LIPID METABOLISM IN THE FRUIT OF *PERSEA AMERICANA* MILL.

I. Studies on the Chemical Composition of Lipids and Their Changes During Fruit Development and Storage

Yoshio KIKUTA

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

Received December 11, 1967

INTRODUCTION

The avocado stores a large amount of lipids in the edible pulp (mesocarp) of the fruit. It would be of interest to elucidate the lipid metabolism in the fruit during growth and storage. From morphological and physiologcial view points of fruit development, SCHROEDER (35) observed that the avocado fruit deviated from most investigated fruits in its method of development, and that its initial growth involved cell division and cell enlargement. Later, cell division became the major factor for enlargement and continued as long as the fruit remained on the tree. Furthermore, a large droplet of reserve lipids was deposited in an idioblast, a large specialized cell, in the mesocarp tissue.

DAVENPORT and ELLIS (15) showed that the accumulation of reserve lipids in the mesocarp during fruit development was accompanied by a decrease in alcohol soluble and insoluble sugars. Such changes are consequently of interest in a study of lipid metabolism in the fruit of avocado.

Recent development of analytical methods for the separation and identification of lipids has materially facilitated studies of lipids and their metabolism, *i. e.*, silicic acid column chromatography (1, 19, 30, 38), thin layer chromatography (8, 31, 32), and gas liquid chromatography (21, 22). Thus in 1965, MAZLIAK (26) could identify six saturated and five unsaturated fatty acids in the mesocarp of avocado fruits. Among these fatty acids he showed that only four acids (palmitic and palmitoleic acids with sixteen carbons and oleic and linoleic acids with eighteen carbons) represented more than 95% of total fatty acids in the fruits. As far as deposited lipids were concerned, more than 60% of the total fatty acids was in the form of oleic acid. Prior to the research of MAZLIAK (26, 27), DAVENPORT and ELLIS (15) indicated that the major fatty acid constituent was a monoenoic acid which was synthesized during a long period of fruit development, while the saturated and polyunsaturated fatty acids were

synthesized only in the primary stage of growth.

In 1967, STUMPF and BARBER (36) demonstrated that mitochondria isolated from avocado mesocarp incorporated labelled acetate into long chain fatty acids in the presence of ATP[1], CoA NADPH, Mn^{++}, and HCO_3^- under aerobic conditions. Palmitic, and oleic acids were the most labelled fatty acids found to accumulate, with the greater radioactivity appearing in the latter, although they did not separate stearic acid from oleic acid fraction.

The biosynthesis of triglyceride in plants was also demonstrated by BARRON and STUMPF (3) in 1962. Microsomes isolated from avocado mesocarp appear to synthesize triglycerides via pathway essentially similar to the system in animal tissue (16). The route, called the KENNEDY and KORNBERG pathway (24), proceeds from glycerophosphate to phosphatidic acid to diglyceride and finally to triglyceride.

Studies on the avocado fruit were undertaken primarily to elucidate chemical composition of the lipid materials and their changes during growth and storage of the fruit.

MATERIALS AND METHODS

Plant materials

The Fuerte variety of Avocado, *Persea americana* MILL. (*P. gratissima* GAERTH.), was used in the studies on the seasonal changes of the lipids in the fruits, but the Hass variety was used in a number of isotope experiments designed for metabolic fates of substrates in the tissue slices. 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 used for experiments.

Crude lipid extraction

Ten grams of freshly grated tissue of avocado mesocarp were homogenized with 10 mℓ of chloroform-methanol mixture (2 : 1, by volume) in a stainless steel ball hammer mill. The resulting mixture was filtered through a Buchner funnel using two layers of WHATMAN No. 42 filter paper. The residue was again extracted with chloroform-methanol mixture and filtered. An additional 10 mℓ of the mixture was used for washing the mill and funnel. The combined extract was evaporated to dryness by using a vacuum rotary evaporator and an air stream. A nitrogen stream was used when it was necessary to avoid oxidation of the lipids in some experiments. The lipids were triturated with diethyl ether and dehydrated with anhydrous sodium sulfate, then reevaporated.

1) Abbreviations used are: ATP (Adenosine-5'-triphosphte); CoA (Coenzyme A); and NADPH (Reduced nicotinamide-adenine dinucleotide phosphate).

The dry lipids were dissolved in *n*-hexane to a volume of 10 mℓ. One half of the sample was dried overnight at 100°C and weighed. The remainder was used for the separation of the classes of lipids described by BARRON and HANAHAN (1).

Preparation and incubation of tissue slices

The technique of HACKETT (18) for preparation of fresh tissue slices and for incubation of the slices under strictly aerobic conditions was used. The tissue slices were prepared by using cork borer No. 7 and hand sectioning the mesocarp tissue from a single fruit. The slices were then infiltrated with a radioactive substrate in 8 mℓ of 0.05 *M* phosphate-bicarbonate buffer, pH 7.0 for 15 minutes under vacuum (10 cmHg). A bach of 10 slices were rinsed once with non-radioactive phosphate-bicarbonate buffer, blotted dry with filter paper and placed on a stainless wire net covered with a cheese cloth in a 125 mℓ respiratory flask. The tips of the cloth were dipped in 20 mℓ of the non-radioactive buffer so that the slices were kept moist during the incubation period. The side arm of the flask was used for trapping respiratory CO_2 in 5% NaOH. After incubation, the slices were transferred to chilled 10% trichloroacetic acid and frozen. The frozen samples were crushed in a mortar and the crude lipids were extracted with chloroform-methanol mixture. The slurry was centrifuged and the precipitate was again homogenized to extract residual lipids. The aqueous and chloroform fractions were separated and washed with chloroform and water, respectively.

Silicic acid column chromatograyhy

The methods of BARRON and HANAHAN (1) and HIRSH and AHRENS (19) were modified for separation of phospholipids. The method described here could separate glycerophospholipids following separation of the neutral lipids. This was accomplished by successive elution with increasing concentrations of methanol in diethyl ether.

Silicic acid was prepared from 100 mesh silicic acid for chromatographic analysis by removal of the fine particles through repeated suspension in N HCl and in distilled water, and decantation of the slower settling particles until approximately one third of the original was removed. The remaining fraction containing the coarse particles was filtered, dried in an oven at 110°C for 24 hours and stored in a closed container. Activation of silicic acid was carried out by successive suspension and decantation for removal of materials soluble in methanol, methanol-ether, benzene, diethyl ether, and *n*-hexane. The resulting silicic acid fraction was dried for 24 hours in an oven at 120°C. All solvents were checked by gas liquid chromatography and by residue measure-

ment after evaporation in a mild air stream.

The standard column was prepared by packing a slurry of 6 g of activated silicic acid in hexane into a chromatographic tube (12mm × 30cm) in which a glass wool plug was placed in the bottom to support the column. An air pressure was applied to the top of the tube to speed the removal of excess solvent, care being exercised not to allow the solvent level to fall below the top of the column.

The sample of the crude lipids, containing a total of 10 to 200 mg, dissolved in hexane, was thoroughly mixed with 0.2 g of silicic acid. Then the sample was quantitatively transferred to the top of the column through a long tipped pipette. Standard lipid was synthesized by esterification of oleic acid and glycerol. Elimination of water from the reaction mixture gave rise to a high yield of triolein concomitant with 1,3-diolein, 1,2-diolein, 1-monoolein and 2-monoolein which were separated by the column followed by identification of primary and secondary hydroxyl group (s) in the synthesized glycerides by infrared absorption spectroscopy (12, 30).

Development of this column proceeded by addition of a series of hexane-ether solvents experimentally selected to give maximum separation of the classes of lipids. The elution schedule was as follows:

Fraction	Elution volume
n-hexane	50 mℓ
15% benzene in hexane (by volume)	30 mℓ
5% diethyl ether in hexane	100 mℓ
15% diethyl ether in hexane	30 mℓ
30% diethyl ether in hexane	40 mℓ
90% diethyl ether in hexane	50 mℓ
90% methanol in diethyl ether	100 mℓ

The glycerophospholipids were successively separated after the elution with 90% diethyl ether in hexane followed by addition of a series of methanol-diethyl ether mixtures:

Fraction	Elution volume
5% methanol in diethyl ether	30 mℓ
20% methanol in diethyl ether	30 mℓ
40% methanol in diethyl ether	30 mℓ
60% methanol in diethyl ether	30 mℓ
methanol	50 mℓ

These solvents were added to the top of the chromatographic column through a reservoir which permitted the application of air pressure. The pressure was

adjusted so that the effluent was collected at 80 mℓ per hour. Subsequent solvents were added just before the last of the preceding solvent passed into the column. Each fraction of the effluent from the column was collected by a Gilson automatic fraction collector.

