Changes in Lipid Components of Seeds during Growth and Ripening of Cacao Fruit

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ABSTRACT

The biosynthesis of lipid in maturing cacao seeds was studied over two cropping seasons (1975-1976) in Brazil using hybrids produced by cross pollination of Catongo with Amelonado cultivars. Seeds were collected at intervals between seed solidification (110-120 days postpollination) and harvesting of ripe fruits at 175-180 days. Lipid accumulated in seeds at a rapid, linear rate after the seeds solidified until near the beginning of ripening, when lipid build up proceeded at a greatly reduced rate. Triglyceride was the major lipid component at all stages of maturity, increasing from 69% to 96% of the total lipid over the period studied. The contributions of other lipid components to total lipids became less prominent as fat accumulated in seeds. However, on an individual seed basis their mass increased throughout development, except for phospholipids and diglycerides, which decreased on a mass/seed basis in the latter stages of fruit maturation. Since the rate of triglyceride production slowed at about the same time, the reduced amounts of phospholipids and diglycerides indicate the classical Kennedy pathway for triglyceride synthesis is operative in seeds of cacao. Fat present early in seed development was more unsaturated than typical cocoa butter of commerce. However, as lipid deposited during the period of active lipid synthesis, starting at about 130 days postpollination, a normal fatty acid composition was quickly established and did not change materially thereafter.

INTRODUCTION

The manufacture of chocolate involves roasting, winnowing and grinding of cocoa beans to a paste or liquor, in which fat is the continuous phase. Depending on the type of chocolate desired, additional cocoa butter, sugar, milk solids and flavorings may be mixed and reground with the chocolate liquor. Fat acts to coat surfaces generated by the grinding of the ingredients, with final viscosity being influenced by the characteristics of the cocoa butter. This phenomenon, while not well understood, may be mediated by the action of surface active materials, such as lecithin, on the crystallization of cocoa butter. The solidification of chocolate, upon cooling, is also directly influenced by cocoa butter. Constancy in the physical characteristics of the fat phase is critically important relative to the manufacturing processes used by the confectioner.

The typical flavors and colors developed in chocolate products depend upon chemical reactions of nonfat cocoa constituents, cocoa butter being only marginally involved. As substitute fats improve, the need for natural cocoa butter decreases. Whether objectives are increasing the nonfat solids of cocoa beans or maximizing the desirable qualities of cocoa butter, better knowledge is needed of cocoa butter composition, biosynthesis and deposition by the plant.

Many researchers have studied the triglyceride composition of cocoa butter (1-5). The major type of triglyceride contains two saturated fatty acids and one unsaturated fatty acid. Unsaturated fatty acids predominantly occupy the middle position on glycerol. In marked contrast to the wealth of data accumulated on the lipid composition of mature cocoa beans, modes of synthesis of the lipids in developing seeds has been studied only to a limited extent. The scarcity of data reflects the fact that cacao is a tropical crop usually grown in regions remote from the laboratory facilities required for analysis.

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Investigations reported herein, and in an accompanying communication (6), were directed toward obtaining basic information about biosynthetic changes occurring in the lipids of developing cacao seeds between pollination and final ripening of cacao fruit. The study of cocoa butter in the past has centered on mature, fermented seeds as received by manufacturers. Thus, data obtained for pooled samples tend to mask differences at the level of individual trees and fruits as influenced by environmental variables, agronomic practices and post-harvest treatment. Seed material for this investigation was collected during a student exchange made between The Pennsylvania State University and Comissao Executiva Do Plano Da Lavoura Cacaueira, Itabuna, Bahia, Brazil. Throughout this report, cacao and cocoa are differentiated in the following manner. Cacao refers to the crop and its constituents up to post harvest handling. Thereafter, cocoa is substituted for cacao.

MATERIALS AND METHODS

Field Trials for Obtaining Experimental Samples

Field trials were designed to obtain samples of cacao which reflected the effects of various stages of seed development on the chemical and physical properties of cocoa butter. Hybrid cacao seeds, of the type currently being planted in Brazil, were produced by pollinating flowers of a Catongo cultivar with pollen from an Amelendado cultivar. Several thousand pollinations were made, after which fruits were collected at progressive stages of development through ripening.

Test plot trials were conducted twice to obtain fruit representative of two growing seasons. The first trial was initiated at the beginning of the Brazilian winter (May 1975). Fruits were collected at 100 and 120 days of growth, and then at 10-day intervals until full maturity at 180 days. Following harvesting from the trees, the outer husk of the fruit (pod) was removed and the seeds were separated from the surrounding pith and seed testa. In the second trial, begun in mid-summer (January 1976), samples were collected at 110 days of growth and then at 10-day intervals until 150 days, when collection was every 5 days through ripening at 175 days. Seeds at 110 days in this trial were similar in appearance to 130-day seeds from the first experiment, the ovules being judged as more than one-half solidified. Prior to this stage of development, the ovules were gel-like in consistency and fat content was very low.

Seeds recovered at each sampling of fruit were freeze dehydrated and stored desiccated over silica gel until transported to The Pennsylvania State University, where the seeds were stored frozen until lipid analyses were initiated nine months later.

