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

Fat Metabolism in Higher Plants*

XIV. FACTORS AFFECTING THE SYNTHESIS OF OLEIC ACID BY PARTICULATE PREPARATIONS FROM AVOCADO MESOCARP

J. B. MUDD† AND P. K. STUMPF‡

From the Department of Biochemistry and Biophysics, University of California, Davis, California

(Received for publication, March 24, 1961)

Numerous investigations have led to the point at which refined enzyme systems may be used to study the biosynthesis of saturated fatty acids (2, 3). The biosynthesis of unsaturated fatty acids has not been so thoroughly elucidated. Formation of unsaturated fatty acids has been observed experimentally *in vivo* (4–9) and *in vitro* (10, 11) but several of these studies showed that incorporated radioactivity was distributed in the fatty acids in proportions quite different from the naturally occurring fatty acid composition. Whenever differences were found, the proportion of unsaturated fatty acids was low.

Early experiments with slices, homogenates, and cell-free preparations from avocado mesocarp produced variable amounts of oleic and saturated fatty acid. It was, therefore, not feasible to compare the avocado system with that in a strain of *Saccharomyces cerevisiae*, which converted saturated fatty acids to unsaturated fatty acids by reactions requiring reduced triphosphopyridine nucleotide and oxygen (11).

This report described conditions under which oleic acid is the principal acid formed from acetate by particulate preparations from avocado mesocarp. It is shown that oxygen is an absolute requirement for oleate biosynthesis in avocado.

EXPERIMENTAL PROCEDURE

Material and Methods—Avocados of the Fuerte variety were customarily used, but MacArthur and Haas variety also were used on occasion as a source of slices, homogenates, and cell-free preparations. In the preparation of the particulate fraction, the mesocarp of one avocado, about 200 g, was ground in a chilled mortar with 250 ml of ice-cold 0.25 M sucrose. The homogenate was pressed through cheesecloth, and the filtrate was centrifuged at 2000 × *g* for 10 minutes in a refrigerated centrifuge. The aqueous layer was drawn from between the sedimented debris and the floating layer of fat and centrifuged again at 15,000 × *g*. The supernatant was decanted, and the pellet (2 to 5 ml) was resuspended in 0.25 M sucrose (5 to 10 ml). This suspension was used as the source of the fatty acid-synthesizing enzymes. The suspension contained 8 to 10 mg of protein per ml as determined by the Nessler method.

The cofactor requirements for the incorporation of acetate into lipid by avocado particulate preparations have been established (10, 12). For the experiments described in this paper, with exceptions noted in the relevant tables and figures, the following amounts of cofactors were used: ATP, 10 μmoles; $KHCO_3$, 30 μmoles; $MnCl_2$, 2 μmoles; GSH, 1 μmole; glucose-6-P, 500 mμmoles; TPN^+, 65 mμmoles; CoA, 50 mμmoles. Other conditions and components of the reaction mixture are as indicated in tables and figures. Reactions were conducted in 12-ml centrifuge tubes with glass stoppers, which were held in a water bath at 31° without shaking. After the desired reaction period, usually 1 hour, 0.1 ml of 2 N HCl was added to the 1.5 ml reaction mixture, and the lipid was extracted in the centrifuge tube, according to the method of Bligh and Dyer (13).

The chloroform solution of lipid was withdrawn, and an aliquot was counted to determine the amount of incorporation of acetate into lipid. The remainder of the sample was saponified by refluxing with 5 ml of 5% ethanolic potassium hydroxide for 15 minutes. Extraction of the alkaline solution with chloroform showed that the incorporation of acetate into nonsaponifiable lipid did not exceed 5%, and because the nonsaponifiable lipid did not interfere with the separation of fatty acids, this step was subsequently omitted. In the routine procedure, the saponification mixture was acidified with 2 ml of 6 N HCl and extracted with chloroform. Without special measures being taken, the recovery of radioactivity in this step was 90 to 95%.

The fatty acids were chromatographed on filter paper impregnated with silicone (Dow Corning 200). With 85% acetic acid as the developing solvent, palmitic and oleic acids were coincident (14). With acetic acid-formic acid-hydrogen peroxide, 6:1:1, as the developing solvent, the unsaturated fatty acids were oxidized, and the products ran close to the solvent front (15). Comparison of chromatograms run in these two solvents permitted the identification of palmitic, stearic, and oleic acids, which were the principal acids labeled.

Organic acids were assayed by the procedure of Hatch and Stumpf.[1] After extraction of lipid from the reaction mixture, the acid aqueous phase was evaporated to 0.1 to 0.2 ml, then 10 ml of acetone added. The precipitated salt was sedimented by centrifugation and the acetone solution decanted. An aliquot was dried on a planchet and counted. The remainder was reduced to a suitable volume for chromatography with pentanol saturated with 5 M formic acid (16) and ethanol-ammonia-water, 80:4:16 (17) as developing solvents.

* For Paper XIII, see (1).

† Fellow of The Jane Coffin Childs Memorial Fund for Medical Research. This investigation has been aided by a grant from the Jane Coffin Childs Memorial Fund for Medical Research. Present address, Department of Plant Biochemistry, University of California, Riverside, California.

‡ Aided in part by a grant from the National Science Foundation (G-4582).

[1] M. D. Hatch and P. K. Stumpf, unpublished experiments.

Lipid before saponification was separated by reversed phase paper chromatography and by chromatography on columns of silicic acid (14, 18).

Oleic acid was oxidized by the method of von Rudloff (19). The oxidation products were separated by the method of Isherwood and Hanes (20).

Radioactivity was counted with a Packard scintillation counter or a Nuclear-Chicago thin window gas flow counter. Chromatograms were counted in a Nuclear-Chicago strip counter, and the percentage composition was determined by measuring the areas of the peaks planimetrically.

