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Author(s)	KIKUTA, Yoshio
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LIPID METABOLISM IN THE FRUIT OF *PERSEA AMERICANA* MILL.

II. Metabolism of Acetate in Relation to Lipid Synthesis

Yoshio KIKUTA

(Department of Botany, Faculty of Agriculture
Hokkaido University, Sapporo, Japan)

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INTRODUCTION

The acetate incorporation into long-chain fatty acids has been demonstrated in a number of plant tissues including maturing peanut seeds, cotyledons of germinating peanut seeds, developing flax seeds, and leaves of spinach, lettuce and castor bean. The long-chain fatty acids synthesized from acetate-1-C¹⁴ by developing flax seeds were labelled in the odd numbered carbon atoms. This observation was consistent with having a synthesis by multiple condensation of either acetate units or malonate units derived by carboxylation of acetate units (32). The cytoplasmic particle preparation from the avocado fruit required ATP¹⁾, CoA, and Mn²⁺ for the synthesis of long-chain fatty acids (42). Carbon dioxide stimulated the rate of synthesis but was not incorporated into fatty acids (41). When a water extract of acetone powder was used, an additional requirement for NADPH could be demonstrated. NADH was ineffective. Even though acetyl-CoA was used as a substrate, ATP was still required. Inhibition by avidin suggested that biotin was involved in the synthesis (3). Malonyl-CoA and acetyl-CoA together were readily incorporated into palmitic and stearic acids, but the system did not require ATP, CoA or HCO₃⁻ and was avidin insensitive (18). This evidence has suggested that the synthesis of long-chain fatty acids by multiple condensation of malonyl-CoA with fatty acyl thioester operates in avocado fruits.

The site of long-chain fatty acid synthesis in the avocado fruit appeared to be in the mitochondria. However, the synthesis of fatty acids in many living organisms is catalyzed by a cytoplasmic enzyme complex. These complexes, which contain at least six or seven different kinds of proteins, are

1) Abbreviations: ATP, adenosine triphosphate; ACP, acyl carrier protein; CoA, coenzyme A; NADH, nicotinamide adenine dinucleotide reduced form; NADPH, nicotinamide adenine dinucleotide phosphate reduced form; Tris, tris (hydroxymethyl) aminomethane.

tightly bound together in *Saccharomyces cerevisiae* (28) and pigeon liver (20, 46) but are readily dissociable in *Clostridium kluyverii*, *Escherichia coli* (14), and in several higher plant systems (10, 11, 34, 38). Among these fatty acid synthesizing complexes, the system from *E. coli* has been most extensively studied. The most interesting aspect is the isolation and characterization of acyl carrier protein (ACP) which binds acyl intermediates during the formation of long-chain fatty acids (2, 14, 26, 28).

In 1964, OVERATH and STUMPF (38) isolated soluble fatty acid synthetase from avocado mesocarp and fractionated it into a heat labile component and a heat stable component. Like a bacterial system, either component alone was inactive in condensing malonyl-CoA to form long-chain fatty acids, but when combined they catalyzed the synthesis of palmitic and stearic acids. ACP (a heat stable component) of *E. coli* and avocado were interchangeable but when the heat stable component from avocado was added to the *E. coli* enzyme system, β -hydroxylauric and β -hydroxymyristic acids were the major component products rather than the expected products for the *E. coli* system, namely vaccenic and stearic acids.

Although the many and complex reactions leading to the formation of saturated fatty acids are now reasonably clear, the origin of oleic acid in plants has remained uncertain. Different preparations appeared to exhibit somewhat different patterns of acetate incorporation into fatty acids and to have different requirements for the reactions. Turning to the studies with microorganisms, BLOCH and his coworkers (7, 8) proposed that aerobic organisms carry out a desaturation requiring NADPH and molecular oxygen, while anaerobic organisms synthesize monounsaturated fatty acids by a different process involving dehydration and elongation of eight and ten carbon β -hydroxy acids (13, 39).

On the other hand, NAGAI and BLOCH (37) have shown in cell-free extracts of *Euglena gracilis* that only thioesters of long-chain saturated fatty acids are desaturated by monooxygen desaturase in the extracts. They concluded that the photoauxotrophic form of *Euglena* synthesized monounsaturated fatty acids by modifications of two mechanisms occurring in non-phosphosynthetic organisms. One of the enzymes, a soluble desaturase, acts on stearyl-ACP but not on shorter chain ACP derivatives nor CoA esters of fatty acids. The second enzyme system of green *Euglena* appears to form oleic acid by elongating already unsaturated acids, as do bacteria, with the difference that in *Euglena* one of the steps requires molecular oxygen. The exact mechanism of this type of desaturation is unknown. By contrast, *Euglena* heterotrophically grown in the dark and lacking chloroplasts, desaturates acyl-CoA thioesters probably by the exact mechanism that is proposed for nonphotosynthetic

organisms.

A fatty acid synthesizing system prepared from spinach leaf chloroplasts has been shown by MUDD and MCMANUS (33, 34, 35) to have requirements very similar to those of the non-mitochondrial system of animals (46), *i. e.*, ATP, CoA, NADPH, Mn^{++} and HCO_3^- , and to be characterized by sulfhydryl groups in the enzyme complex and is supposed to follow the similar pathway of the soluble system. Photosynthetic reduction of $NADP^+$ could be utilized for the synthesis of fatty acids by this system which formed saturated fatty acids together with some unsaturated fatty acids.

STUMPF and JAMES (43, 44, 45) reported the presumably similar system in lettuce chloroplasts and the remarkable stimulation by light was postulated as being due to generation of ATP, NADPH and molecular oxygen. This system incorporated acetate largely into palmitic, palmitoleic and oleic acids and time activity curves strongly suggested that neither palmitic and stearic acids could serve as precursors of the corresponding Δ^9 -unsaturated fatty acids.

In experiments with castor bean leaves, JAMES (21, 22) confirmed that palmitic and stearic acids were not precursors of oleic acid since the radioactivity of oleic acid synthesized from acetate-1- C^{14} quickly rose to a greater extent than in the saturated fatty acids, and only the C_8 , C_{10} , C_{12} and C_{14} saturated acids gave rise to labelled oleic acid in the leaves. However, oleic acid was the precursor of linoleic acid which in turn might be the precursor of linolenic acid (16, 22, 29).

This separation of the synthetic pathways for the saturated and unsaturated fatty acids has been confirmed by further experiments with the enzyme system isolated from avocado. Although the particulate preparation from avocado mesocarp (36) showed an absolute requirement for oxygen for the synthesis of oleic acid as it did in the system from *Saccharomyces cerevisiae* (28), this could not be ascribed to a desaturation of stearic acid since saturated acids added to the enzyme system were recovered unchanged. Another interesting point is that a high concentration of malonate favored the synthesis of oleic acid (4, 36).

Moreover, acetate-1- C^{14} or acetyl-1- C^{14} -CoA contributed to both saturated and unsaturated fatty acids whereas malonyl 1,3- C^{14} -CoA was incorporated only into the saturated fraction (4). A sonicated system of the avocado particulate preparation demonstrated that biotin was required since it was sensitive to avidin which completely inhibited stearic acid synthesis. An amount of avidin required for the inhibition of the stearic acid formation had little effect on the formation of oleic acid. In 1965, YANG and STUMPF (47) summarized evidence for the possible metabolic separation of pathways in the synthesis

of saturated and unsaturated fatty acids by the avocado mesocarp.