Thin layer chromatography

Five grams of silica gel (Applied Scientific Co.) were spread over each 20 × 20 cm glass plate. The plates were activated at 110°C for 30 minutes. The two types of developing solvent systems (31) used for separation and identification of the classes of lipids were diethyl ether: *n*-hexane: acetic acid (10 : 90 : 1, by volume) for neutral lipids; and chloroform: methanol: water (85 : 15 : 3, by volume) for phospholipids and glycolipids. Compounds were detected (32) by spraying 1.0% iodine-methanol solution over the plate. The iodine vapour could be used to detect almost any organic compound. Bromocresol green gave spots for all lipids. Special reagents used for characterization of lipids were ninhydrin for the amino group in cephaline, diphenylamine for glycolipids and inositides, $SnCl_2$–HCl for lecithin, perchloric acid-molybdate reagent for phosphatidic acid and other phospholipids. Detection of phosphatidyl glycerol was carried out by periodate oxidation of adjacent hydroxyl groups followed by SCHIFF's base formation (23).

Saponification and esterification procedures

The lipid sample was saponified with 5% methanolic KOH by refluxing for about 30 minutes. 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 residue was again dissolved in 10% methanol in ether. An equal volume of 1.0% diazomethane 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.

Gas liquid chromatography and C^{14} counting

The gas liquid chromatography units of Varian Aerograph Inc. HY–FI model 550 oven and HY–FI model 600 D electronometer were used with a Honywell-Brown 0.1mV 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

40 mℓ per minute flow, and hydrogen at 15 mℓ per minute flow.

The 90% 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 (22). The collected effluent was eluted with chloroform and transferred to planchets for radioactive counting.

All radioactivity determinations were made using a Tracer Lab. Compumatic II scaler with a gas flow Geiger-Mueller tube of a thin end window type. For counting lipids, a thin layer (less than 0.1 mg/cm^2) of the sample was mounted to avoid self absorption of C^{14}. The specific radioactivity of fatty acids was determined by gas liquid chromatographic analysis of the fatty acid methyl esters follwed by C^{14} counting of the collected effluent under each peak.

Infrared absorption spectroscopy

Infrared absorption spectroscopy was employed for the qualitative analysis of the classes of lipids isolated from avocado mesocarp with a Beckman infrared spectrometer (IR-8). The purified sample lipids were placed on the NaCl plates as liquid films. The spectra obtained were identified by their characteristic absorption band assigments (12, 29, 30).

Since lipids contain methylene and methyl groups, their infrared spectra have bands at 2850–2950 cm^{-1} (νCH, usually two bands resolved), at *ca* 1465 cm^{-1} (δCH of methylene and methyl) and at 1380 cm^{-1} (δCH of methyl). The carbonyl group is notably important in lipid, producing strong absorption in the region 1660–1760cm^{-1}. A fatty acid as a carboxylic acid, the band at 1760 cm^{-1} (monomer) or 1710 cm^{-1} (dimer) which is associated with 2500–3000 cm^{-1} band characteristic of νOH and the bands at 1420 cm^{-1} and 1200–1300 cm^{-1} are due to coupling between OH bending and C–O stretching of dimer hydrogen bonding. While in glycerides as in other esters, there is a band at 1735 cm^{-1} for the carbonyl group and two at 1050–1300 cm^{-1} corresponding to asymmetric and symmetric stretching of C–O–C ester bond (*i. e.*, 1190 cm^{-1} for RCOOR, 1165 cm^{-1} $RCOOCH_3$).

OH stretching occurs in lipids in a broad band from 2500 to 3600 cm^{-1} depending on the position of the hydroxy group. However, the resolution in this region was not enough to estimate the position of the OH group in the lipid. On the other hand, the C–O stretching vibration could be resolved into 3 maxima: 1050 cm^{-1} for the primary OH, 1100 cm^{-1} for the secondary OH, and 1150 cm^{-1} for the tertiary OH.

Complex lipids are difficult to characterize mainly because of the great variety of possible isomers. Crystalline samples gave sharper bands than the spectra of liquids. Triglyceride could be distinguished from diglyceride and monoglyceride by the presence or absence of the absorption band at 3200–3650 cm^{-1} (νOH). Bands at 3200–3650 cm^{-1} (νOH) and 2850–2950 cm^{-1} (νCH_3–) could serve for the identification of di- and monoglycerides. The bands at 1050–1150 cm^{-1} for C–O vibration could also distinguish between di- and monoglycerides: 1-monoglyceride having secondary-OH and primary-OH, 2-monoglyceride primary-OH only, 1, 3-diglyceride secondary-OH, and 1, 2-diglyceride primary-OH.

The most interesting band of glycolipid spectra is that of the hydroxy group. The α-sugar has a characteristic band at 844 ± 8 cm^{-1} and the β-sugar has one at 891 ± 7 cm^{-1}. Variable C–O–C stretching appeared at 1030–1150 cm^{-1}. Three other bands at 770 cm^{-1}, 844 cm^{-1} or 891 cm^{-1} and 920 cm^{-1}.

The infrared absorption spectra of a number of phospholipids have been published but there are many gaps and discrepancies in the data (12, 30.) The main discrepancy was caused by impurities in the samples. A common feature in the infrared spectra of phospholipids is the absorbance by a phosphate group at 1050 cm^{-1} for νP–O–C or at 1250 cm^{-1} for νP=O.

RESULTS

A. *Separation and identification of lipids*

The lipids extracted from avocado mesocarp were separated by silicic acid column chromatography as well as thin layer chromatography. The isolated lipids were characterized by infrared absorption spectroscopy and the fatty acid composition was determined by gas liquid column chromatography.

The separation of avocado lipids by thin layer chromatography was carried out using silica gel adsorbent with two solvent systems. The results are summarized as follows:

a) *n*-Hexane: diethyl ether: acetic acid (140 : 60 : 1, by volume)

Lipid	*Rf*
Triglyceride	0.9
Free fatty acid	0.7
1, 3-Diglyceride	0.5
Pigment	0.35
1, 2-Diglyceride	0.3
Pigment	0.25
Monoglyceride	0.1
Phospholipids and glycolipids	origin

When the proportion of hexane was increased, the above *Rf* values were decreased and carotinoids appeared in front of the triglyceride. The two pigments in the above table were chlorophylls.

b) Chloroform : methanol : water (85 : 25 : 3, by volume)

Lipid	*Rf*
Neutral lipids	1.0
Pigments	0.9
Phosphatidic acid	0.8
Glycolipid II	0.7
Phosphatidyl ethanolamine	0.6
Phosphatidyl glycerol	0.5
Glycolipid I	0.4
Phosphatidyl choline	0.3
Lysophosphatidic acid	0.2
Phosphatidyl inositol	0.1
Unknowns	0.05

The unknowns may be lysophosphatides and sphingolipids.

Identification of the neutral lipids

The silicic acid column chromatography of the crude lipid extract obtained from avocado mesocarp is presented in figure 1. Three major independent peaks and several minor peaks were obtained by this procedure. They will be discussed in the order of appearance.

Fraction I (Eluted with *n*-hexane and with 15% benzene in hexane). Hydrocarbons and waxes are supposed to elute in this fraction (1, 38). No further investigation was made concerning carotinoid pigments, waxes and steroids.

Fraction II (Eluted with 5% diethyl ether in hexane). The major peak was triglyceride. The minor peak in this fraction was free fatty acid contaminated with a small amount of triglyceride. Although the authentic compounds of triolein and oleic acid could be resolved by this procedure, for the separation of free fatty acid from natural products it was necessary to wash the preparation with diethyl ether-alcoholic KOH solution. Thin layer chromatograms showed that the major peak gave one spot, and minor peak two spots, triglyceride and free fatty acid.