RESULTS

Intracellular Location of Fatty Acid Synthesis—Because of numerous reports that the enzymes catalyzing fatty acid synthesis are soluble (21, 22), the localization of fatty acid synthesis in avocado mesocarp was re-examined. The data in Table I show that the activity was associated with a particulate fraction sedimenting between 2,000 and 15,000 × *g*. This association was true for preparations made either in sucrose or phosphate-bicarbonate. The supernatant is inactive alone, and when combined with the particles is actually inhibitory. The acetate incorporated into fatty acids was found in triglycerides (60 to 70%) and phospholipid (30 to 40%); only occasionally was labeled free fatty acid detected. When some particles were sedimented between 2,000 and 6,000 × *g* and others between 6,000 and 15,000 × *g*, most of the particles and most of the activity was in the heavier fraction. Both fractions, however, synthesized 45 to 50% palmitic and the remainder oleic acid. In addition to catalyzing the synthesis of fatty acids and glycerides, the enzymes of the particulate fraction catalyze the interconversion of intermediates of the tricarboxylic acid cycle, and β-oxidation of long chain fatty acids.

In early experiments with acetate-1-C^{14}, the results of which are shown in Table II, a surprising distribution of the incorporated label was found. The labeled acids were predominantly saturated. Stearic acid which is found only in trace amounts in avocado, was well represented. Oleic acid, which forms about 70% of avocado fatty acids, seldom accounted for more than 30% of the incorporated activity. Furthermore, the amount of labeling in oleic acid was unpredictable from experiment to experiment. The unusual distribution was apparently not an artifact brought about in the isolation of the particles, because tissue slices and homogenates showed the same anomalies. Before experiments on the mechanism of oleate biosynthesis were performed, it was desirable to establish experimental conditions under which oleate was the principal acid labeled. These conditions were arrived at empirically.

Position of Double Bond in Octadecenoic Acid—After incubation of the particulate preparation with acetate-1-C^{14}, the labeling of an octadecenoic acid was evident from the gas chromatographic and paper chromatographic separation of the reaction products. These methods did not, however, distinguish the position of the double bond. It was, therefore, shown that the product was Δ9-octadecenoic acid by cleaving the labeled acid with permanganate-periodate and tentatively identifying the reaction products as azelaic and pelargonic acids. A sample of biosynthesized radioactive fatty acids (59,000 d.p.m.) containing 39% oleic acid as determined by paper chromatography (14, 15), was oxidized (19). The oxidation products (38,000 d.p.m.) were chromatographed in two systems. With propanol-ammonia (7:3) as the developing solvent (20), 10% of the radioactivity cochromatographed with azeleate, and the remainder with the monocarboxylic acids (pelargonate, palmitate, stearate). With 85% acetic acid as the developing solvent (14), 30% of the radio-

TABLE I

Localization of acetate incorporating ability

Reaction mixtures contained: phosphate buffer, pH 8.0, 50 μmoles; acetate-1-C^{14} (800,000 d.p.m.), 250 mμmoles; cofactors as in material and methods; particle suspension and supernatant as indicated; final reaction volume, 3.0 ml; reaction period, 2 hours.

Experiment	Acetate incorporated
	mμmoles
1. Grinding in 0.25 M sucrose	
Particle suspension, 1 ml	1.34
Supernatant, 1 ml	0.05
Particles, 1 ml + supernatant, 1 ml	0.68
2. Grinding in 0.1 M phosphate-bicarbonate pH 8.0	
Particle suspension, 1 ml	3.74
Supernatant, 1 ml	0.02
Particles, 1 ml + supernatant, 1 ml	2.97

TABLE II

Distribution of radioactivity incorporated by avocado preparations from acetate-1-C^{14}

Reaction mixtures for Experiments 1 to 3 contained: of phosphate buffer, pH 8.0, 100 μmoles; acetate-1-C^{14}, (1.6×10^6 d.p.m.), 500 mμmoles; double the quantities of cofactors in material and methods; and 1-g slices of avocado mesocarp or an equivalent amount of homogenate. The slices were cut from a cylinder of avocado mesocarp taken with a 5-mm cork borer. Reaction mixtures for Experiments 4 and 5 contained 50 μmoles phosphate buffer, pH 8.0, 50 μmoles; acetate-1-C^{14} (800,000 d.p.m.), 250 mμmoles; cofactors as in material and methods; particles suspension, 0.5 ml; final volume, 1.5 ml. Methyl esters were prepared from extracted lipid by interesterification with 5% HCl in methanol, and separated by gas chromatography. Effluent esters were trapped in chloroform on glass wool in a glass bulb. They were eluted with chloroform into a scintillation counter vial, the solvent was evaporated, and scintillation mixture was added. Radioactivity was measured in a Packard scintillation counter. Recovery of radioactivity was about 60%.

Material	Acetate incorporated	Distribution of label*		
		Palmitate	Stearate	Oleate
	mμmoles	%	%	%
1. Slice	20.0	48.5	10.5	31.5
2. Slices	25.0	38	13	45
Homogenate	36.5	27	14.5	54
3. Homogenate	4.3	54.5	22	17.5
4. Particles	39.0	68.5	6	12.9
5. Particles	36.0	42	21.5	23
Normal fatty acid composition†		12	0	74

* Some label in acids of chain length less than 16, and in linoleate.

† Also palmitoleic 2%; linoleic 12%.

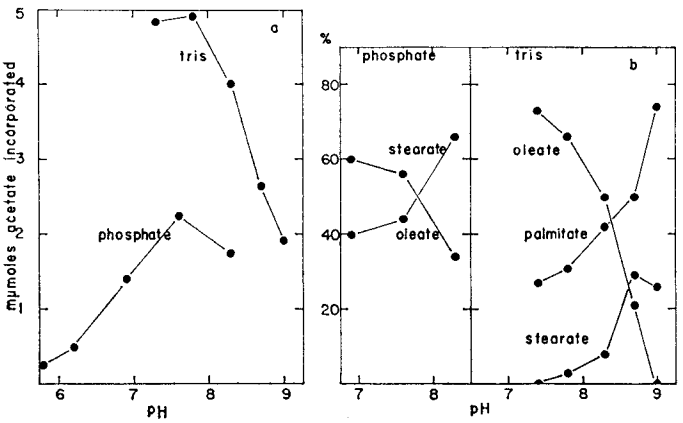

FIG. 1. *a*. Effect of pH on the incorporation of acetate into lipid. *b*. Effect of pH on distribution of label. Reaction mixtures contained: Tris-HCl, 100 μmoles, or phosphate buffer, 500 μmoles; acetate-1-C^{14} (400,000 d.p.m.) 50 mμmoles; particle suspension, 0.3 ml; other cofactors and conditions as in "Material and Methods;" final volume, 1.5 ml.