Contrary to much information concerning the fatty acid synthesizing system of avocado preparations, little is known about the lipid synthesis *in vivo* in the fruit of avocado which predominantly forms oleic acid and stores triglyceride exclusively. The metabolic fate of acetate utilized by avocado mesocarp is dependent upon the stage of development of the fruit, since acetate, by way of acyl CoA, is incorporated into tricarboxylic acids, into fatty acids and carotenoids, and into sugars and proteins. As was shown in previous paper (25), the incorporation of acetate into organic acid was nearly constant over a long period of fruit development while, in the developing fruit which rapidly accumulating lipids, most of acetate contributed to the assimilation sequence leading to the formation of triglyceride.

Studies on the metabolism of acetate were undertaken primarily to elucidate the fatty acid synthesis and the tricarboxylic acid cycle operation in the tissue slices of avocado fruit.

MATERIALS AND METHODS

Plant materials

The HASS variety of avocado, *Persea americana* Mill. (*P. gratissima* GAERTH.), was used in a number of isotope experiments designed for metabolic fates of substrates in tissue slices and subcellular particulate fractions. The fruits were grown on the Riverside campus of the University of California, and after harvest were stored at 5°C for at least 24 hours before being used for experiments.

Chemicals

All chemicals were purchased from commercial sources. Sodium acetate-1-C¹⁴ (7 mC/mM), acetate-2-C¹⁴ (7 mC/mM), citrate-1, 5-C¹⁴ (33 mC/mM), and succinate-1, 4-C¹⁴ (25 mC/mM) were obtained from Calbiochem Co., Calif. Coenzyme A and ATP were obtained from Biochemicals Inc., Wis., and NADP and NAD from C.F. Boehringer & Soehne GmbH, Mannheim, Germany. Glucose-6-phosphate and glutrthione were also purchased from Calbiochem Co., Calif.

Preparation and incubation of tissue slices

The technique described in the previous paper of this series (25) was used. The tissue slices were prepared by using cork borer No. 7 and hand sectioning the mesocarp tissue from a single chilled fruit. The tissue slices were then infiltrated with a radioactive substrate in 5 ml of 0.05 M phosphate buffer, pH 7.0 for 15 minutes under vacuum (7 to 10 mmHg). A batch of 10 slices was

rinsed once with non-radioactive buffer, blotted dry with filter paper, and placed on a stainless wire net covered with cheese cloth in a 125 ml respiratory flask. The tips of the cloth were dipped in 20 ml of the non-radioactive phosphate buffer solution so that the slices were kept moist during the incubation period. The side arm of the respiratory flask was used for trapping respiratory carbon dioxide in 5% NaOH. After incubation, the slices were transferred to chilled 10% trichloroacetic acid and then frozen. The frozen samples were crushed in a mortar and the crude lipid was extracted with chloroform-methanol (2 : 1, by volume). The slurry was centrifuged in a clinical centrifuge and then the precipitate was again homogenized to extract residual lipids with additional chloroform-methanol mixture. The aqueous and chloroform fractions were washed with chloroform or distilled water, respectively. The residues were extracted with 5% trichloroacetic acid. The resulting debris was hydrolyzed overnight in *N* NaOH at 50°C. Only the chloroform fraction was subjected to further analysis.

Preparation of cytoplasmic particles

A procedure by MUDD and STUMPF (36) was employed for the examination and incubation of the enzyme activity of the lipid synthesis by the cytoplasmic particulate fraction.

The peeled mesocarp tissue (*ca.* 100 grams) from a single fruit stored in crushed ice was grated into a glass container and dispersed in 200 ml of 0.4 *M* sucrose containing 4 *mM* glutathione, 1 *mM* MgSO₄ and 20 *mM* Tris-HCl buffer, at pH 8.0. The Brei was squeezed in a 4-layered cheese cloth bag with a hand press. The extract was centrifuged at 1000 × *g* for 10 minutes. The supernatant was again centrifuged at 10,000 × *g* for 30 minutes. After the centrifugation at 10,000 × *g*, the pellet was suspended with 0.4 *M* sucrose-tris buffer in a Potter homogenizer tube with a Teflon pestle. The suspension was again centrifuged at 10,000 × *g* for 30 minutes. The collected pellet was homogenized and taken up in 0.4 *M* sucrose-tris buffer, pH 7.0.

All the processes were carried out in a room at 4°C with equipment that had been in the cold room for at least 12 hours before the preparation. The protein concentration of cytoplasmic particles was determined with a biuret reagent (15).

Reaction mixture contained 10 μmoles of ATP, 50 mμmoles of CoA, 70 mμmoles of NADP⁺ and 0.5 μmoles of glucose-6-phosphate and 30 μmoles of HCO₃⁻. Total volume of reaction mixture was adjusted to 1 ml with 0.4 *M* sucrose-tris buffer, and 0.2 to 0.4 ml of cytoplasmic particulate fraction (2-4 mg nitrogen) containing 5 *mM* glutathione and 2 *mM* MgSO₄ in 0.4 *M* sucrose-0.02 *M* tris-HCl buffer, pH 7.0. Glucose-6-phosphate could be replaced by

isocitrate or malate.

The reactions were initiated in 15 ml centrifuge tubes at 28°C by the addition of radioactive substrates and were stopped by the addition of 4 ml chloroform-methanol hydrochloric acid (200:100:1, by volume) and lipids were extracted in the centrifuge tubes by the method of BLIGH and DYER (6).

Silicic acid column chromatography

The methods of BARRON and HANAHAN (2) and HIRSH and AHRENS (19) were modified for the separation of glycerophospholipids.

Development of the silicic acid column proceeded by addition of a series of n-hexane/diethyl ether/methanol solvents experimentally selected to give maximum separation of the classes of lipids.

C¹⁴ radioactivity determination

All radioactivity determinations were made using a Tracer Lab. Com-pumatic II scalar. For counting lipids, a thin layer (less than 0.1 mg/cm²) of the sample was mounted to avoid self absorption of C¹⁴.

For BaC¹⁴O₃, either infinite thickness of the sample (more than 20 mg/cm²) or a calibration made from the self absorption curve was necessary to determine the radioactivity. The respiratory C¹⁴O₂ trapped in NaOH solution was heated to just below the boiling point, then 1 ml of 2 N barium chloride solution was added to form the BaC¹⁴O₃ precipitate which could be obtained by centrifugation. All precipitates were washed with hot water and methanol, then the BaCO₃ precipitate, suspended in methanol, was transferred to a planchet, dried and weighed. It was convenient that the count of infinite thickness of the precipitate directly gave a specific activity of respiratory carbon dioxide.

Gas liquid chromatography

The gas liquid chromatography units of Varian Aerograph Inc. HY-FI model 550 oven and HY-FI model 600 D electrometer were used with a HONEYWELL BROWN 0.1 mV potentiometric recorder. A 150 cm × 3 mm copper column of 15% diethylene glycol succinate on Chromosorb W, 60-80 mesh, was operated at 180°C with nitrogen carrier gas having a flow rate of approximately 100 ml per minute. The injection block was heated to 240°C. The effluent was detected by means of flame ionization with oxygen supplied at 10 lb psi, 40 ml per minute flow and hydrogen at 6 lb psi, 15 ml per minute flow.