Fraction III (Eluted with 15% diethyl ether in hexane). The effluent from the column was identified as 1, 3-diglyceride from its infrared absorption spectrum, which showed the presence of a secondary hydroxy group. However, the neutral lipid had two spots on a thin layer chromatographic plate

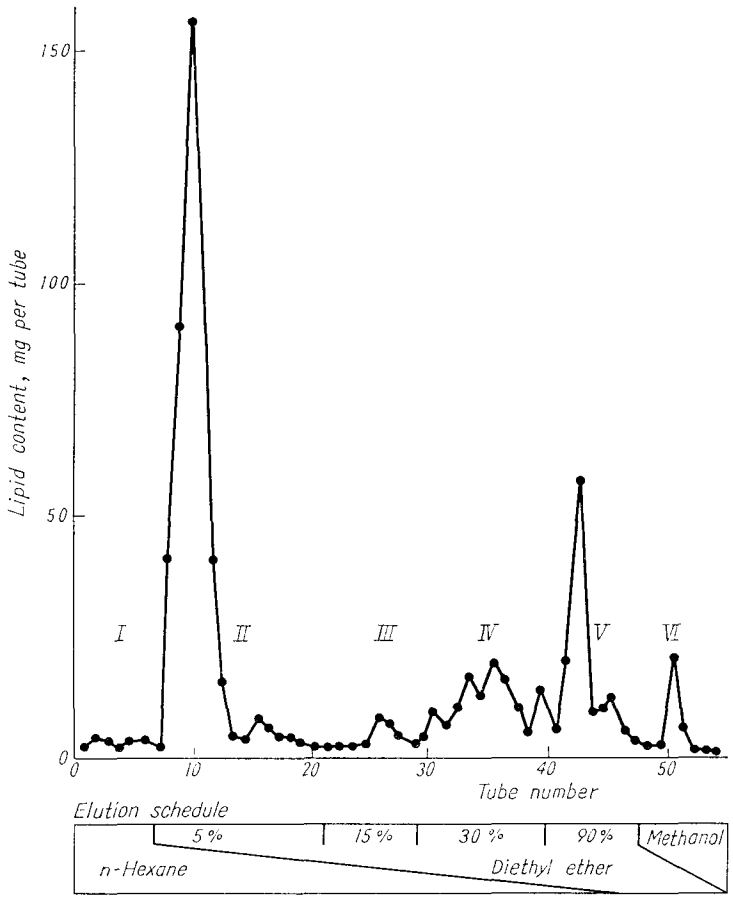

Fig. 1. Silicic acid column chromatography of total lipid extract from the mesocarp of Fuerte avocado fruit.

corresponding to 1, 2- and 1, 3-diglycerides. The latter was a minor constituent and probably an artifact. The fatty acid composition of this fraction showed that the presence of polyunsaturated fatty acids was greater than that of acids found in the following fraction.

Fraction IV (Eluted with 30% diethyl ether in hexane). Several peaks appeared in this fraction. The first three peaks were uniformly a yellow oil, which contained mostly 1, 2-diglyceride. The following peak, a dark green oil, was readily crystallized at room temperature. This material was a mixture of chlorophyll and glycolipid. The infrared spectrum revealed that the lipid contained hexose residue besides a diglyceride residue in the molecule (33). Gas liquid chromatographic analysis indicated that 76.1% the fatty acid was

TABLE 1. Percentage composition of fatty acids in various classes of lipids found in avocado mesocarp tissue

A. Fuerte avocado fruit harvested November 8, 1966

Lipid	Percent fatty acid composition[1]							
	16:0	16:1	18:0	18:1	18:2	18:3	20:0	UK
Hydrocarbons								
Triglyceride	25.4	7.0	0.5	54.3	12.3		0.5	
Free fatty acid	20.3	9.7	0.4	43.7	22.5	3.0		0.4
Diglyceride I	15.0	9.0		45.0	28.0	3.0		
Diglyceride II	18.4	3.9	0.7	64.8	12.2			
Glycolipid I	6.7	2.5	1.6	13.1	76.1			
Momoglyceride	17.1	7.2	2.7	43.2	24.3	1.0	0.9	3.6
Glycolipid II	3.8	2.2	1.2	12.8	74.1	6.0		
Phospholipid	16.9	4.4	3.3	20.5	36.1	9.8		9.0

B. Hass avocado fruit harvested April 14, 1966

Lipid	Percent fatty acid composition							
	16:0	16:1	18:0	18:1	18:2	18:3	20:0	UK
Triglyceride	17.7	7.8	0.2	61.2	12.0		1.1	
Diglyceride	12.6	6.2	0.4	67.4	11.2	0.8	1.0	0.8
Monoglyceride	35.2	7.0	2.4	37.5	5.4	6.3	1.0	5.2
Phospholipid I[2]	18.8	5.6	5.6	52.5	8.0	9.0	0.2	0.3
Phospholipid II	23.4[3]	1.9	0.6	39.7	14.2	17.0	0.2	3.0
Phospholipid III	16.8	3.7	1.0	24.4	30.5	22.8	0.4	0.4
Phospholipid IV	8.2	2.8	0.6	50.9	5.1	32.0	0.1	0.3

1) Symbols are: 16:0; palmitic, 16:1; palmitoleic, 18:0; stearic, 18:1; oleic, 18:2; linoleic, 18:3; linolenic, 20:0; arachidic, UK; unknown fatty acids.
2) Phospholipid I, II, III, and IV represent phosphatidic acid, phosphatidyl glycerol, phosphatidyl ethanolamine, and phosphatidyl choline, respectively.
3) Including *trans*-hexadecenoic acid.

linoleic acid, 6.7% palmitic acid, 2.5% palmitoleic acid, 1.6% stearic acid, 13.1% oleic acid. The same type of glycolipid was found in fraction V except that the infrared spectrum was not identical for hexose residue.

Fraction V (Eluted with 90% diethyl ether in hexane). Monoglyceride appeared in the first major peak and a glycolipid in the minor peak. The fatty acid composition of the glycolipid was mainly the same as the glycolipid described in the fraction IV, *i.e.*, 74.1% linoleic acid, 6.0% linolenic acid,

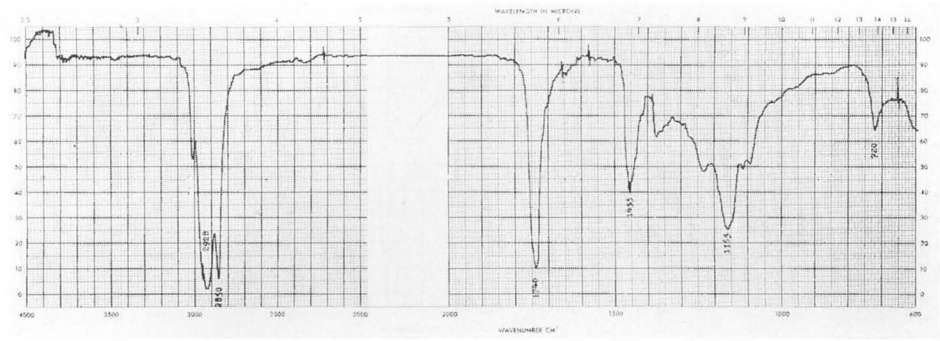

Fig. 2. (A)

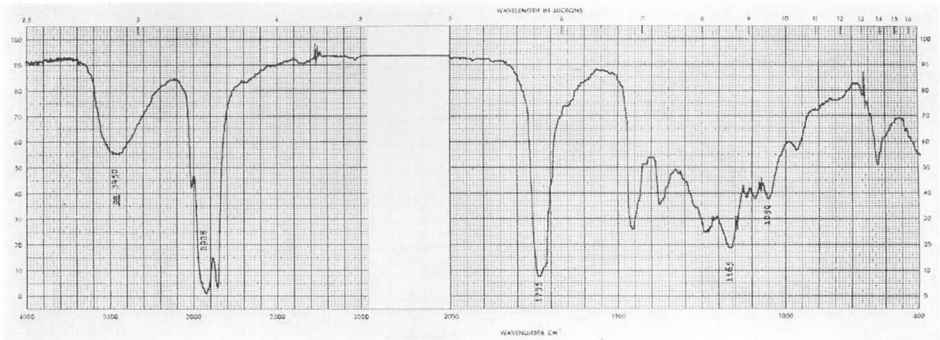

Fig. 2. (B)

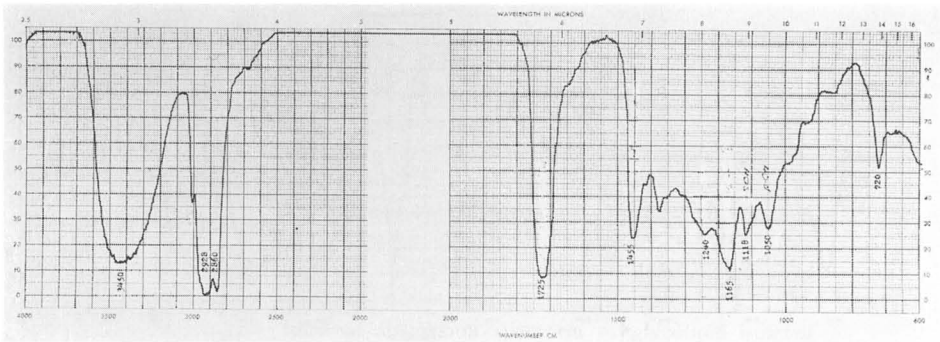

Fig. 2. (C)

12.8% oleic acid, 3.8% palmitoleic acid, 3.8% palmitic acid, and 2.2% stearic acid.