TABLE III

Effect of temperature on incorporation of acetate into fatty acids

Reaction mixtures contained: Tris-HCl, pH 7.8, 100 μmoles; acetate-1-C^{14} (800,000 d.p.m.), 250 mμmoles; particle suspension, 0.3 ml; other cofactors and conditions as in material and methods; final volume, 1.5 ml.

Experiment	Temperature	Acetate incorporated	Distribution of label		
			Palmitate	Stearate	Oleate
			%	%	%
1	2°	0.08			
	13°	0.29			
	25°	2.49	72.5	27.5	0
	31°	4.94	48	34	18
	39.5°	8.11	38	24	38
2	23°	3.77	77.5	22.5	0
	31°	8.84	52.5	28.5	19
	40°	19.98	32.5	37.5	30
	46°	20.80	32.5	29.5	38

activity chromatographed with pelargonic and azelaic acids, which are not separated in this solvent, and the remainder with palmitate and stearate. Beside demonstrating that the unsaturated product was Δ9-octadecanoic acid, these results showed synthesis *de novo* of the long chain fatty acid rather than addition of a C2 unit to a pre-existing carbon chain.

Effect of pH—The incorporation of acetate into lipid by avocado particles had a pH optimum about 8. At final concentrations of 0.067 M, results in phosphate and Tris buffers were in good agreement, but variation in phosphate concentration changed the amount of incorporation; at higher concentrations of phosphate, incorporation was inhibited (Fig. 1). Tris is inhibitory in the avian liver system (22), and has also been found to inhibit fatty acid synthesis catalyzed by extracts of acetone powder of avocado mitochondria.[2] The effect of changing the concentration of phosphate in experiments with avocado mitochondria may be related to effects on mitochondrial swelling (23). The incorporation of acetate into oleate was favored at the optimal pH, and declined rapidly as the pH was raised. The decline in oleate was compensated by an increase in the percentages of palmitate and stearate (Fig. 1).

Effect of Temperature—Incorporation of acetate into lipid increased as the temperature was raised. At 46°, there was indication that some heat inactivation had taken place as there was only a slight increase at that temperature over the incorporation at 40° (Table III). Saponification of the lipid and separation of the fatty acids showed that the incorporation into oleate was favored at the higher temperatures. Time course of acetate incorporation was followed at two temperatures (Fig. 2). This data established no precursor-product relationship among the different fatty acids, although it is noticeable that oleate production declined at later times, and this decline was matched by an increase in the rate of synthesis of the saturated fatty acids, particularly stearate.

Effects of Variations in Concentrations of Acetate, Bicarbonate, and Sucrose—Variation in acetate concentration and bicarbonate concentration influenced the amount of radioactive lipid formed, but did not affect the composition of the radioactive fatty acids. Sucrose concentration changes had no effect either on incorporation of acetate into total lipid or on distribution of incorporated activity.

Effect of Varying Particle Concentration—Variation in the amount of particle suspension added to the reaction mixture consistently resulted in inhibition of lipid synthesis at higher particle concentrations (Fig. 3). The distribution of radioactivity in the fatty acids also was affected by variations in particle concentration; as particle concentration increased, the proportion and amount of oleate declined sharply (Fig. 3). These observations suggested that the particle suspension might contain a regulatory factor which inhibited lipid synthesis in general and oleate synthesis in particular.

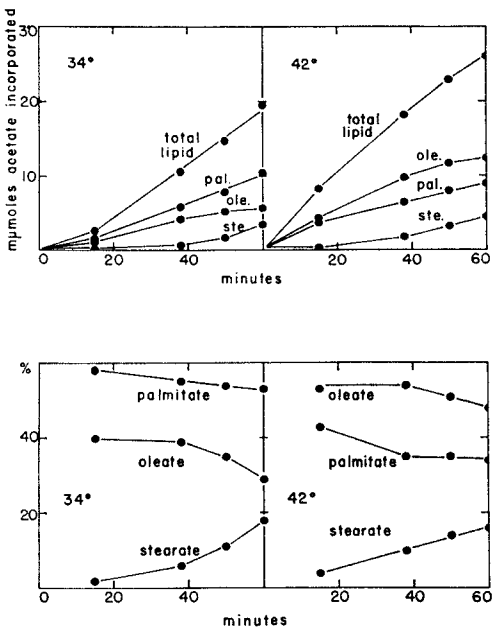

FIG. 2. Time course of acetate incorporation at different temperatures. Reaction mixtures contained Tris-HCl, pH 7.8, 100 μmoles, and acetate-1-C^{14} (400,000 d.p.m.), 59 mμmoles. Other cofactors and conditions as indicated in "Methods and Materials," except for variation in temperature and duration of reaction; final volume, 1.5 ml.

[2] E. J. Barron and P. K. Stumpf, unpublished experiments.