Ninety per cent of the effluent was recovered from a gas splitter so positioned between the outlet of the chromatographic column and the inlet of the flame ionization chamber that 10% of the total effluent could be recorded on the chart. The collection tubes were packed with glass wool and moistened

with chloroform (24). The collected effluent was eluted with chloroform and transferred to planchets for radioactive counting.

The recovery of the radioactivity from the column varied from 60 to 90%, probably due to the following factors: 1) Incomplete vaporization at the injection block, 2) Absorption by the liquid phase of the column, 3) Aerosol formation at the collection tube, 4) Back pressure from the outlet caused by inserting a tightly packed collection tube, and 5) Impurities in the sample, *i. e.*, the degradation products during isolation and methylation, and the contaminations of unsaponified lipids and hydroxy fatty acids which required high retention volumes. The following precautions were practised to obtain maximum recoveries: 1) The injection block was heated to 240°C and the sample was diluted 10 to 20% in benzene. 2) Two to 3 μg of the samples of fatty acid methyl esters were used for each injection with a volume of 5 to 15 μl , and the total radioactivity was maintained at more than 5000 cpm per injection. 3) The collection tube was 6 inches long and 4) was loosely packed with glass wool to within one inch of the end. 5) The column was not loaded for at least two hours before use. In general it was necessary to analyze the same sample 3 or more times in order to establish reliable means. The data express the specific radioactivity for the common fatty acids, *i. e.*, palmitic, palmitoleic, stearic, oleic, linoleic, linolenic and several unknown fatty acids including arachidic acid, and the percentage distribution of label among the common fatty acids recovered by this method.

Saponification and esterification procedures

The lipid sample was saponified with 5% methanolic KOH by refluxing for 3 hours. This alcoholic solution was washed once with ether, then acidified with 2 N HCl. The resulting fatty acids were extracted three times with diethyl ether. The combined ether extract was evaporated, and the fatty acids were again dissolved in 10% methanol in ether. An equal volume of one per cent diazomethane in ether was added to esterify the fatty acids. In this way the fatty acids from avocado lipids were converted to their respective methyl esters. After dissolving the esters in benzene, they were ready for gas liquid chromatography.

Permanganate oxidation procedure

Five mg of oleic acid isolated by gas liquid chromatography (together with carrier) was added to 4 ml of acetone containing 20 mg of potassium permanganate. Five hundredths ml of 10% KOH solution was added and the mixture was heated under reflux until the purple color of permanganate disappeared (30 min. to 3 hrs.). At the end of this time the mixture was allowed to cool

and was acidified carefully with sulfuric acid. The mixture was hydrolyzed for 30 minutes, and cooled. Any excess oxidant was removed by the addition of a little sodium bisulfite solution. The resulting acids were extracted with diethyl ether and methylated by diazomethane. The yield was *ca.* 50% for azelaic acid and *ca.* 30% for pelargonic acid. Gas liquid chromatography was manually programmed between 130°C and 180°C for the isolation of pelargonic and azelaic acids.

RESULTS

A. Relationship in the synthesis of lipids and water soluble acids by tissue slices

Mature Hass avocado fruit tissue slices could metabolize acetate under

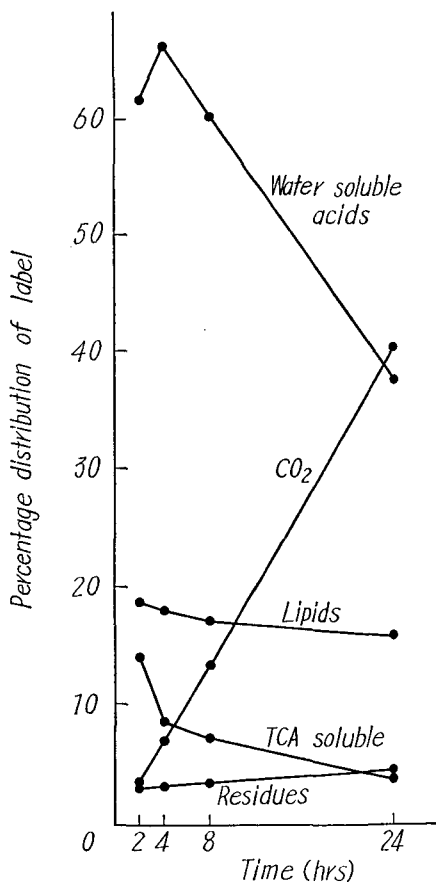


Figure 1. Acetate-1-C¹⁴ metabolism by the tissue slices from mature avocado fruit.

the experimental conditions described previously. Elimination of atmospheric CO_2 from the reaction chamber gave slight enhancement in water soluble acid incorporation while lipid synthesis was not affected in mature fruit tissue slices. This effect could be corrected by the use of the phosphate-bicarbonate buffer in the reaction chambers. Figure 1 shows time course studies on the acetate metabolism by the tissue slices. The vacuum infiltrated acetate was used up in two to four hours at which time the maximum incorporation of acetate was found. The acetate was converted partly to lipids and water soluble acids, partly to trichloroacetic acid soluble and insoluble materials and

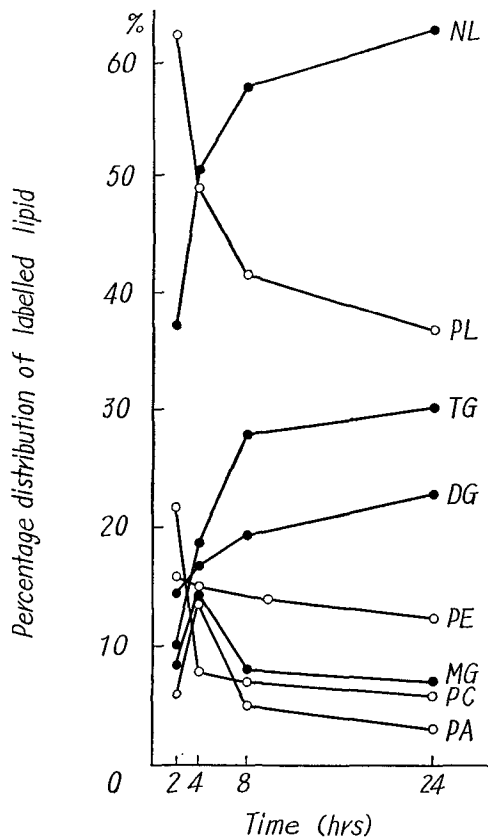


Figure 2. Acetate- 1-C^{14} incorporation into classes of lipids by the tissue slices from mature avocado fruit¹⁾.

1) The following symbols are used in figures:
 NL, Neutral lipids; TG, Triglyceride; DG, Diglyceride; MG, Monoglyceride; FA, Free fatty acid; PL, Phospholipids; PA, Phosphatidic acid; PG, Phosphatidyl glycerol; PE, Phosphatidyl ethanolamine; PI, Phosphatidyl inositol; and PC, Phosphatidyl choline.