Fraction VI (Eluted with methanol). In this layer chromatographic analysis of this fraction, the material applied on the silica gel plate stayed at the origin when developed in *n*-hexane: diethyl ether: acetic acid (140 : 60 : 1,

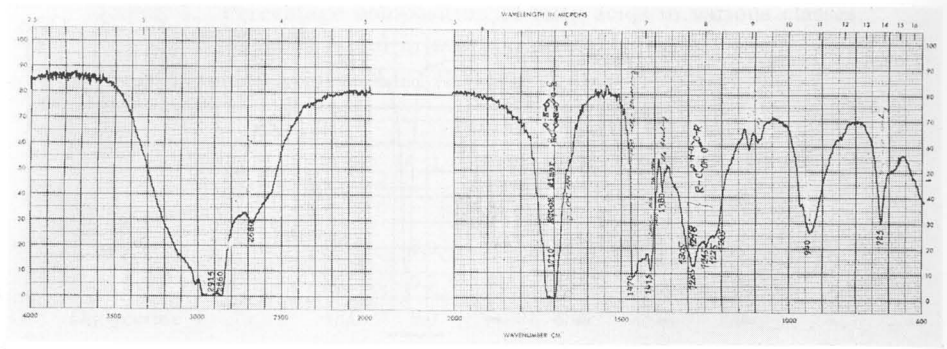

Fig. 2. (D)

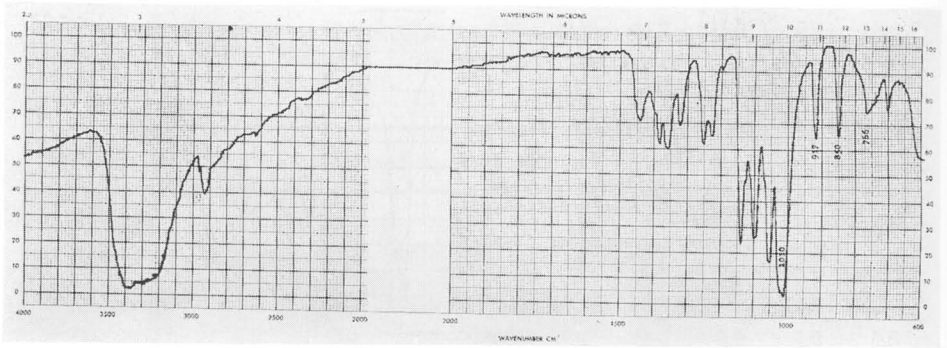

Fig. 2. (E)

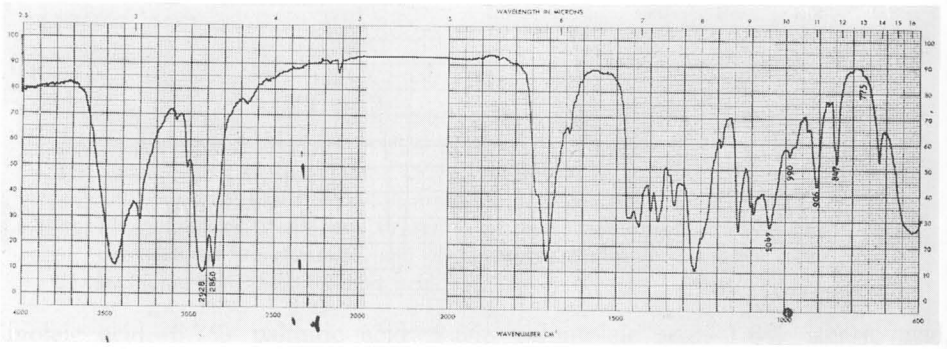

Fig. 2. (F)

by volume), but migrated when developed in chloroform : methanol : water (85 : 25 : 3, by volume). Five major phospholipids separated in this fraction.

The fatty acid composition of the lipids in the various fractions is shown in Table 1 and the infrared spectra of triglyceride, diglyceride, monoglyceride, glycolipid, phospholipid and oleic acid are shown in figure 2.

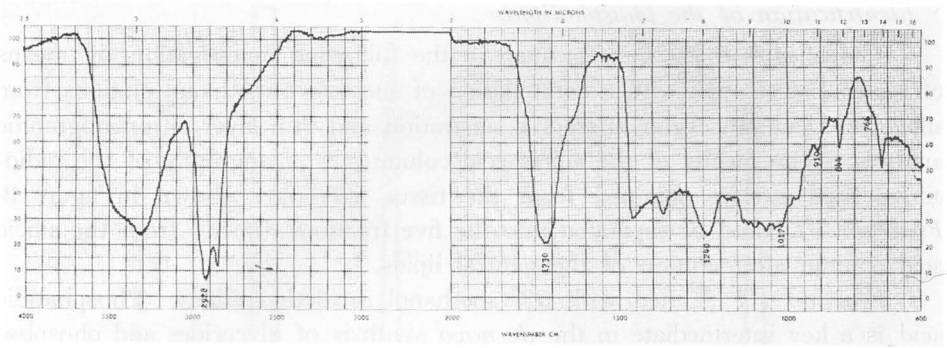

Fig. 2. (G)

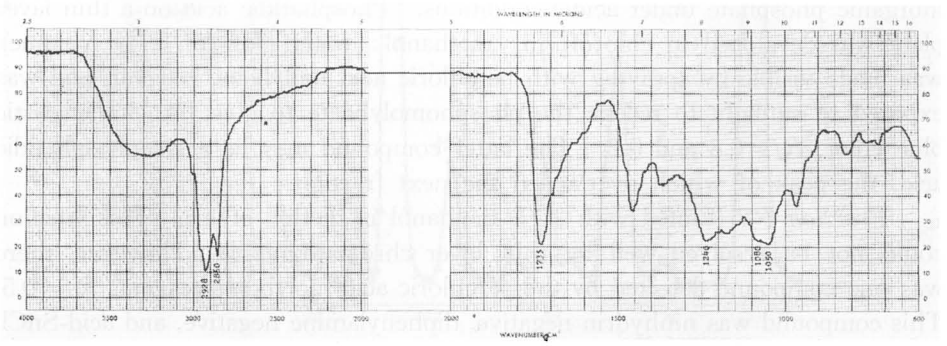

Fig. 2. (H)

Fig. 2. (A) Infrared absorption spectrum of triglyceride isolated from Fuerte avocado fruit.

(B) Infrared absorption spectrum of diglyceride isolated from Fuerte avocado fruit.

(C) Infrared absorption spectrum of monoglyceride isolated from Fuerte avocado fruit.

(D) Infrared absorption spectrum of oleic acid obtained from commercial source.

(E) Infrared absorption spectrum of dextrose obtained from commercial source.

(F) Infrared absorption spectrum of glycolipid isolated from Fraction IV of avocado lipids.

(G) Infrared absorption spectrum of glycolipid isolated from Fraction V of avocado lipids.

(H) Infrared absorption spectrum of phospholipid isolated from Fraction P5 of avocado lipids.

Identificatian of the phospholipids

The phospholipids were isolated in the following investigation by means of tracer experiments with a combination of successive methanol elutions from silicic acid column chromatographic separation and thin layer chromatographic analysis. The results of the silicic acid column chromatography of the radioactive lipid extract obtained from the tissue slices are shown in figure 3. *Fraction VI* could be separated into the five fractions directly from the silicic acid column after elution of the neutral lipids.