Inhibition by Free Fatty Acids—An emulsion was made of lipid extracted from a particulate preparation from avocado mesocarp. Incorporation of acetate into lipid by a freshly prepared suspension of particles was inhibited by the emulsion, the degree of inhibition being proportional to the amount of emulsion added. The crude lipid was separated into neutral lipid and phospholipid (18), and these fractions were tested for inhibitory activity. Table IV shows that the inhibitory activity was in the neutral lipid fraction. The glycerides were then separated from free fatty acids by extraction with chloroform from an alkaline aqueous phase. The aqueous phase was acidified, and the free fatty acids were extracted. Comparison of the inhibitory action of these fractions showed that the free fatty acids strongly

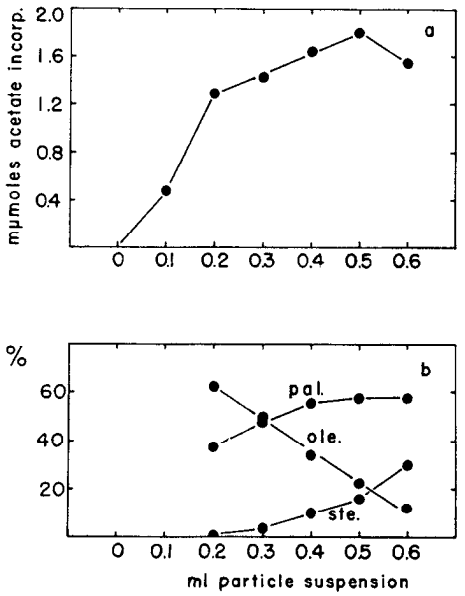

FIG. 3. *a*. Effect of particle concentration on incorporation o acetate into lipid. *b*. Effect of particle concentration on distribu tion of label in fatty acids. Reaction mixtures as in Fig. 2 except for variation in particle concentration. *pal*, palmitate; *ole*, oleate; *ste*, stearate.

TABLE IV

Effect of neutral lipid and phospholipid on incorporation of acetate

Reaction mixtures contained: phosphate buffer, pH 8.3, 50 μmoles; acetate-1-C^{14} (800,000 d.p.m.), 250 mμmoles; particle suspension, 0.3 ml; amounts of lipids in emulsion as indicated; other cofactors and conditions as in materials and methods; final volume, 1.5 ml.

Additions	Amount of lipid	Acetate incorporated
	mg	*mμmoles*
None		8.5
Neutral lipid	0.39	6.9
Neutral lipid	0.78	5.2
Neutral lipid	1.56	4.6
Neutral lipid	2.34	4.6
None		8.6
Phospholipid	0.12	8.6
Phospholipid	0.23	11.2
Phospholipid	0.46	12:1
Phospholipid	0.69	13.5

TABLE V

Effect of triglyceride and free fatty acid on incorporation of acetate

Reaction mixtures as in Table IV.

Additions*	Amount of addition	Acetate incorporated
		mμmoles
None		7.0
Triglyceride	490	5.4
Triglyceride	980	5.0
Triglyceride	1960	4.5
Triglyceride	2940	4.1
None		6.8
Free fatty acid	72	4.9
Free fatty acid	144	4.6
Free fatty acid	288	2.9
Free fatty acid	432	2.1

* A molecular weight of 926 was assumed for triglyceride, and 280 for free fatty acid.

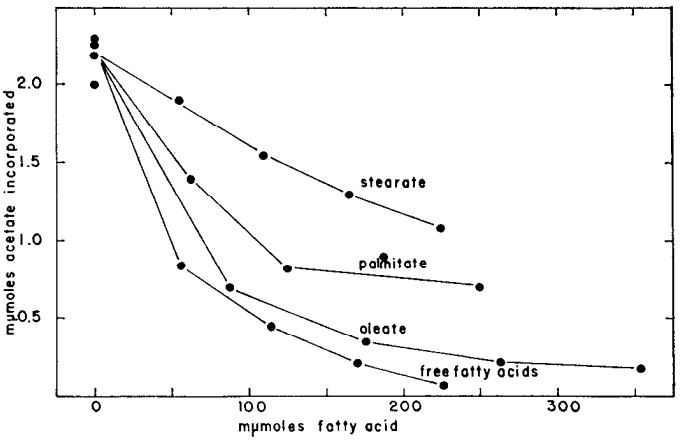

FIG. 4. Inhibition of acetate incorporation by free fatty acids. Reaction mixtures contained: phosphate buffer, pH 8.0, 50 μmoles; acetate-1-C^{14}, 250 mμmoles; particle suspension, 0.3 ml; cofactors and conditions as in "Material and Methods;" final volume, 1.5 ml. Acids added as potassium salts.

inhibited incorporation of acetate into long chain fatty acids (Table V). When the free fatty acid fraction was compared with authentic fatty acids, it corresponded most closely to oleic acid (Fig. 4). Oleate was used for further study on the inhibitory effect. Although oleate strongly inhibited incorporation of acetate into fatty acids, it did not affect the distribution of the incorporated activity.

Inhibition by oleate was alleviated but not completely reversed by albumin, which is a known scavenger for free fatty acids. The site of inhibition by oleate is suggested by the parallel inhibition of incorporation of acetate into lipid and organic acids (Table VI). Since both of these incorporations require the intermediate participation of acetyl-CoA, it appeared that oleate interfered with its formation. Inasmuch as it is known that the particulate preparation incorporates long chain fatty acids into glycerides by reactions requiring ATP and CoA, the most likely explanation of the effect of fatty acids is that they efficiently compete for ATP and CoA with acetate. According to this interpretation, it should be possible to reverse the effect of oleate on incorporation of acetate into lipid by increasing the

TABLE VI

Inhibition by oleate of acetate incorporation into lipid and organic acids

Reaction mixture contained: Tris-HCl, pH 7.8, 100 μmoles; acetate-1-C^{14} (800,000 d.p.m.), 100 mμmoles; particle suspension, 0.3 ml; cofactors and conditions as in material and methods; and potassium oleate as indicated; final volume, 1.5 ml.