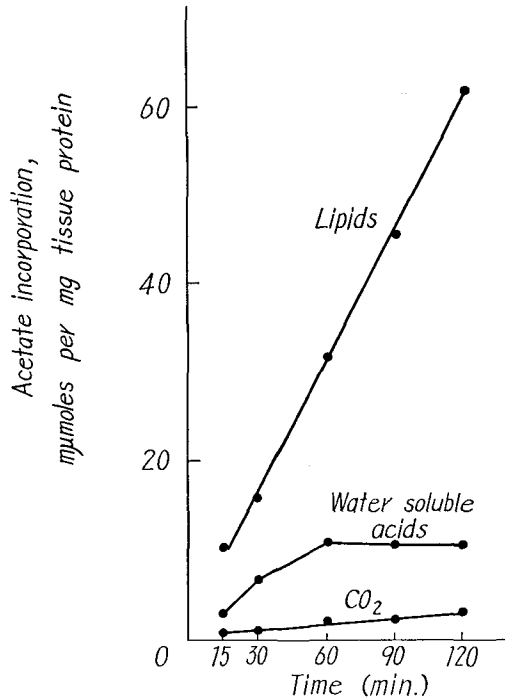


Figure 3. Assimilation of acetate-1-C¹⁴ by the tissue slices from developing avocado fruit.

partly to CO₂. In addition, it was presumed to be present in the tissue that free acetate and other volatile compounds were lost during extraction procedures. The organic acids were eventually oxidized to carbon dioxide, while lipids were slowly transformed from phospholipids to neutral lipids during the next eight hours (fig. 2). On the other hand, acetate was heavily incorporated into lipids, mainly the triglyceride fraction, by the developing avocado mesocarp tissue. but the same amount of acetate incorporation was found in the water soluble acid fraction (fig. 3 and fig. 4).

Interestingly, the particulate fraction prepared from the same developing avocado fruit shows a different pattern of acetate assimilation. The water soluble acid fraction was high but lipid synthesis was remarkably reduced to about 4% of the level of activity found in the tissue slices on the same nitrogen basis (fig. 5). It seems to be possible either that the enhanced lipid synthesis in developing fruit was a matter of the soluble fraction of the tissue (38), or the enzyme systems were so labile that the lipids could not be properly formed (34), and/or there was a deficiency of the acceptor for the esterification of

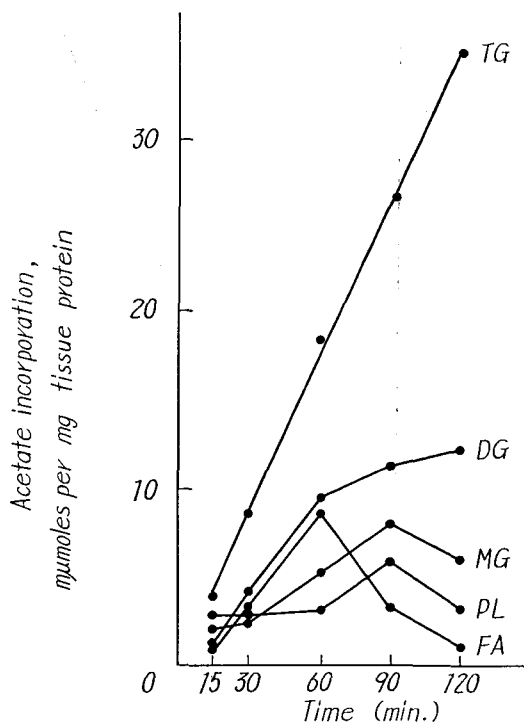


Figure 4. Incorporation of acetate-1-C¹⁴ into classes of lipids by the tissue slices from developing avocado fruit.

synthesized fatty acid which may inhibit the synthesis of lipid (34). Figure 6 shows the accumulation of free fatty acids by this particulate enzyme system. It will be discussed detail later.

The release of CO₂ from specifically labelled acetate was determined over the experimental period using the apparatus described in materials and methods. The results are shown in figure 7. The curves have essentially shown the theoretical agreement on the existence of the complete operation of TCA cycle except that the production of C¹⁴O₂ from acetate-2-C¹⁴ was slightly high.

Because of a lag in the production of C¹⁴O₂ from acetate-2-C¹⁴, there was a delay in appearance of the label in carboxyl groups of malic acid. Probably CO₂ was not released until the second turn of the cycle. It is clear from the comparison with figure 8, that the appearance of C¹⁴O₂ from acetate-1-C¹⁴ occurred only after the malic acid had become labelled, because succinate-1, 4-C¹⁴ readily released C¹⁴O₂ at the same initial rate of C¹⁴O₂ production from citrate-1, 5-C¹⁴ of equal specific activity.

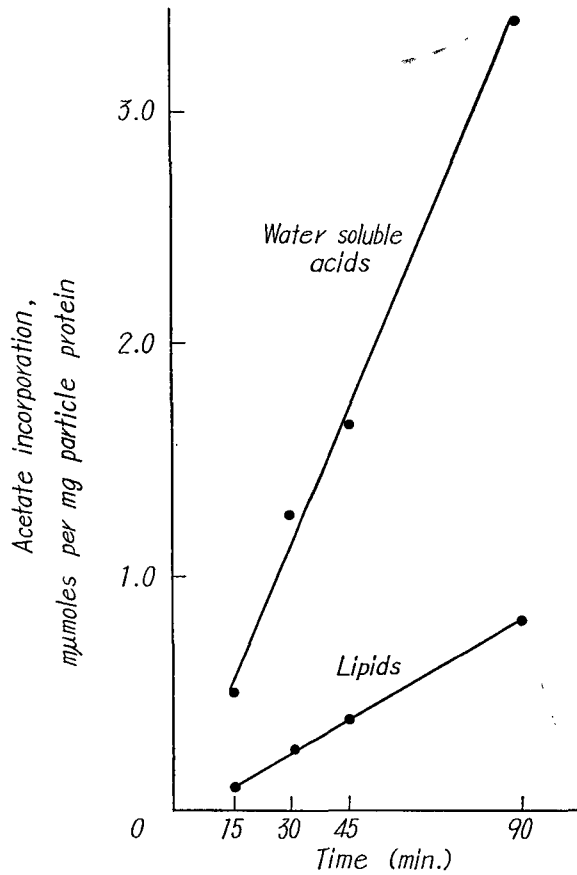


Figure 5. Assimilation of acetate-1- C^{14} into water soluble acids and lipids by cytoplasmic particle enzyme system isolated from mesocarp of avocado fruit.

A further important point in figure 7 is that the time taken for the production of a particular amount of $C^{14}O_2$ from acetate-1- C^{14} of the same specific activity in the initial 60 minutes, and the rate of CO_2 evolution from acetate-2- C^{14} tends to be close to the rate of that from acetate-1- C^{14} . Such a figure is expected on theoretical grounds. Furthermore, there was no label found in the lipid fraction from succinate-1, 4- C^{14} and citrate-1, 5- C^{14} .

These results indicate that the complete TCA cycle operation with a subsidiary pathway of malate decarboxylation appeared in the developing fruit and no evidence of citrate cleavage to provide acetyl CoA for lipid synthesis, nor isocitrate cleavage was found.