Fraction P1 (Eluted with 5% methanol in diethyl ether). Phosphatidic acid is a key intermediate in the *de novo* synthsis of glycerides and phosphoglycerides (16, 24). This compound is detected by hydrolytic removal of inorganic phosphate under acidic conditions. Phosphatidic acid on a thin layer plate was developed in chloroform : methanol : water (85 : 25 : 3, by volume), was made visible by spraying with perchloric acid molybdate reagent, and was exposed to sunlight to reduce the phosphomolybdate to give the characteristic blue color, $Rf=0.8$ and 0.2. The latter compound may be a lysophosphatidic acid, the peak of which overlapped the next fraction.

Fraction P2 (Eluted with 20% methanol in diethyl ether). This fraction could not be resolved well by thin layer chromatography. However, there was one compound detected by the perchloric acid molybdate reagent, $Rf=0.5$. This compound was ninhydrin negative, diphenylamine negative, and acid-$SnCl_2$ reagent negative. The periodate SCHIFF's base reagent gave three spots including the above compound. It may be concluded that this compound is phosphatidyl glycerol, although only 14% of the fatty acids in this fraction was *trans*-3-hexadecenoic acid positioned between palmitic and palmitoleic acids detected by gas liquid chromatography. There was a large amount of unknown lipids presented in this fraction including lysophosphatidic acid and a glycolipid.

Fraction P3 (Eluted with 40% methanol in diethyl ether). The major constituent in this fraction was a ninhydrin positive compound, $Rf=0.6$, perhaps phosphatidyl ethanolamine. Another compound, $Rf=0.2$, was also ninhydrin positive. The presence of phosphatidyl serine is uncertain in plants (23).

Fraction P4 (Eluted with 60% methanol in diethyl ether). Very little occurred in this fraction and it was difficult to identify. It was diphenylamine positive and acid molybdate positive, $Rf=0.1$. The principal constituent was tentatively identified as phosphatidyl inositol. Another lipid in this fraction was ninhydrin positive, $Rf=0.00$ to 0.05.

Fraction P5 (Eluted with 100% methanol). The lipid in this fraction was indicated to be phosphatidyl choline by the reagent of hydrochloric acid-

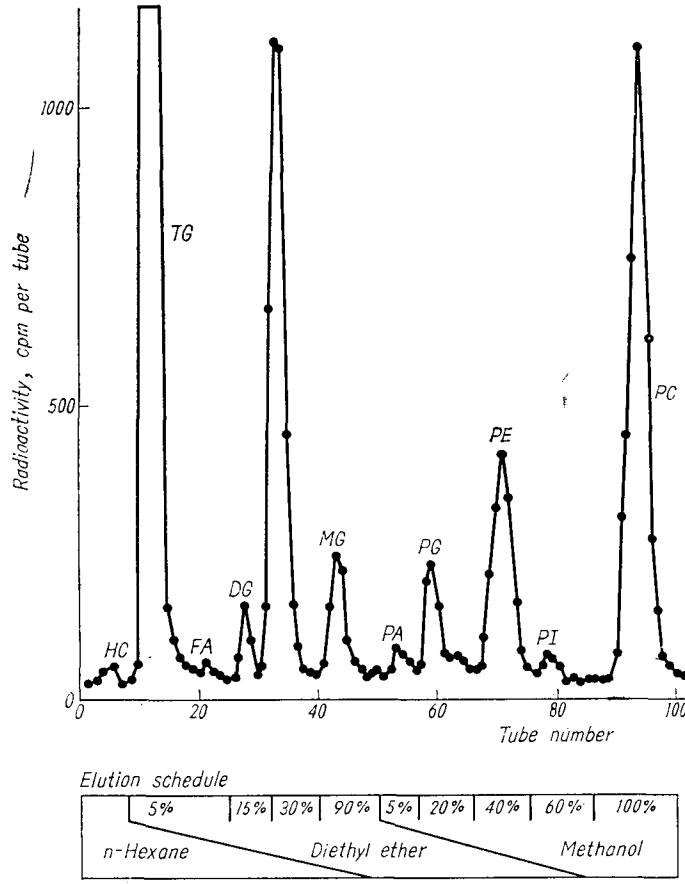

Fig. 3. Silicic acid column chromatography of total lipid extract from the tissue slices of Hass avocado fruit incubated with acetate-1-C^{14} for 120 minutes. Symbols used are: HC (Hydrocarbons), TG (Triglycerides), FA (Free fatty acids), DG (Diglycerides), MG (Monoglycerides), PA (Phosphatidic acid), PG (Phosphatidyl glocerol), PE (Phosphatidyl ethanolamine), PI (Phosphatidyl inositol), and PC (Phosphatidyl choline).

$SnCl_2$ solution, $Rf=0.3$. Approximately 2.8 μmoles of fatty acid per gram of the tissue could be isolated from the developing fruit, while a mature fruit had 5.5 μmoles of fatty acid produced by saponification of this fraction.

B. Changes in lipids during fruit development and storage

The lipid content of avocado mesocarp during the fruit growth has been

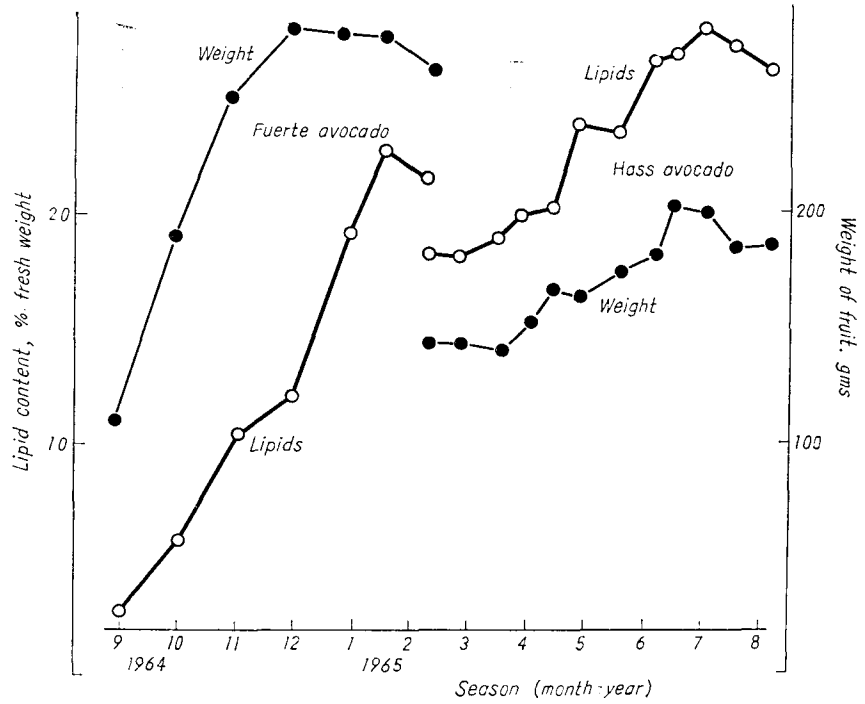

Fig. 4. Seasonal changes in lipids and weight of Fuerte and Hass avocados.

plotted in figure 4. Fruit weight are also shown. Although the Fuerte and Hass avocado differed in size and lipid content at a given time, it was apparent that the growth of both varieties almost ceased before the fruit showed its maximum accumulation of reserve lipid. As the lipid content of the mesocarp increased during fruit development, the content of water was reduced.