Oleate added	Acetate incorporated	
	Lipid	Organic acids
mμmoles	*mμmoles*	
0	5.85	7.60
78	2.45	3.40
156	2.25	2.65
234	1.80	2.50
312	1.45	2.10
390	1.10	2.55

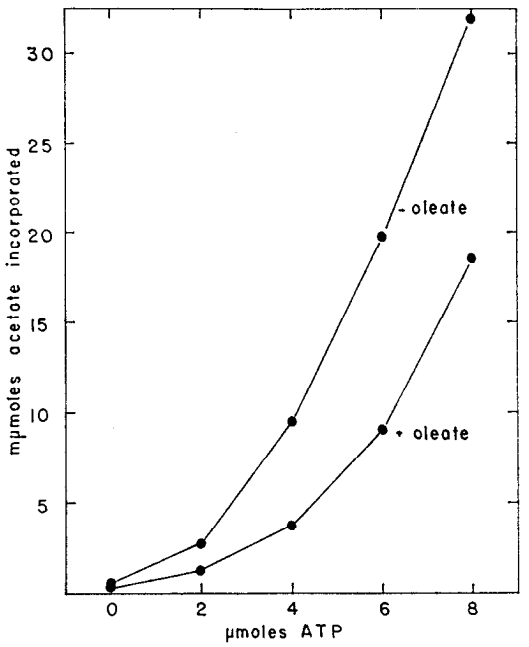

FIG. 5. Oleate inhibition at different ATP concentrations. Reaction mixtures as in Table VII except for variation in ATP concentration and addition of potassium oleate, 120 mμmoles, as indicated.

concentration of ATP. Attempts have been made to do this but results have not given a definite answer. Fig. 5 shows the effect of various ATP concentrations in the presence and absence of oleate. It is evident that there are endogenous ATP-consuming reactions from the fact that the response curve does not extrapolate to zero incorporation for zero ATP. A further difficulty is the high ATP concentrations required to saturate the system. However, the increase in ATP concentration from 6 to 8 μmoles per reaction gives approximately the same response in acetate incorporation whether oleate is present or not. This is an indication that the oleate effect is reversed by higher ATP concentrations.

Inhibition by Supernatant—The inhibitory effect of fatty acids had not explained the preferential effect of inhibition of oleate biosynthesis when particle concentration was raised. Another possibility was suggested by the observation that the supernatant obtained after spinning down the particulate fraction, not only inhibited incorporation of acetate into total lipid, but also inhibited incorporation into oleate particularly. Although the supernatant showed no activity in lipid synthesis, it was active in the formation of organic acids (Table VII). By adding varying amounts of the supernatant to a constant amount of particles in a reaction mixture the effect of increased particle concentration could be simulated (Table VIII). Boiling the supernatant removed its inhibitory effect on gross incorporation, but did not completely remove its inhibition of oleate biosynthesis. Washing the particle suspension by resuspension in 0.25 M sucrose and resedimentation was tried as a means of removing the soluble enzymes that contaminated the unwashed pellet. The washed particles showed a more linear relationship of incorporation and particle concentration, but the washed particles still showed the

TABLE VII

Inhibition of oleate biosynthesis by supernatant fraction

Reaction mixtures contained: Tris-HCl, pH 7.8, 100 μmoles; acetate-1-C^{14} (400,000 d.p.m.), 100 mμmoles; other cofactors and conditions as materials and methods; final volume, 1.5 ml. Particles were sedimented between 2,000 and 15,000 × *g*; "microsomes" were sedimented between 15,000 and 140,000 × *g*; supernatant represents soluble proteins remaining after sedimentation of "microsomes."

Addition	Acetate incorporated		Distribution of label		
	Organic acids	Lipid	Palmitate	Stearate	Oleate
	mμmoles		%	%	%
1. Particles, 0.2 ml	6.55	1.70	34	3	63
2. "Microsomes," 0.2 ml	0.75	0.25			
3. Supernatant, 0.2 ml	7.55	0.05			
4. Item 1 + item 2	5.95	2.10	35	23	42
5. Item 1 + item 3	5.15	1.05	36	52	12
6. Item 2 + item 3	7.50	0.35			

TABLE VIII

Effect of supernatant on incorporation and distribution of acetate

Reaction mixtures as in Table VII except for additions. Particles prepared as in material and methods. Supernatant, remaining after sedimentation of particles.

Additions	Acetate incorporated	Distribution of label		
		Palmitate	Stearate	Oleate
	mμmoles	%	%	%
Particles, 0.2 ml	2.25	30	0	70
Particles, 0.2 ml + supernatant, 0.05 ml	1.85	29	6	65
Particles, 0.2 ml + supernatant, 0.10 ml	1.65	35	12	53
Particles, 0.2 ml + supernatant, 0.15 ml	1.35	57	19	24
Particles, 0.2 ml + supernatant, 0.20 ml	0.95	50	30	20
Supernatant, 0.2 ml	0			
Boiled supernatant, 0.2 ml	0			
Boiled supernatant, 0.2 ml + particles, 0.2 ml	2.10	43	0	57

decline in proportion of labeled oleate at the higher particle concentrations.

Effect of Aeration—The possibility remained that some cofactor or endogenous substrate was being exhausted at the higher particle concentrations. Because the enzymic activity of the supernatant fraction appeared to be in catalyzing tricarboxylic acid cycle activity, it seemed likely that oxygen was being consumed. Reactions were therefore run at two different particle concentrations with and without aeration. The results (Table IX) showed that aeration maintained oleate synthesis even at high particle concentrations. There is a tendency for aeration to reduce gross incorporation probably by surface inactivation of the enzymes.

Absolute Oxygen Requirement for Oleate Biosynthesis—The experiences described above made it possible to choose conditions of pH, temperature, and particle concentration which would produce appreciable label in oleate when radioactive acetate was supplied to avocado particles. With this system the effect of anaerobiosis on the biosynthesis of oleate was tested. The experiments were carried out in Thunberg tubes which were evacuated and the atmosphere replaced either with air or helium. The results (Table X) clearly proved that oxygen is an absolute requirement for oleate biosynthesis.

