaction mixture.[3] It is apparent that the majority of the synthesized fatty acids has been incorporated into glyceride and phospholipid moieties. On hydrolysis of the

[3] Unfortunately, the particle acetone powder extract contains the tricarboxylic acid cycle that affords a drain on the substrates used for fatty acid synthesis. With acetate-1-C^{14} and acetyl-1-C^{14}-CoA (and to a lesser extent malonyl-1,3-C^{14}-CoA) as the substrate, the formation of citric, glutamic, succinic, and malic acids has been shown by paper chromatography. From 15 to 40% of the radioactivity from the substrate added may be found in these compounds. Because the fatty acid-synthesizing activity of the extract declines sharply by dialysis, it has not been possible to remove the tricarboxylic acid cycle intermediates and thereby prevent the drain of acetyl-CoA away from fatty acid synthesis.

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total lipid, palmitic and stearic acids occur as the predominate acids (Table IV). A trace of arachidic acid also is formed. At the beginning of these studies, no unsaturated fatty acid was synthesized; however, some of the more recent enzyme preparations have catalyzed the synthesis of a small amount of an unsaturated fatty acid that has been tentatively identified as oleic acid. Thus, when enzymically synthesized free fatty acids are chromatographed in a solvent system consisting of acetic acid-formic acid-H_2O_2, there is a decrease in the palmitic acid peak with a concurrent appearance of a hydroxy acid peak.

Malonyl-CoA has been observed in the reaction mixture when aged enzyme (extract particle acetone powder aged 24 hours in deep freeze) was incubated with either acetate-1-C^{14} or acetyl-1-C^{14}-CoA as the substrate. After extraction with chloroform-methanol 1 ml of concentrated NH_4OH was added to the aqueous, phase, and ammonolysis was carried out for 10 minutes at 45°. The aqueous phase was evaporated down to 0.1 to 0.2 ml; then 8 ml of acetone were squirted in to mix the small amount of water rapidly with the acetone.[4] After the salts were centrifuged down, the acetone supernatant was evaporated to a very small volume, and samples were cochromatographed with synthetic monomalonamide in ethanol-NH_3-H_2O and pentanol saturated with 5 M formic acid. The synthesis of acetyl-CoA from acetate-1-C^{14} and CoA and the products of the malonyl-CoA deacylase (free malonic) and decarboxylase (acetyl-CoA) have been observed by the same technique.

To prove that there is a synthesis *de novo* of fatty acids and not simply a chain elongation of existing fatty acids, chromic acid oxidation of the biosynthesized fatty acids was carried out (21). By this procedure, the hydrocarbon chain is oxidized to CO_2 until the penultimate CH_3CH_2-group is reached, and this is then converted to acetic acid. After chromic oxidation, the acetic acid was steam-distilled into concentrated ammonia. To a 4 ml sample was added 0.05 ml of 1 M Hyamine in methanol, and the sample was taken to dryness with the addition of small amounts of methanol at frequent intervals. The dried sample was dissolved in toluene-phosphor solution and counted by the Packard liquid scintillation spectrometer. Table VI shows that the expected isotope content in the acetic acid derived from the terminating C_2 unit of the long chain fatty acid was obtained, thereby indicating a synthesis *de novo* of fatty acids to palmitic acid and stearic acid. No evidence could be obtained to suggest chain lengthening with C^{14}-palmitic acid and cold acetate as substrate, because labeled stearic acid was never formed. Palmitic acid was activated as the CoA derivative because it was readily incorporated into glycerides, providing both ATP and CoA were present. No short chain fatty acids appeared as intermediates during the synthesis of palmitic acid and stearic acid.

Table V

Reaction product produced by particle acetone powder extract and type of fatty acids isolated after hydrolysis

A. Relative percent of activity in reaction products. Experiment 1. Lipid extracts of five standard reaction mixtures combined. Experiment 2. Lipid extracts of six standard reaction mixtures combined. Reactions and separations carried out as described in Fig. 1 and text.

Fraction	Experiment 1		Experiment 2	
	C.p.m.	%	C.p.m.	%
Hydrocarbons	2,400	2.0	5,565	2.0
Free fatty acids	7,080	5.0	10,210	5.0
Monoglycerides	5,890	4.0	1,830	1.0
Diglycerides	15,610	11.0	9,250	4.5
Triglycerides	47,730	33.0	57,200	29
Phospholipids	62,040	44.0	116,060	58

B. Types and percentages of fatty acids synthesized.

Sample	Palmitic	Stearic	Arachidic	Oleic
	%	%	%	%
1. January	41	59	Approximately 1	
2. March	37	63	Approximately 1	
3. July	16	79		5

Table VI

Chromic acid oxidation

Each sample was digested for 6 hours with 300 mg of chromic acid and 3 ml of sulfuric acid (1 ml of concentrated H_2SO_4 and 2 ml of H_2O) under gentle reflux with a double-walled condensor. From 1 to 2 mmoles of acetic acid were added, and the mixture was steam-distilled. Theoretical calculations were based on the fact that these fatty acid samples had 25% palmitic and 75% stearic acid as determined by paper chromatography.

	C.p.m.	Theoretical 25% C_{16} 75% C_{18}	Theoretical
			%
(*a*) Initial fatty acids	90,400		
Acetic acid	11,125	10,655	104
(*b*) Initial fatty acids	139,000		
Acetic acid	16,380	15,915	102
(*c*) Initial fatty acids	183,000		
Acetic acid	20,250	21,060	96

DISCUSSION

The fatty acid-synthesizing systems in the avocado mesocarp seem to differ from the systems reported by others (1–4) working with animal systems in that the sole site of synthesis is associated with "mitochondrial" particles. In addition, the site for triglyceride and phospholipid synthesis seems to be in the "microsomal" particle. There is no evidence that there is a soluble enzyme complex in the cytoplasm for the synthesis of fatty acids. Whereas fresh mitochondria readily synthesize palmitic, stearic, and oleic acids, in the water-soluble extracts of acetone powders obtained from these "mitochondrial" particles, the capacity to form oleic acid has been mostly lost. Also of interest is the consistent failure to elongate added palmitate to stearate by the addition of acetyl-CoA or malonyl-CoA to palmitate in the presence of ATP and CoA, although the system readily synthesizes stearate from a mixture of acetyl-CoA and malonyl-CoA.

Of interest from the comparative point of view is that the actual mechanism of synthesis seems to be identical with that described in mammalian and yeast preparations. The avidin sensitivity of the system, the incorporation of malonyl-CoA into fatty acids, and the requirement for CO_2 and ATP for the incorporation of acetyl-CoA all support the current concept of saturated fatty acid synthesis.

[4] Extraction procedure developed by Dr. M. D. Hatch of this department.

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SUMMARY

Water extracts of acetone powders of mitochondrial particles of avocado mesocarp readily incorporate acetate into palmitate and stearate in the presence of carbon dioxide, adenosine triphosphate, coenzyme A, Mn^{++}, and reduced triphosphopyridine nucleotide-generating system. The system is very sensitive to avidin. Acetylcoenzyme A and malonylcoenzyme A together are readily incorporated into palmitate and stearate, but the system does not require adenosine triphosphate, coenzyme A, or carbon dioxide and is avidin-insensitive. The site for triglyceride synthesis seems to be the "microsomal" fraction.

Acknowledgment—We wish to thank Miss Susan Woodbury, Mrs. George Ann Brady, and Mr. H. Sah for their assistance in the preparation of acetone powders of the avocado particles.

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