The marked increase in lipid content in Fuerte avocado fruit took place mainly over a four months' period (October to January). In the Hass avocado, the lipid accumulation activity seemed to be prolonged for about nine months. The large increase in lipids during fruit development was found to be mainly a large increase in the triglyceride fraction (figure 5). The phospholipid fraction remained constant over the examined period of fruit growth. The monoglyceride fraction appeared to be slightly decreased with concomitant increase in the diglyceride fraction. The data of figure 6 indicate that it was principally oleic acid which was being synthesized during this period. Palmitic, palmitoleic, and linoleic acids increased slightly while linolenic acid remained constant. The fatty acid composition of the total lipid from Fuerte avocado

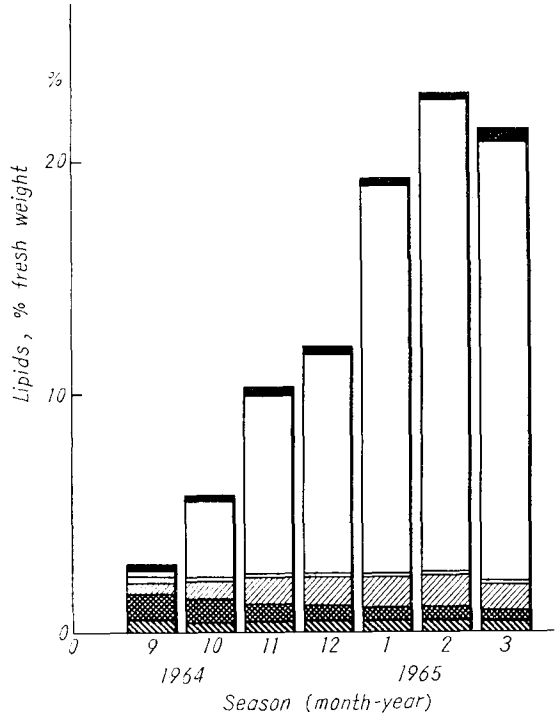

Fig. 5. Seasonal changes of classes of lipids in the mesocarp of Fuerte avocado fruit.
Fractions from top to bottom in each histogram are: hydrocarbons, triglycerides, free fatty acids, diglycerides, monoglycerides, and phospholipids.

mesocarp has been plotted with respect to fruit weight in figure 7. Twenty fruits were sampled from September to December. Drastic changes between linoleic and oleic acids were obserbed while palmitic acid together with palmitoleic acid remained constant. It seems probable that these changes were due to the initiation of reserve lipid formation in the mesocarp of the fruit, while in younger fruit the bulk of fatty acids had arisen from phospholipids and glycolipids which were uniquely associated with the structural membranes of the fruit cells (7, 37). Thus, early rise of linoleic and linolenic and unknown fatty acids described by MAZLIAK (26) and DAVENPORT and ELLIS (15) may be associated with these lipids.

The changes in lipid content during the ripening and storage period were determined and are shown in Table 2 for two lots of fruit harvested on January 19, 1965, or on February 17, 1965. The fruits were stored for 9,

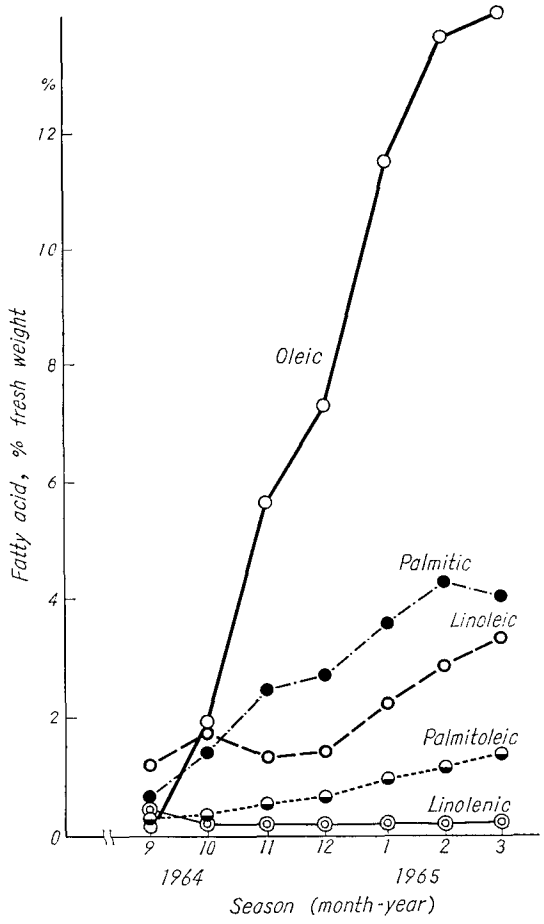

Fig. 6. Seasonal changes of fatty acid composition in the fruit of Fuerte avocado.

or 14 days, respectively. There were slight increases in the total lipids during storage in both lots of fruit. However, there were large increases in the monoglyceride and free fatty acid fractions, with some relative decreases in the diglyceride and phospholipid fractions. This sequence of change suggests that the rate of triglyceride formation was exceeded by the increased rate of degradation of triglyceride to form monoglyceride and free fatty acid in the fruit during storage. The reverse situation could explain the accumulation of the reserve lipids in the developing fruit (figure 5).

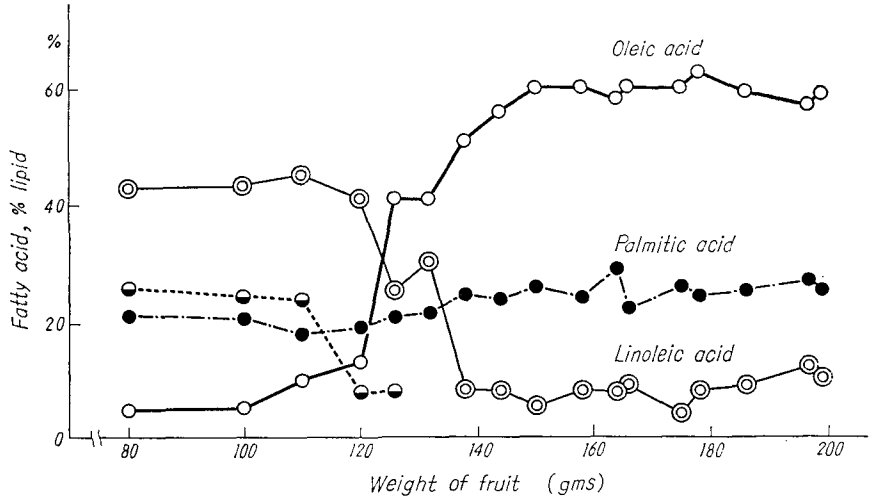

Fig. 7. Fatty acid composition of total lipids in Fuerte avocado.

TABLE 2. Percentages of various classes of lipids in Fuerte avocado mesocarp tissue after storage

Date harvested	January 19, 1965			February 17, 1965		
Days after harvest	1	9		1	14	
Class	%	%	diff.	%	%	diff.
Triglyceride	16.40	17.10	0.70	19.96	19.51	− 0.45
Diglyceride	1.63	1.28	− 0.35	1.29	1.24	− 0.05
Monoglyceride	0.50	1.33	0.38	0.78	2.26	1.48
Free fatty acid	0.12	0.29	0.17	0.10	0.50	0.40
Phospholipid	0.32	0.30	− 0.02	0.39	0.20	− 0.19
Others[1)]	0.23	0.25	0.02	0.28	0.31	0.03
Total lipids	19.20	20.55	1.35	22.80	24.02	1.22
Weight loss		5.0			7.5	

1) Other substance extracted with chloroform: methanol (2:1) included hydrocarbons and sterols.

C. *Assimilatian of acetate in the fruit of avocado*

As was shown in previous figures, the avocado fruit has the ability to synthesize as much as 200 mg of lipid per gram of fresh mesocarp during a four-month's period. The metabolic fate of acetate utilized by avocado mesocarp is dependent upon the stages of development of fruit, since acetate, by way of acetyl CoA, is incorporated into organic acids in TCA cycle, into

TABLE 3. Seasonal changes in the incorporation of acetate into water soluble acids and lipids by the tissue slices from the mesocarp of developing Hass avocado

25 μC of acetate-1-C^{14} (7mC/mM) were used for the vacuum infiltration of 8 g of the tissue slices with 8 mℓ of 0.05 M phosphate-bicarbonate buffer, pH 7.0. Data show mμmoles of acetate incorporated per hour per g fresh weight.

Date harvested	Dry weight	Acids	Lipids	Phospholipid
	%	mμmol.	mμmol.	mμmol.
Oct. 6, 1965	13.6	46.5	235.0	13.0
Apr. 14, 1966	16.3	44.5	367.0	73.5
Nov. 25, 1966	19.0	33.0	249.0	60.2
Feb. 9, 1967	22.4	49.0	94.0	12.5

glycerolipids, into pigments and even into sugars and proteins (5, 6). Table 3 shows the incorporation of acetate-1-C^{14} into lipids and water soluble acids by Hass avocado tissue slices related to the maturity of the fruits. Although Hass avocado requires eighteen months for the development of the fruit, the

TABLE 4. Effects of aeration on the assimilation of acetate by the tissue slices from mature and developing Hass avocado fruits

25 μC of acetate-1-C^{14} (7 mC/mM) were used for the vacuum infiltration of 8 g of the tissue slices with 8 mℓ of 0.05 M phosphate-bicarbonate buffer, pH 7.0. Air or nitrogen was passed through the reaction chambers at the rate of 60 mℓ per minute during the incubation period. A mature fruit was picked on March 3, 1966 and a developing fruit was picked on April 14, 1966. Data expressed cpm acetate incorporated per g fresh weight per hour and per cent distribution of the label.