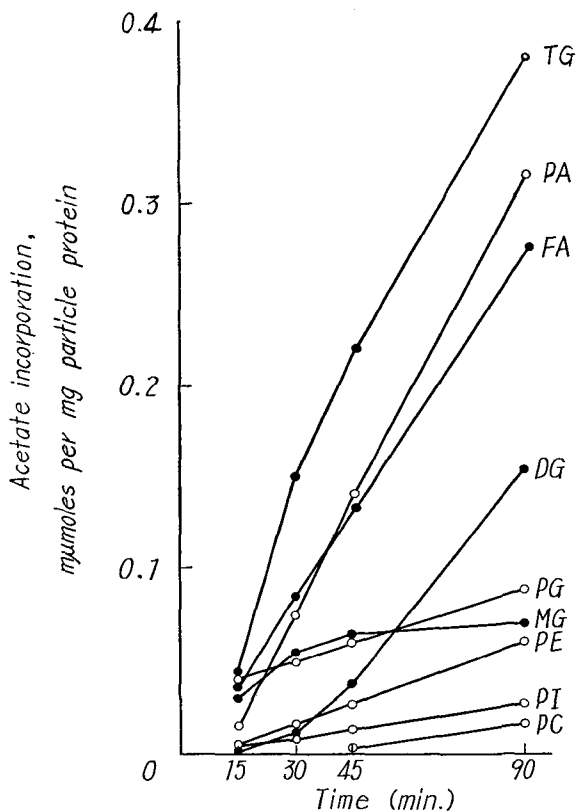


Figure 6. Acetate-1-C¹⁴ incorporation into classes of lipids by cytoplasmic particle enzyme system isolated from mesocarp of avocado fruit.

B. Acetate incorporation into classes of lipids

The vacuum infiltrated acetate-1-C¹⁴ was used up by the assimilation processes during two to four hours' incubation of the tissue slices prepared from the mesocarp of avocado fruit (fig. 1). A mature fruit assimilated approximately 20% of the acetate into lipids which were gradually transformed from phospholipids to neutral lipids, especially triglyceride. The changes of distribution of label indicated that losses of phosphatidyl ethanolamine and phosphatidyl choline fractions were involved, while the diglyceride fraction remained constant during the incubation period (fig. 2).

On the other hand, the tissue slices from the developing fruit showed rapid incorporation of acetate into triglyceride as much as 40 times faster than that into triglyceride by the mature fruit (fig. 4). Although the developing

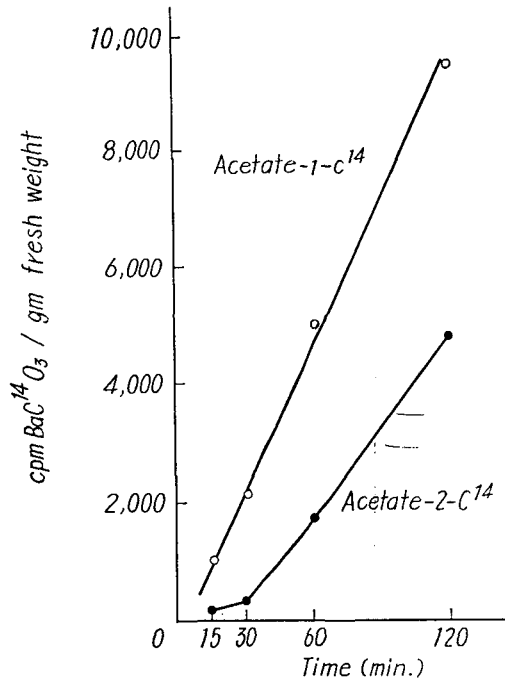


Figure 7. $C^{14}O_2$ evolution from acetate-1- C^{14} and acetate-2- C^{14} of equal specific activity (5 mC/mM) by the tissue slices of avocado fruit.

fruit showed accumulation of label in phosphatidyl ethanolamine and phosphatidyl choline fractions, on a percentage basis the label in these compounds decreased.

C. Comparative studies on the acetate incorporation into lipids by tissue slices and by a particulate fraction isolated from a single fruit

Subcellular particles isolated from mesocarp of an avocado fruit were simultaneously compared with tissue slices. A developing fruit was cut into two parts. One half of the fruit was used for preparation of the tissue slices and the other was used for isolation of a particulate preparation according to the methods described earlier. It is shown in figure 6 that the utilization of phosphatidic acid was limited in the particulate fraction, even though triglyceride was formed. Free fatty acid was also accumulated by this subcellular system. Moreover, mono-, di- and triglyceride formation was reduced to 50% in comparison with the results of tissue slices of avocado mesocarp (fig. 4). From this evidence, it seems probable that triglyceride is not synthesized by the particulate fraction but by a soluble or microsomal fraction which also

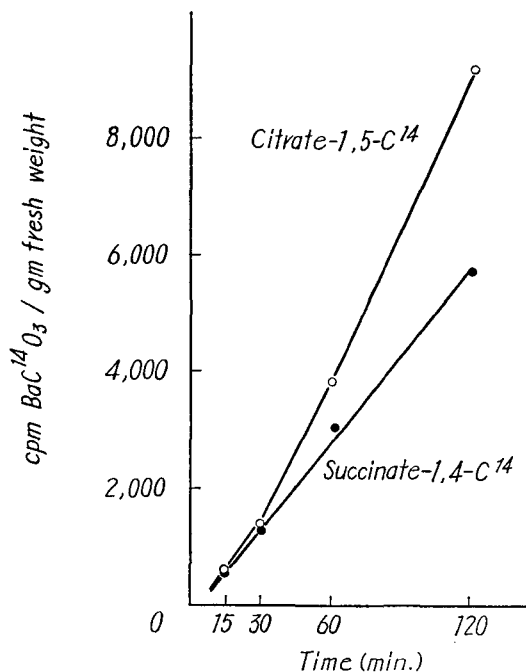


Figure 8. $C^{14}O_2$ evolution from citrate-1,5- C^{14} and succinate-1,4- C^{14} of equal specific activity (1 mC/mM) by the tissue slices of avocado fruit.

provide a phosphatidic acid phosphatase to form diglyceride and monoglyceride. Diglyceride and monoglyceride may be phosphorylated by mitochondrial enzyme, ATP: diglyceride kinase, as described by BRADBEER and STUMPF (9). Interestingly, the phosphatidyl glycerol fraction was labelled, followed by labelling in phosphatidyl ethanolamine, phosphatidyl inositol and phosphatidyl choline fractions by the cytoplasmic particulate system.

D. Synthesis of fatty acids and their incorporation into lipids

Tables 1 and 2 show the effect of nitrogen atmosphere on the synthesis of fatty acid during two hours' incubation of the tissue slices. The chromatographic effluents under the radioactive peak were pooled even though the segregation of non-radioactive lipids was troublesome in the interpretation of the data. Then isolated fatty acids were esterified and analyzed by gas liquid chromatography followed by radioactivity determination. In general, the label was increased by anaerobiosis in saturated fatty acids, *i. e.*, palmitic and stearic acids. While the synthesis of oleic and linoleic acids was reduced approximately 50 per cent under the nitrogen atmosphere. This is not only due to

TABLE 1. Effects of aeration on the synthesis of fatty acid by the tissue slices from the mesocarp of Hass avocado

25 μC of acetate-1- C^{14} (7 mC/mM) were used for vacuum infiltration of 8 gms of the tissue slices with 8 ml of 0.05 M phosphate-bicarbonate buffer, pH 7.0. Air or nitrogen stream was passed through the reaction chambers at the rate of 60 ml per minute (9 lb psi) during incubation period. A developing fruit was harvested on April 14, 1966 and tested immediately. After 2 hours' incubation, avocado lipids were extracted and separated by silicic acid column chromatography into the classes of lipids; then fatty acid composition and respective radioactivity were determined. Data are expressed in cpm acetate-1- C^{14} incorporated per μg of fatty acid.