Effect of Cyanide and Azide—Incorporation of acetate into long chain fatty acids was inhibited only to a slight extent by cyanide (2×10^{-3} M) and azide (2×10^{-3} M). This slight inhibition was not associated with decrease of oleate formation. It seems that the requirement for oxygen in the biosynthesis of oleate, therefore, does not involve a pathway of electron transport such as the cytochrome system. Attempts to substitute for oxygen with artificial electron acceptors have been thwarted by the inhibitory effects of the electron acceptors.

Metabolism of Long Chain Fatty Acids—Several of the experiments reported above and particularly those demonstrating the oxygen requirement suggest a precursor-product relationship of stearate and oleate. Palmitate and stearate were tested as substrates for a hypothetical desaturating enzyme. Both acids were readily activated as indicated by their rapid incorporation into phospholipid and triglyceride, but saponification and separation of the fatty acids showed that no detectable transformations had taken place. Experiments with decanoic acid also resulted in a rapid ATP- and CoA-dependent incorporation of label into glycerides. In this case also, saponification and identification of the labeled fatty acids indicated that the decanoic acid had been incorporated into complex lipids without chain lengthening.

TABLE IX

Effect of aeration on distribution of incorporated acetate in long chain fatty acids

Reaction mixtures as in Table VII except as indicated in this table. Aeration by slowly bubbling air through the reaction mixture.

	Acetate incorporated	Distribution of label			
		Palmitate	Stearate	Arachidate	Oleate
	mμmoles	%	%	%	%
Particles, 0.2 ml	6.5	30	5	0	65
Particles, aerated, 0.2 ml	1.5	27	0	0	73
Particles, 0.5 ml	8.0	45	28	4	23
Particles, aerated, 0.5 ml	4.6	29	0	0	71

TABLE X

Effect of oxygen on distribution of incorporated acetate in long chain fatty acids

Reaction mixtures contained: Tris-HCl, pH 7.8, 100 μmoles; acetate-1-C^{14} (800,000 d.p.m.), 250 mμmoles; cofactors as in material and methods; particle suspension, 0.3 ml; final volume, 1.5 ml; reaction time 1 hour at 40°. Reactions run in Thunberg tubes. The tubes were evacuated, and the atmosphere was replaced either with air or with helium.

	Acetate incorporated	Distribution of label		
		Palmitate	Stearate	Oleate
	mμmoles	%	%	%
Air	11.9	53	1	46
Air	10.4	48.5	5	46.5
Helium	11.8	61	39	0
Helium	10.9	66.5	33.5	0

TABLE XI

Incorporation of acetate into organic acids and fatty acids

Reaction mixtures as in Table VII (particle suspension, 0.2 ml).

	Acetate incorporated	Distribution of label
	mμmoles	%
Lipid	1.70	
Palmitate		34
Stearate		3
Oleate		64
Organic acids*	6.55	
Citrate		70
Malate		8
Succinate		12

* Other compounds tentatively identified: α-ketoglutarate 5%, glutamate 5%.

Interrelationship of Organic and Fatty Acid Metabolism—Table VI showed that there was considerable incorporation of acetate into water-soluble acids. Incorporation into this fraction usually exceeded the incorporation into lipid. The composition of the radioactive organic acids was variable, but citrate was always predominant. Some typical results are presented in Table XI. Predictably, malonate inhibited incorporation of acetate into the organic acid fraction, but of the acids formed, succinate formed a higher proportion than in the absence of malonate. Citric acid also inhibited the incorporation of acetate into organic acids. These two acids also had a striking effect on incorporation of acetate into lipid. Citrate stimulated at lower concentrations but inhibited when the concentration was raised. Citrate always lowered the proportion of incorporated radioactivity found in oleate. Malonate slightly inhibited the incorporation of acetate into fatty acids, but always increased the incorporation into oleate (Table XII).

TABLE XII

Effect of citrate and malonate on acetate incorporation into organic acids and fatty acids

Reaction mixtures as in Table VII except as indicated in this table (particle suspension, 0.2 ml).

	Amount of addition	Acetate incorporated		Distribution of label			
		Organic acids	Lipid	Palmitate	Stearate	Arachidate	Oleate
	μmoles	*mμmoles*		%	%	%	%
No additions		6.45	8.55	30	30	0	40
Citrate	5	3.15	10.8	31	51	4	14
Citrate	10	2.80	11.4	28	54	3	15
Citrate	20	2.10	9.45	29	47	2	22
Citrate	30	2.10	7.45	27	48	2	23
No additions		6.60	8.20	26	33	0	41
Malonate	5	6.25	8.50	23	12	0	65
Malonate	10	5.35	8.15	24	14	0	62
Malonate	30	3.70	7.10	21	9	0	70

DISCUSSION

The intracellular localization of fatty acid synthesis is still subject for discussion and experimentation, although perhaps not in that order. Work in Gurin's laboratory indicated that fatty acid synthesis by rat liver took place in the mitochondria (24). It was possible to solubilize the enzymes by grinding liver tissue in phosphate buffer rather than sucrose (25), or by lysing the mitochondria in water (26). On the other hand, the enzymes in rabbit mammary gland were always found in the soluble fraction (21). Most of the work done by Wakil *et al.* (22) has been with a soluble system, but they have recently rediscovered mitochondrial synthesis of fatty acids (27). Abraham *et al.* (28) have a fatty acid-synthesizing system which requires the cooperative efforts of microsomes and a soluble fraction. Although it has previously been reported that in avocado, fatty acid synthesis is a property of a particulate fraction (10), the variability in synthesizing systems briefly noted above prompted a re-examination.