Atmosphere / Fraction	Air		Nitrogen	
	cpm	%	cpm	%
Mature fruit:				
Water soluble acids	55,100	75.5	21,800	78.2
Total lipids	15,375	24.5	7,050	21.8
Neutral lipids	4,060	26.4	4,500	63.9
Phospholipids	11,315	73.6	2,550	36.1
Developing fruit:				
Water soluble acid	44,500	11.0	38,000	13.2
Total lipids	347,000	89.0	250,000	86.8
Neutral lipids	293,600	80.0	208,000	65.8
Phospholipids	73,400	20.0	42,000	34.2

incorporation of acetate into organic acids of TCA cycle members is nearly constant over a long period of development of fruit. However, in the developing fruit which was rapidly accumulating lipid, most of acetate contributed to the assimilation sequence leading to the formation of triglyceride.

Under anaerobic conditions, rates of incorporation of acetate into both the lipid and the water soluble acid fractions were reduced, but the anaerobic condition was especially inhibitory to lipid synthesis (Table 4). A mature fruit assimilated approximately 20% of acetate into lipids during four hours' incubation period. On the other hand, the tissue slices from the developing fruit showed rapid incorporation of acetate into lipids as much as twenty times

TABLE 5. Specific radioactivity of fatty acid components in the classes of lipids synthesized from acetate-1-C^{14} by the tissue slices of avocado mesocarp

25 μC of acetate-1-C^{14} (7 mC/mM) were used for the vacuum infiltration of 8 g of the tissue slices in a total volume of 16 mℓ, with 0.05 M phosphate-bicarbonate buffer, pH 7.0. The tissue slices were incubated for 2 hours in the reaction chamber described in materials and methods. Specific radioactivity is expressed as cpm per μg of fatty acid isolated from the tissue slices of avocado fruit.

Fraction	Fatty acid in mesocarp slices				
	16:0[1)]	16:1	18:0	18:1	18:2
Triglyceride					
% Component	23.9	7.2	0.9	56.3	11.7
% Labelled	19.7	3.7	5.2	67.1	4.3
cpm μg acid	763	667	6436	1156	532
Diglyceride					
% Component	29.9	12.3	0.3	48.8	8.7
% Labelled	18.4	7.9	5.2	63.3	5.1
cpm μg acid	807	833	21973	1678	683
Monoglyceride					
% Compont	33.2	11.8	0.7	43.4	10.9
% Labelled	21.9	7.3	7.4	51.9	11.5
cpm μg acid	1856	1247	21287	2403	516
Phospholipids					
% Component	29.6	12.8	0.9	38.9	17.8
% Labelled	21.6	7.0	6.5	51.2	13.7
cpm μg acid	2553	1861	25083	4562	2100

1) 16:0, 16:1, 18:0, 18:1, and 18:2 represent palmitic, palmitoleic, stearic, oleic and linoleic acids, respectively. Other minor fatty acids are dismissed.

faster than that into lipids by the mature fruit.

Specific radioactivity of fatty acids in the classes of lipids synthesized from acetate-1-C^{14} by the developing avocado tissue slices clarified precursor-product relationships among monoglyceride, diglyceride and triglyceride (Table 5). The formation of these glycerides was mainly based on the esterification of oleic acid synthesized *de novo*. High content of palmitic acid is characteristic of the developing fruit. The phospholipid fraction showed the highest specific radioactivity among examined fractions, and stearic acid has the highest specific radioactivity among the common fatty acids. A high content of palmitic acid is characteristic of the developing fruit.

DISCUSSION

The avocado fruit deviated from most investigated fruits in its method of development. SCHROEDER (35) observed that its initial growth involved cell division and cell enlargement. Later, cell division became major factor for enlargement and continued as long as the fruit remained on the tree. Since the growth of the fruit almost ceased before the fruit showed its maximum accumulation of reserve lipids, a decrease in water content as a percent of fresh weight of the fruit during development results from a displacement of water by lipids.

During the phase of rapid lipid synthesis, oleic acid was predominantly synthesized, and eventually deposited in triglyceride in the mesocarp tissue of the fruit. The early rise in linoleic acid appeared to be associated with glycolipid which was a unique component in chloroplast in young fruit (7, 33) and with phospholipid fraction which did not change during development of the fruit. This evidence supports the observation of an early establishment of the presence of polyunsaturated fatty acids in fruit, which then remain fairly constant throughout subsequent development (15).

Since the fruits are havested until they are consumed, they continuously undergo a loss in weight, which is largely a loss of water from the fruits. However, ERICKSON and KIKUTA (17) observed that there appeared to be a slight loss of reserve lipids during ripening and an increase during storage following ripening of Hass avocado fruits. This change appears to be responsible for the maturity of the harvested fruit. Changes in classes of avocado lipids were observed during storage. There were reductions in the rate of triglyceride synthesis and diglyceride content as well as marked increases in monoglyceride and free fatty acid fractions, suggesting that lipids were involved to some extent in metabolic changes during the ripening process.

DAVENPORT and ELLIS (15) studied fatty acid composition during storage

indicating that the saturated fatty acids and the polyunsaturated fatty acids tended to rise. MAZLIAK (27) also examined the changes of fatty acid composition of the fruit under artificially modified atmosphere and drew the general conclusion that in all the classes of lipids and in all the regions of the fruit, the percentage of unsaturated fatty acid was greater as the concentration of oxygen was increased in the atmosphere in which the fruit ripening, while saturated fatty acid was increased under oxygen poor or carbon dioxide rich atmosphere. Interestingly, short-chain fatty acids in the phospholipid fraction were increased in the presence of high oxygen pressure. These results support the concepts developed by BLOCH with *Clostridium*, *Saccharomyces* and *Euglena* (9, 10), MUDD and STUMPF with mitochondria from avocado mesocarp (28), and by JAMES in preparations of leaves and chloroplasts (20) that oxygen is absolutely required for oleic acid synthesis.

A water extract of an acetone powder prepared by BARRON and STUMPF (2, 3) from the avocado fruit mitochondrial fraction could not produce oleic acid in spite of the ready formation of stearic acid. The system available 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 (28) 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 rise of labelling in stearic acid from vacuum infiltrated acetate-1-C^{14} does not mean that oxidative desaturation reaction takes place in the stearic acid molecule to form oleic acid. The specific radioactivity of stearic acid was the highest fatty acid among those acids synthesized during the incubation period, but the stearic acid synthesized *de novo* was readily incorporated into complex lipids, especially into triglycerides. 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 vacuum infiltration period.

The formation of long-chain unsaturated fatty acids from corresponding saturated fatty acids in animals was established by the work of SCHOENHEIMER and RITTERBERG (34). 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 pyridine nucleotide, as NADPH,

(10). In contrast to yeast, anaerobically grown *Clostridium butyricum*, which synthesized unsaturated fatty acids, can not utilize long-chain fatty acids as sources for their unsaturated analogues, but the labels from octanoate-1-C^{14} and decanoate-1-C^{14} are incorporated into both saturated and unsaturated fatty 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 strictly sensitive. Acetyl CoA was incorporated into stearic and oleic acids but malonyl CoA was mainly incorporated into stearic acid without inhibition by avidin (4). The complete mechanism for the enzymatic synthesis of oleic acid in higher plants has still remained uncertain.

The evidence for the existance of phosphatidic acid in plants was the presence of large amounts of its phospholipid in cabbage leaves (14). But it was found later that this was mainly an artifact caused by an activation of phospholipase *D* during isolation procedures (19, 25, 33). Recently some evidence was presented suggesting the presence of phosphatidic acid and its role in plants (3, 11, 13). From the data presented, it may be concluded that synthesis of triglyceride from diglyceride and monoglyceride by esterification of unsaturated fatty acids, principally oleic acid, is the main route in the accumulation of the reserve lipids in the mesocarp of avocado fruit.

SUMMARY

Studies on the lipid metabolism in the fruit of avocado, *Persea americana* Mill., were undertaken primarily to elucidate the pattern of the fatty acid synthesis as a predominant feature during growth of the fruit and the changes in the classes of lipids in the mesocarp of the fruit.

The lipids extracted from avocado mesocarp were separated by silicic acid column chromatography as well as thin layer chromatography. The isolated lipids were characterized by infrared absorption spectroscopy and the composition of fatty acid was determined by gas liquid chromatography.