Fraction	Specific radioactivity of fatty acid						Overall**
	16:0	16:1	UK*	18:0	18:1	18:2	
Triglyceride							
Air	242	199		2054	210	120	212
Nitrogen	180	253		3568	113	69	155
Diglyceride							
Air	169	117		2886	226	153	240
Nitrogen	165	85		2558	125	43	151
Monoglyceride							
Air	1013	458	857	2846	452	101	598
Nitrogen	675	231	750	4015	232	78	379
Phospholipid A***							
Air	861	325	522	1333	506	165	579
Nitrogen	723	289	666	3829	229	69	347
Phospholipid B							
Air	1916	1287	1028	4044	1340	183	1145
Nitrogen	920	977	1555	7070	860	141	936
Phospholipid C							
Air	3679	4140	3232	32273	10635	1641	8519
Nitrogen	3408	1458	2639	18819	5285	449	4398

16:0, 16:1, 18:0, 18:1, and 18:2 represent palmitic, palmitoleic, stearic, oleic and linoleic acids, respectively.

* An unknown fatty acid, positioned between 16:1 and 18:0 is not likely mergaric acid (17:0).

** Overall specific activity of the lipid fraction is expressed in cpm per μg of total fatty acid in the fraction.

*** The major constituents of the phospholipids are phosphatidic acid and phosphatidyl glycerol in PA, phosphatidyl ethanolamine in PB and phosphatidyl choline in PC. fractions.

TABLE 2. Effects of aeration on the percentage distribution of labelled fatty acid synthesized by the tissue slices from mesocarp of Hass avocado

Fraction	Per cent distribution of labelled fatty acid								
	S.C*	16:0	16:1	U.K	18:0	18:1	18:2	18:3	20:0**
	%	%	%	%	%	%	%	%	%
Triglyceride									
Air	1.1	17.7	5.4		4.0	67.3	4.2		0.3
Nitrogen	6.3	18.9	6.9		12.1	53.7	4.3		2.9
Diglyceride									
Air	3.1	13.9	2.3	0.8	6.0	60.7	4.0	8.9	0.3
Nitrogen	4.2	16.6	2.4	0.0	8.8	60.6	1.7	5.5	0.2
Monoglyceride									
Air	2.4	36.5	3.6	1.2	5.0	46.8	1.4	3.0	0.1
Nitrogen	1.9	37.7	2.9	1.1	8.2	43.9	1.3	3.0	0.3
Phospholipid A									
Air	4.0	23.1	2.5	1.2	2.4	52.6	2.8	4.9	6.5
Nitrogen	6.0	30.2	2.9	1.7	9.0	42.1	2.2	3.1	2.8
Phospholipid B									
Air	2.0	29.6	3.6	1.4	3.4	55.1	2.7	1.3	0.9
Nitrogen	3.6	20.9	3.8	2.3	7.3	54.5	3.0	3.1	1.5
Phospholipid C									
Air	3.8	9.1	1.5	0.9	7.9	72.5	2.5	1.3	0.5
Nitrogen	1.2	13.2	1.2	1.8	6.5	73.0	1.3	0.9	0.9

* Short chain fatty acids.

** Arachidic and other unknown fatty acids.

	Normal fatty acid composition of lipids								
	S.C	16:0	16:1	U.K	18:0	18:1	18:2	18:3	20:0
	%	%	%	%	%	%	%	%	%
Triglyceride	0.1	15.9	4.3		0.5	69.7	9.5		
Diglyceride	0.2	15.3	4.1	0.1	0.5	72.5	5.9	1.4	
Monoglyceride	0.5	21.5	3.9	0.5	1.1	65.8	5.7	1.0	
Phospholipid A	0.4	15.5	3.5	1.0	0.8	64.1	10.9	3.6	0.2
Phospholipid B	0.4	17.5	4.1	1.4	1.1	54.7	19.3	1.3	0.2
Phospholipid C	0.4	17.1	3.6	3.1	1.3	60.5	13.2	0.5	0.3

the requirement of molecular oxygen for the synthesis of the unsaturated fatty acids but also due to the reduction of total acetate incorporation into the assimilation processes of the tissue slices. There was a marked contrast between the original percent composition of fatty acid and the label distribution among fatty acids synthesized, and this phenomenon was accentuated in a nitrogen atmosphere.

The specific radioactivity of each fraction indicates that approximately 25 per cent of total formation of neutral lipids was inhibited by the anaerobic conditions, although the heavy inhibition occurred in phospholipid C (phosphatidyl choline) fraction but not in phospholipid B (phosphatidyl ethanolamine) fraction. It is interesting that the conversion of glycerides in the sequence of monoglyceride to triglyceride is indicated by the data in Tables 1 and 2 which show the highest specific radioactivity of fatty acid isolated from the monoglyceride fraction. The distribution of label in monoglyceride, diglyceride, and triglyceride fractions indicates that the incorporation of palmitic acid into monoglyceride was as much as 36.5 per cent, and furthermore, approximately 50 per cent of esterification was accomplished by the unsaturated fatty acids in this step. The further incorporation of oleic acid into the diglyceride and triglyceride fractions was the predominant feature.

The percentage composition of fatty acid in the diglyceride fraction and the phospholipid A (phosphatidic acid) fraction is similar, though the overall specific radioactivity of the diglyceride fraction is less than the half of that of the phospholipid A (phosphatidic acid) fraction and also the same as that of the triglyceride fraction, while the phospholipid fractions had high specific radioactivity among examined fractions. This evidence indicates that the degradation of triglycerides to form diglycerides or the degradation of phospholipids followed by a subsequent synthesis of neutral lipids occurred in the tissue slices. Thus, the equilibration of the label between the triglyceride and diglyceride fractions is derived. It is clear that oleic acid accumulated in the triglyceride fraction and the phospholipid C (phosphatidyl choline) fraction, both of which were the major constituents of the tissue lipids being representatives of glycerides and glycerophosphatides, respectively.

Since an unexpected rise of stearic acid among commonly occurring fatty acids was observed in the synthesized glycerolipids by the tissue slices, the time course studies on the fatty acid synthesis were conducted over a four-hour period. Figure 9 shows that palmitoleic, and arachidic acids were the least labelled acids among those examined. On the other hand, palmitic and oleic acids were quickly labelled and later diluted with endogenous substrate or equilibrated with non-radioactive fatty acids.