The particulate fraction in which fatty acid-synthesizing activity has been found is sedimented at centrifugal forces considered to sediment mitochondria. The particles have enzymic activities usually associated with mitochondria, such as the ability to interconvert intermediates of the tricarboxylic acid cycle, and the ability to oxidize long chain fatty acids. It has not been excluded that the fatty acid-synthesizing activity of the particles may be due to enzymes adsorbed on the mitochondria or to aggregated microsomes. It is to be noted that the particulate fraction, in forming glycerides, can readily trap fatty acids that are formed. This property may be of importance in facilitating fatty acid synthesis by removing the reaction product. But it may equally well be a handicap in the attempts to observe interconversions of fatty acids. Fatty acids that are added to the reaction mixture are rapidly incorporated into triglycerides, and as such have no opportunity for chain elongation or desaturation. It is apparently necessary to prepare, from avocado particles, enzymes which will synthesize free fatty acids including oleic acid. Extracts of acetone powders of avocado particles do make free fatty acids, but it has now been definitely shown that these acids are entirely saturated.[2]

The enzyme systems for the formation of unsaturated fatty acids have been described by Bloomfield and Bloch (11), who were able to show a requirement for TPNH and oxygen. The soluble system of Bernhard *et al.* (29) also converted stearic acid to oleic acid, but cofactor requirements were not elucidated. Other reports of desaturation *in vitro* of long chain fatty acids must be questioned because of the unspecific method of assay by reduction of methylene blue (30, 31). Although the avocado system is complicated, the results obtained with it, showing that oleate biosynthesis is absolutely dependent on oxygen and is insensitive to cyanide and azide, are similar to those obtained with enzyme preparations from *Saccharomyces cerevisiae* (11).

We arrived empirically at our results on the experimental conditions necessary for the incorporation of acetate into oleate. With the exception of the pH effect, the conditions all illustrate the oxygen requirement for oleate biosynthesis. Low particle concentrations reduce the demand for oxygen in extraneous reactions, and the effect of higher temperatures may be explained as raising the rate of oxygen diffusion in the unshaken reaction mixtures. It is probable that the practice of flushing reaction vessels with nitrogen, presumably to prevent fatty acid oxidation, has been responsible for the low labeling of oleate in some experiments (10, 32).

Inhibition of fatty acid biosynthesis by free fatty acids has been observed by Langdon (33). We agree that the requirement of free fatty acid for ATP and CoA is the fundamental effect, but utilization of ATP is probably more important than utilization of CoA. In the avocado system, because activated long chain fatty acids are incorporated into glycerides, CoA is released. There is no such return of ATP.

This information on fatty acid synthesis may be considered in conjunction with information on the effect of fatty acids on phosphorylation and swelling of mitochondria. Sacktor (34) observed that decline of P:O ratio on aging of mitochondria could be prevented by the presence of bovine serum albumin. Recently, this observation has been extended by showing that the decline in P:O ratio can be reproduced by addition of oleate to mitochondria, and both effects can be reversed by albumin (35). Scholefield (36) and Pressman and Lardy (37) observed that fatty acids uncoupled oxidative phosphorylation. Somewhat later, Lehninger and Remmert (38) showed that a factor, enzymically released from mitochondria, stimulated swelling of the mitochondria and uncoupled oxidative phosphorylation. The effects of the factor could be duplicated by oleate. It was shown that mitochondrial swelling caused by various agents including oleate, could be reversed by ATP, magnesium ions, and albumin (39).

In these three cases, mitochondrial swelling, phosphorylation, and fatty acid synthesis, the basic effect of free fatty acids may be the consumption of ATP as they are activated. Apparently, the lipids of the mitochondria are attacked by lipolytic enzymes, and ATP is necessary to repair them. Turnover of ATP is, therefore, accelerated by free fatty acids and in some cases the increased rate may be responsible for stimulation of ATP-P^{32} exchange (40).

Langdon (33) finds that even albumin-bound fatty acids inhibit fatty acid synthesis to some extent. Inasmuch as it is known that crystalline samples of serum albumin contain fatty acids (41, 42), it is not surprising that in the study on fatty acid synthesis, albumin in the absence of added oleic acid caused some inhibition. It also provides an explanation for the incomplete reversal of oleate inhibition of fatty acid synthesis, and the incomplete reversal of the effect of oleate on P:O ratio (35).

There are several reports of stimulation of fatty acid synthesis

by citrate, isocitrate, and malonate. The stimulatory effect of citrate could not be replaced by TPNH, or by TPNH-generating systems (43). It was concluded that the effect of citrate was by virtue of its supplying carbon dioxide necessary of the formation of malonyl-CoA (44). It is probable that stimulation by malonate was due to decarboxylation similarly producing carbon dioxide (45). However, malonate and citrate have affected fatty acid synthesis by the avocado system in the presence of saturating levels of bicarbonate. They undoubtedly influence tricarboxylic acid cycle activity. In the avocado system, acetate may be utilized either to form fatty acids or organic acids. Citrate may block entry of acetate into the tricarboxylic acid cycle, thus making more acetate available for fatty acid synthesis. It has a second effect of lowering the proportion of oleate formed, presumably because oxygen is depleted in the oxidation of the added citrate. Malonate also inhibits the tricarboxylic acid cycle activity, but the potential increase in fatty acid synthesis is counteracted by the formation of malonyl-CoA, which dilutes the radioactive malonyl-CoA formed from radioactive acetate. In the presence of malonate, the proportion of labeled oleate increased because of the inhibition of oxygen consuming reactions associated with the tricarboxylic acid cycle.

It is interesting that malonate and citrate, normal products of acetate metabolism by avocado particles, can affect fatty acid synthesis in the ways described. In the absence of hormonal control, fatty acid synthesis may be controlled indirectly by these two acids.

SUMMARY

1. Fatty acids are synthesized from acetate by a particulate preparation from avocado mesocarp, sedimenting between 2,000 and 15,000 × *g*. The preparation also incorporates acetate into citric, succinic, malic, α-ketoglutaric, and malonic acids.

2. The fatty acids are in the form of triglycerides and phospholipids.

3. The pH optimum for acetate incorporation is 8. The proportion of incorporated acetate in oleate declines markedly above this pH.

4. The formation of oleate is absolutely dependent on oxygen. Conditions that tend to increase the availability of oxygen favor the formation of oleate. These conditions include higher temperatures (up to 46°), and the use of dilute suspensions of the particles.