During the phase of rapid lipid synthesis in the developing fruit, oleic acid was predominantly formed, and eventually deposited in triglycerides in the mesocarp tissue. The tissue slices from the mesocarp could metabolize radioactive acetate into lipids which were then transformed from phospholipids to triglycerides, and into water soluble organic acids. Palmitic, palmitoleic, stearic, and oleic acids were the most labelled fatty acids found to accumulate in the tissue slices. From the kinetic evidence, it may be concluded that the synthesis of triglyceride from diglyceride and monoglyceride by the esterification of unsaturated fatty acids, principally oleic acid, is the main route in the

accumulation of lipids in the mesocarp tissue from the avocado fruit.

ACKNOWLEDGMENT

The author wishes to express the deepest appreciation to Dr. Louis C. ERICKSON for making this study possible and to Dr. Takashi TAGAWA for his kind advice in the preparation of the manuscript. He extends his appreciation to Dr. John B. MUDD for valuable advice and discussion relative to the biochemical studies of lipids in plant. The education of the author was furthered by the staff members of the Department of Botany of the Faculty of Agriculture, Hokkaido University and of the Department of Biochemistry of the University of California, Riverside.

Literature cited

1. BARRON, E. J. and D. J. HANAHAN. 1958. Observation on the silicic acid chromatography of the neutral lipides of rat liver, beef liver and yeast. Jour. Biol. Chem. 231: 493–503.
2. BARRON, E. J., C. SQUIRES and P. K. STUMPF. 1961. Fat metabolism in higher plants: XV. Enzymic synthesis of fatty acids by an extract of avocado mesocarp. Jour. Biol. Chem. 236: 2610–2614.
3. BARRON, E. J. and P. K. STUMPF. 1962. Fat metabolism in higher plants. XIX. The biosynthesis of triglycerides by avocado mesocarp enzymes. Biochim. Biophys. Acta 60: 329–337.
4. BARRON, E. J. and P. K. STUMPF. 1962. Evidence for separate pathways for the biosynthesis of saturated and unsaturated fatty acids by an avocado enzyme system. Jour. Biol. Chem. 237: pc 613.
5. BEEVERS, H. 1961. Metabolic production of sucrose from fat. Nature 191: 433–436.
6. BENEDICT, C. R. and H. BEEVERS. 1962. Formation of sucrose from malate in germinating castor beans. II. Reaction sequence from P-enolpyruvate to sucrose. Plant physiol. 37: 176–178.
7. BENSON, A. A. 1964. Plant membrane lipids. Ann. Rev. Plant Physiol. 15: 1–16.
8. BENSON, A. A. and B. MARUO. 1958. Plant phospholipids. I. Identification of the phosphatidyl glycerols. Biochim. Biophys. Acta 27: 189–195.
9. BLOCH, K., P. BARONOWSKY, H. GOLDFINE, R. LENNARZ, R. LIGHT, A. T. NORRIS and G. SCHEUERBRANDT. 1961. Biosynthesis and metabolism of unsaturated fatty acids. Federation Proc. 20: 921–927.
10. BLOOMFIELD, D. K. and K. BLOCH. 1960. The formation of Δ^9-unsaturated fatty acids. Jour. Biol. Chem. 235: 337–345.
11. BRADBEER, C. and P. K. STUMPF. 1960. Fat metabolism in higher plants. XIII. Phosphatidic acid synthesis and diglyceride phosphokinase activity in mitochondria from peanut cotyledons. Jour. Lipid Research 1: 214–220.
12. CHAPMAN, D. 1965. Infrared spectroscopy of lipids. Jour. Amer. Oil Chem. Soc. 42: 353–371.

13. CHENIAE, G. M. 1965. Phosphatidic acid and glyceride synthesis by particles from spinach leaves. Plant Physiol. 40: 235–243.
14 CHIBNALL, A. C. and H. J. CHANNON. 1927. The ether soluble substances of cabbage leaf cytoplasm. II. Calcium salts of glyceride phosphoric acid. Biochem. Jour. 21: 233–246.
15. DAVENPORT, J. B. and S. C. ELLIS. 1959. Chemical changes During growth and storage of the avocado fruit. Aust. Jour. Biol. Sci. 12: 445–454.
16. DAWSON, R. C. M. 1966. The metabolism of animal phospholipids and their turnover in cell membranes. Essay in Biochemistry 2: 69–115.
17. ERICKSON, L. C. and Y. KIKUTA. 1964. Ripening Hass avocado in high and low humidities. Calif. Avocado Soc. Year Book 48: 107–108.
18. HACKETT, D. P. 1953. The osmotic change during auxin-induced water uptake by potato tissue. Plant physiol. 27: 279–284.
19. HIRSCH, J. and E. H. AHRENES, JR. 1958. The separation of complex lipide mixtures by the use of silicic acid chromatography. Jour. Biol. Chem. 233: 311–320.
20. JAMES, A. T. 1963. The biosynthesis of long chain saturated and unsaturated fatty acids in isolated plant leaves. Biochim. Biophys. Acta 70: 9–19.
21. JAMES, A. T. and A. J. P. MARTIN. 1956. Gas-liquid chromatograyhy. The separation and identification of the methyl esters of saturated and unsaturated acid from formic to *n*-octadecanoic acid. Biochem. Jour. 63: 144–152.
22. KARMEN, A., LAURA GUIFFRIDA and R. L. BOWMAN. 1962. Radioassay by gas liquid chromatography of lipids labelled with carbon-14. Jour. lipid Research 3: 44–52.
23. KATES, M. 1959. Chromatographic studies of the lipid components of leaves. Biochem. Biophys. Research Commun. 1: 238-243.
24. KENNEDY, E. P. 1957. Biosynthesis of Phospholipids. Federation Proc. 16: 847–852.
25. MCCARTY, R. E. and A. T. JAGENDORF. 1965. Chloroplast damage due to enzymatic hydrolysis of endogenous lipids. Plant Physiol. 40: 725–735.
26. MAZLIAK, P. 1965. Les lipides de l'avocat. I. Composition en acides gras des diverses parties du fruit. Fruits 20: 49–57.
27. MAZLIAK, P. 1965. Les lipides de l'avocat. II. Variation de la composition en acides gras des lipides du pericarpe selon la composition de l'atmosphere autor des fruits en maturation. Fruits 20: 117–120.
28. MUDD, J. B. and P. K. STUMPF. 1961. Fat metabolism in higher plants. XIV. Factors affecting the synthesis of oleic acid by particulate preparation from avocado mesocarp. Jour. Biol. Chem. 236: 2603–2609.
29. NAKANISHI, K. 1962. Infrared absorption spectroscopy, Plactical: 1–233. Nankodo Co. Ltd.
30. NELSON, G. J. 1962. The lipid composition of normal mouse liver. Jour. Lipid Research 3: 256–262.
31. NICHOLS, B. W. 1963. Separation of lipids of photosynthetic tissues. Improvement in analysis by thin layer chromatography. Biochim. Biophys. Acta 70: 417–322.
32. RANDERATH, K. 1963. Thin-Layer Chromatography. 126–156. Verlag Chemie-

Academic Press.

33. SASTRY, P. S. and M. KATES. 1963. Lipid components of leaves. III. Isolation and characterization of mono- and digalactosyl diglycerides. Biochim. Biophys. Acta 70 : 214-216.
34. SCHOENHEIMER, R. and D. RITTERBERG. 1936. Deuterium as an indicator in the study of intermediate metabolism. V. The desaturation of fatty acids in the organism. Jour. Biol. Chem. 113 : 505-510.
35. SCHROEDER, C. A. 1953. Growth and development of the Fuerte avocado fruit. Amer. Soc. Hort. Sci. 61 : 103-109.
36. STUMPF, P. K. and G. A. BARBER. 1957. Fat metabolism in higher plants. IX. Enzymic synthesis of long chain fatty acids by avocado particles. Jour. Biol. Chem. 227 : 407-417.
37. WINTERMANS, J. F. G. M. 1960. Concentrations of phosphatides and glycolipids in leaves and chloroplasts. Biochim. Biophys. Acta 44 : 49-54.
38. ZILL, L. P. and E. A. HARMON. 1962. Lipids of photosynthetic tissue. I. Silicic acid chromatography of the lipids from whole leaves and chloroplasts. Biophim. Biophys. Acta 57 : 573-583.