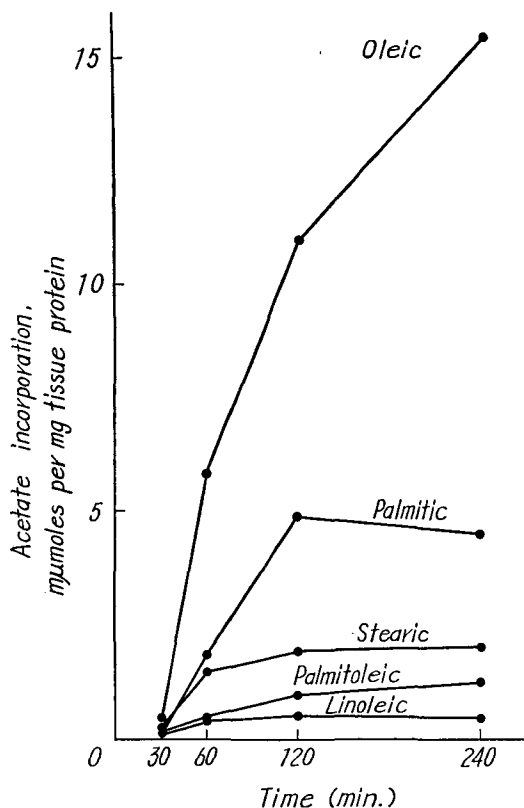


Figure 9. Fatty acid synthesis from acetate-1-C¹⁴ by the tissue slices of developing avocado fruit.

Stearic acid was synthesized almost as rapidly as oleic acid after vacuum infiltration of acetate (figure 10). This relationship continued for one hour then the percentage labelling in stearic acid decreased during the remainder of the experiment which lasted four hours, while the percentage labelling in oleic acid remained high throughout the experiment. The specific radioactivity of stearic acid was highest after one hour's incubation whereas that of oleic acid consistently increased during the experiment period. This observation suggests that there is a direct desaturation of stearic acid to form oleic acid. Although 5 to 10% of the label from an infiltrated stearate-1-C¹⁴ appeared in oleic, linoleic as well as palmitic acids, the rate of incorporation was not consistent in the tissue slices.

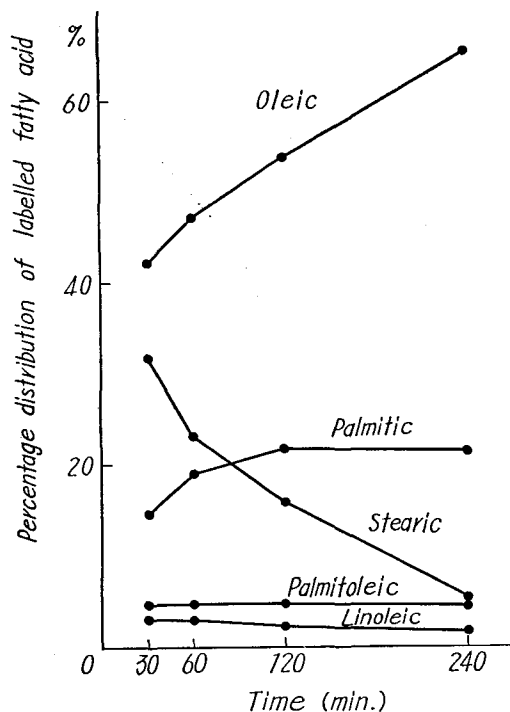


Figure 10. Changes in percentage distribution of labelled fatty acid synthesized from acetate-1-C¹⁴ by the tissue slices of avocado fruit.

E. Specific radioactivity of oleic acid synthesized from acetate-1-C¹⁴

After the failure of several attempts concerning the direct conversion of stearic acid to oleic acid by the tissue slices, the homogenates, and the particles isolated from mesocarp tissue of avocado fruit, chemical degradation of oleic acid was carried out by means of permanganate cleavage of the central double bond in the oleic acid molecule into pelargonic and azelaic acids. The oxidation products were separated by gas liquid chromatography followed by isotope counting. The calculated specific activity of synthesized oleic acid can be seen in figure 11 and Table 3.

Obviously the rapid incorporation of label was by chain elongation of oleic acid precursor with acetate-1-C¹⁴ during the initial two hours of incubation. However, specific activity of oleic acid was gradually increased over the eighteen hours examined. In contrast is the cessation of formation of stearic acid which could not be the direct precursor for oleate. The unknown fatty acid positioned between C₁₆ and C₁₈ might serve as the precursor. The specific activity of

TABLE 3. Changes of specific radioactivity of permanganate oxidation products of oleic acid synthesized by the tissue slices from mesocarp of avocado fruit

Oleic acid synthesized from acetate-1-C¹⁴ by the tissue slices was isolated by gas chromatography, oxidized by permanganate in acetone and the isolated fatty acids were methylated to inject on the column of the gas chromatograph. The oven temperature was programmed from 130°C (pelargonic acid) to 180°C (azelaic acid). Data are expressed as specific radioactivity, cpm/ μ g fatty acid.

I.

Time, min.	cpm/ μ g fatty acid				
	7	30	60	120	240
Pelargonic acid (a)	56	89	72	208	386
Azelaic acid (b)	138	202	481	694	571
(a+b)/2	97	145	277	451	478
Oleic acid	78	144	180	420	460
Stearic acid	956	1216	7500	9810	8340

II.

Time, hour	cpm/ μ g fatty acid			
	5	9	10	20
Pelargonic acid (a)	536	704	848	1240
Azelaic acid (b)	520	288	240	152
(a+b)/2	528	496	544	696
Oleic acid	456	568	584	720
Stearic acid	7710	8420	7930	7260
Unknown acid	2550	1656	1504	880

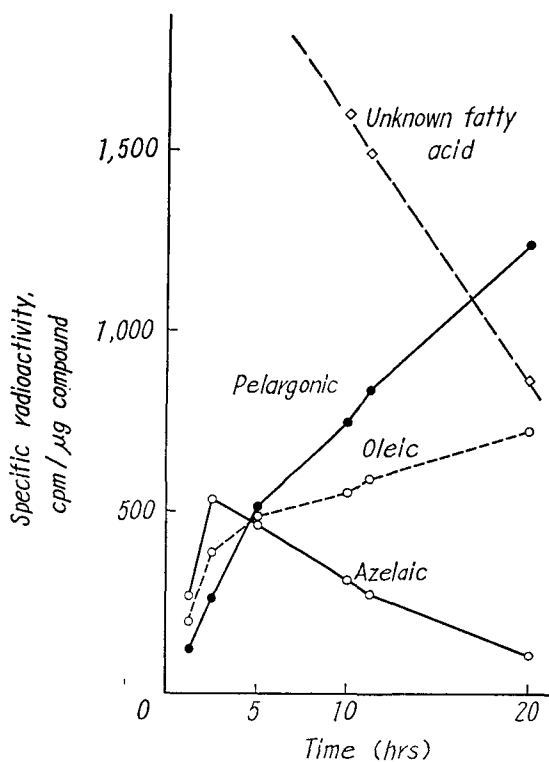


Figure 11. Time course studies on the oleic acid permanganate oxidation products synthesized from acetate-1-C¹⁴ by the tissue slices of avocado fruit.

the latter compound decreased with the response to the increase of pelargonic acid activity. However, the structure of the unknown compound was not determined.