5. Free fatty acids, particularly oleate, inhibit incorporation of acetate into fatty acids. Incorporation into acids of the tricarboxylic acid cycle is also inhibited. The inhibition is probably due to the utilization of adenosine triphosphate.

6. Malonate and citrate inhibited incorporation of acetate into acids of the tricarboxylic acid cycle. Malonate increased the proportion of label found in oleate, whereas citrate lowered the proportion.

7. When decanoate, palmitate, and stearate were supplied to the particulate fraction, they were incorporated unchanged into triglyceride and phospholipid. There was no desaturation or chain elongation of these acids.

REFERENCES

1. Bradbeer, C., and Stumpf, P. K., *J. Lipid Research*, **1**, 214 (1960).
2. Wakil, S. J., Titchener, E. B., and Gibson, D. M., *Biochim. et Biophys. Acta*, **34**, 227 (1959).
3. Brady, R. O., Bradley, R. M., and Trams, E. G., *J. Biol. Chem.*, **235**, 3093 (1960).
4. Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, **113**, 505 (1936).
5. Dauben, W. G., Hoerger, E., and Petersen, J. W., *J. Am. Chem. Soc.*, **75**, 2347 (1953).
6. Simmons, R. O., and Quackenbush, F. W., *J. Am. Oil Chem. Soc.*, **31**, 441 (1954).
7. Coppens, N., *Nature (London)*, **177**, 279 (1956).
8. Kurtz, E. B., and Miramon, A., *Arch. Biochem. Biophys.*, **77**, 514 (1958).
9. Crombie, W. M., and Ballance, P. E., *Nature (London)*, **183**, 1195 (1959).
10. Stumpf, P. K., and Barber, G. A., *J. Biol. Chem.*, **227**, 407 (1957).
11. Bloomfield, D. K., and Bloch, K., *J. Biol. Chem.*, **235**, 337 (1960).
12. Squires, C. L., Stumpf, P. K., and Schmid, C., *Plant Physiol.*, **33**, 365 (1958).
13. Bligh, E. G., and Dyer, W. J., *Can. J. Biochem. Physiol.*, **37**, 911 (1959).
14. Mangold, H. K., Lamp, G. B., and Schlenk, H., *J. Am. Chem. Soc.*, **77**, 6070 (1955).
15. Buchanan, M. A., *Anal. Chem.*, **31**, 1616 (1959).
16. Buch, M. L., Montgomery, R., and Porter, W. L., *Anal. Chem.*, **24**, 489 (1952).
17. Long, A. G., Quayle, J. R., and Stedman, R. J., *J. Chem. Soc.*, 2197 (1951).
18. Borgström, B., *Acta Physiol. Scand.*, **25**, 101 (1952).
19. von Rudloff, E., *Can. J. Chem.*, **34**, 1413 (1956).
20. Isherwood, F. A., and Hanes, C. S., *Biochem. J.*, **55**, 824 (1953).
21. Hele, P., Popjak, G., and Lauryssens, M., *Biochem. J.*, **65**, 348 (1957).
22. Wakil, S. J., Porter, J. W., and Gibson, D. M., *Biochim. et Biophys, Acta*, **24**, 453 (1957).
23. Tapley, D. F., *J. Biol. Chem.*, **222**, 325 (1956).
24. Shaw, W. N., Dituri, F., and Gurin, S., *J. Biol. Chem.*, **226**, 417 (1957).
25. Dituri, F., Shaw, N. W., Warms, J. V. B., and Gurin, S., *J. Biol. Chem.*, **226**, 407 (1957).
26. Van Baalen, J., and Gurin, S., *J. Biol. Chem.*, **205**, 303 (1953).
27. Wakil, S. J., McLain, L. W., Jr., and Warshaw, J. B., *J. Biol. Chem.*, **235**, PC31 (1960).
28. Abraham, S., Matthes, K. J., and Chaikoff, I. L., *J. Biol. Chem.*, **235**, 2551 (1960).
29. Bernhard, K., von Bülow-Köstner, J., and Wagner, H., *Helv. Chim. Acta*, **42**, 152 (1959).
30. Shapiro, B., and Wertheimer, E., *Biochem. J.*, **37**, 102 (1943).
31. Jacob, A., *Compt. Rend. Soc. Biol.*, **242**, 2180 (1956).
32. Zebe, E. C., and McShan, W. H., *Biochim. et Biophys. Acta*, **31**, 513 (1959).
33. Langdon, R. G., in K. Bloch (Editor), *Lipid metabolism*, John Wiley and Sons, New York, 1960, p. 238.
34. Sacktor, B., *J. Gen. Physiol.*, **37**, 343 (1953).
35. Helinski, D. R., and Cooper, C., *J. Biol. Chem.*, **235**, 3573 (1960).
36. Scholefield, P. G., *Can. J. Biochem. Physiol.*, **34**, 1227 (1956).
37. Pressman, B. C., and Lardy, H. A., *Biochim. et Biophys. Acta*, **21**, 458 (1956).
38. Lehninger, A. L., and Remmert, L. F., *J. Biol. Chem.*, **234**, 2459 (1959).
39. Lehninger, A. L., *J. Biol. Chem.*, **234**, 2465 (1959).
40. Ahmed, K., and Scholefield. P. G., *Nature (London)*, **186**, 1046 (1960).
41. Goodman, D. S., *J. Am. Chem. Soc.*, **80**, 3892 (1958).
42. Kendall, F. E., *J. Biol. Chem.*, **138**, 97 (1941).
43. Porter, J. W., Wakil, S. J., Tietz, A., Jacob, M. I., and Gibson, D. M., *Biochim. et Biophys. Acta*, **25**, 35 (1957).
44. Gibson, D. M., Titchener, E. B., and Wakil, S. J., *Biochim. et Biophys. Acta*, **30**, 376 (1958).
45. Nakada, H. I., Wolfe, J. B., and Wick, A. N., *J. Biol. Chem.*, **226**, 145 (1957).