DISCUSSION

Contrary to the avocado fruit mitochondrial particles which mainly incorporated acetate into water soluble acids, and into fatty acids, the tissue slices prepared from a developing fruit strikingly assimilated acetate into the lipids, especially triglycerides, while a small amount of water soluble acid was found. The distribution of the label in synthesized fatty acids has been shown to be 60% in oleic acid of the triglyceride fraction.

Since the intracellular localization of fatty acid synthesis is not clear, particularly in avocado, it is difficult to examine a suitable enzyme system isolated from avocado mesocarp. Much work concerning lipid synthesis in

plants has described the mitochondrial system from avocado fruit, the chloroplast preparation of spinach or preparation of castor bean leaves. Mitochondrial preparations from avocado mesocarp are rich in green pigment and glycolipid which indicates contamination with chloroplasts or chloroplast fragments. YANG and STUMPF (47) described a lipid synthesis system in the soluble fraction from avocado mesocarp. OVERATH and STUMPF (38) studied the characterization of this system which could be separated into two components quite similar to those of the *E. coli* system founded by GOLDMAN, ALBERT and VAGELOS (14). The heat stable components (ACP carrier protein) are interchangeable between avocado and the *E. coli* systems (38). Even though this soluble fatty acid system is present in avocado mesocarp, it could not account for all of the oleic acid synthesis. On the other hand, it could not exclude the possibility of oleic acid synthesis by the soluble system.

A water extract of acetone powder prepared by BARRON and STUMPF (3, 4) from the mitochondrial fraction could not produce oleic acid in spite of the ready formation of stearic acid. The only available system for oleic acid synthesis is the particulate fraction, with which the enzyme seems to be firmly associated.

Elaborate studies on the requirements for oleic acid synthesis by mitochondria-like particles described by MUDD and STUMPF (36) indicated that the presence of malonic acid, diluted particle concentration, elevated temperature of reaction mixture, and low pH favored oleic acid synthesis. These conditions also favor evolution of molecular oxygen or availability of this gas, and therefore lend support to the concept of the absolute requirement of molecular oxygen for oleic acid synthesis.

The early rise of labelling in stearic acid from vacuum infiltrated acetate- $1-C^{14}$ does not mean that an oxidative desaturation reaction takes place in the stearic acid molecule to form oleic acid. The specific activity of stearic acid became higher after one to two hours' incubation but kept constant during subsequent incubation. Further, the infiltrated stearate- $1-C^{14}$ was not shown to be converted to oleic acid, but rapidly incorporated into triglyceride, although free stearic acid was still present in the tissue slices after more than 8 hours' incubation.

It could be concluded that the synthesized stearic acid could arise from acetate condensation with palmitic acid. Perhaps the condensation was accelerated in the relatively anaerobic condition prevailing during the vacuum infiltration period. Oleic acid synthesis was slow but was consistent over the entire experimental period.

The formation of long-chain unsaturated fatty acids from corresponding

saturated fatty acids in animals was established by the work of SCHOENHEIMER and RITTENBERG (40). Studies in BLOCH's laboratory have shown that in yeast the formation of Δ^9 -unsaturated fatty acids is catalyzed by an enzyme system acting on the CoA derivatives of corresponding saturated fatty acids and requiring molecular oxygen and reduced triphosphopyridine nucleotide (8). In contrast to yeast, anaerobically grown *Clostridium butyricum*, which synthesized unsaturated acids, can not utilize long-chain saturated acids as sources for their unsaturated analogues, but the labels from octanoate-1-C¹⁴ and decanoate-1-C¹⁴ are incorporated into both saturated and unsaturated acids. Since the long-chain fatty acids were not randomly labelled, a chain elongation process, combined with the introduction of a double bond is the predominant mode of incorporation. Mechanism for the enzymatic synthesis of oleic acid in plants has still remained unknown.

Gas liquid chromatographic analysis of the linear relationship between logarithmic retention time and number of carbons in the chain of the fatty acid (23) revealed the presence of short chain monenoic acids but not that of dienoic and trienoic acids except linoleic and linolenic acids. JAMES (22) reported that only the short-chain saturated fatty acids, *i. e.*, caprylic, capric, lauric and myristic acids, gave rise to labelled oleic acid in isolated castor bean leaves. Two separate pools of the long-chain fatty acids were suggested. In one, the short-chain fatty acid was converted to unsaturated fatty acid, notably to oleic acid followed by linoleic and linolenic acids. The separate mechanism of saturated and unsaturated fatty acid synthesis was confirmed in avocado system that oleic acid synthesis was not sensitive to avidin while stearic acid synthesis was sensitive. Acetyl CoA was incorporated into stearic and oleic acids but malonyl CoA was mainly incorporated into stearic acid without inhibition by avidin (4).

It is well known that the acids of the tricarboxylic acid cycle regulate fatty acid biosynthesis (30). The mechanism proposed is an allosteric change of the biotin enzyme for the carboxylation reaction (31). High concentration of citrate enhanced the fatty acid synthesis by avocado particle enzymes and the presence of malonate gave rise to oleic acid synthesis (36). No finding is reported on the direct desaturation of saturated fatty acids in higher plants (4, 22, 36), nor elimination of a water molecule from 9-hydroxystearic acid (27).

The incorporation of acetate into tricarboxylic acid occurred in the particle enzyme from avocado (5, 36) and in chloroplast preparations (33, 34, 35). Malonic acid can arise from malonyl CoA (17) and the oxidative decarboxylation of oxaloacetate (12) in the plant. It may be suggested that the accumulation of malonate or malonyl CoA gives rise to a synthesis *de novo* of oleic

acid under aerobic conditions by an induction of conformational changes in the long-chain fatty acid synthetase system during the chain elongation reaction and also regulates tricarboxylic acid oxidation. Under anaerobic conditions, only chain elongation may take place.

SUMMARY

Studies on the lipid metabolism in the fruit of avocado, *Persea americana* MILL., were undertaken primarily to elucidate the pattern of acetate utilization followed by the fatty acid synthesis as an important process in the mesocarp of avocado. The vacuum infiltrated acetate-1-C¹⁴ was used up by the assimilation processes during two to four hours' incubation of the tissue slices. A mature fruit assimilated approximately 20 per cent of acetate into lipids which were gradually transformed from phospholipids to neutral lipids, especially to triglyceride. On the other hand, the tissue slices from the developing fruit showed rapid incorporation of acetate into triglyceride as much as 40 times faster than that into triglyceride by the mature fruit.

Subcellular particles isolated from the mesocarp of avocado fruit were simultaneously compared with tissue slices. It was shown that the utilization of phosphatidic acid was limited in the particulate system, even though triglyceride was formed. Free fatty acid was also accumulated by this system. Moreover, neutral lipid formation was reduced to 50 per cent in comparison with the results of the tissue slices of the avocado mesocarp.

The tissue slices could metabolize radioactive acetate into water soluble organic acids which were then oxidized to carbon dioxide via the tricarboxylic acid cycle. Palmitic, stearic and oleic acids were the most-labelled fatty acid found to accumulate in the tissue slices. Oleic acid was formed by way of chain elongation of already unsaturated short-chain fatty acids, as was the fact that the azelaic acid was rapidly labelled whilst the pelargonic acid was gradually labelled after the permanganate oxidation of the labelled oleic acid synthesized from acetate-1-C¹⁴ by the mesocarp tissue slices of avocado fruit.

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