Analytical Methods

Moisture and Fat Contents. Two seeds from the center of each of ten pods were weighed into a tared beaker and lyophilized 48 hr. The sample was allowed to equilibrate to constant weight in a desiccator yielding the total solids content. Fat content was determined by Soxhlet extraction with hexane (7), except for immature seeds from the early

TABLE I

Moisture, Dry Matter and Lipid Contents of Seeds of Cacao at Various Stages of Fruit Development and Ripening over Two Growing Seasons

Age days post pollination	Growth[a] stage	Fresh wt. g/seed	Dry matter g/seed	Moisture %	Lipid	
					mg/Seed	% of Dry matter content
			Experiment 1			
100	-b	.081	.033	95.9	0.1	2.9
120	-b	.449	.023	94.2	0.7	3.2
130	1	.641	.072	88.7	10.0	13.8
140	2	.960	.261	75.6	87.7	33.6
150	3	1.139	.434	61.8	201.6	46.4
160	4	1.412	.711	50.3	356.2	50.1
170	5	1.605	.977	39.1	516.5	52.9
180	7.5	1.900	1.224	35.6	646.7	52.8
			Experiment 2			
110	1	.804	.102	87.4	33.0	16.2
120	2	1.006	.265	73.6	107.1	40.4
130	3	1.138	.452	60.2	226.7	50.1
140	4	1.322	.633	52.1	334.1	52.8
150	5	1.565	.957	38.8	531.4	55.5
155	5.5	1.608	1.000	38.1	548.4	54.8
160	6.0	1.669	1.082	35.2	598.3	55.3
165	6.5	1.757	1.138	35.2	609.6	53.6
170	7.0	1.807	1.193	34.0	636.9	53.4
175	7.5	1.774	1.177	33.7	638.3	54.2

[a]Growth stage assigned according to solids content (dry matter) of individual seeds.

[b]Growth stage not assigned as collected samples were not subjected to complete analysis.

stages of growth. These samples, containing appreciable amounts of polar lipids, were analyzed for total lipid content by extraction with chloroform/methanol (2:1, v/v), and after treatment to remove nonlipid contaminants (8), the extract was dried under a stream of nitrogen. Mature beans were also extracted in this manner when the objective was the recovery of a total lipid fraction for the quantification of individual lipid components. Hereafter, this fraction is referred to as "lipid extract."

Triglycerides. Lipid extract (1 mg) was subjected to thin layer chromatography (TLC) on precoated (0.25-mm thick) silica gel 60 plates (Scientific Products, Obetz, OH) using a solvent system of petroleum ether, ethyl ether, and acetic acid (60-40-1, v/v/v). The triglyceride area on a developed plate was scraped into a screw-cap test tube, and the triglycerides were extracted into 5 ml chloroform by swirling the tube and sedimenting the absorbant by centrifugation. A lipid-free region on the TLC plate was treated similarly. This sample was used in the reference beam of the spectrophotometer to eliminate interference from the binder in the silica gel, which was extracted into the solvent.

An aliquot of the chloroform extract was dried under N_2, and a standard volume of chloroform was added to bring the concentration of the triglycerides within the range of infrared spectrophotometric measurement (9). The solution was scanned from 1850 cm^{-1} to 1650 cm^{-1} (Perkin Elmer Model 597). The peak at 1742 cm^{-1} was measured by triangulation, and triglyceride concentration was established by comparison to a calibration curve prepared using known weights of commercial cocoa butter, which was 98 percent triglyceride.

Diglycerides. A colorimetric procedure (10) was used for diglyceride quantification, after isolation by TLC. The small amount of diglyceride present in cocoa butter necessitated the loading of thin-layer plates with approximately 25 mg of lipid extract. Development and recovery of the diglyceride fraction were as described for triglyceride quantification.

Monoglycerides. A chromotropic acid procedure (11) was employed for monoglyceride measurement. Cocoa butter (0.5 g) was treated with perchloric acid to convert 2-monoglyceride to 1-monoglyceride, and, after periodic acid oxidation, formaldehyde was measured colorimetrically by reaction with chromotropic acid.

Sterols. Cocoa butter sterols (free, nonesterified) were quantitatively isolated using digitonin-impregnated columns (12). Modifications to the procedure included reduction of sample size to 0.5 g and the inclusion of 200 μg of cholesterol as an internal standard. The sterols were separated by gas chromatography (Hewlett Packard Model 5750 gas chromatography) on a 6 mm x 2 mm ID glass column packed with 3 percent SP 2250 on 100/120 mesh Supelcoport (Supelco Inc., Bellefonte, PA).

Weight of sterols present was calculated by the method of internal standardization (13).

Free Fatty Acids. A weighed sample of lipid extract (0.5 g) was dissolved in 50 ml diethyl ether and titrated to a phenolphthalein endpoint with 0.009 N ethanolic potassium hydroxide. Free fatty acid content was calculated as oleic acid and expressed as percent weight of the total cocoa butter.

Phospholipids. Aliquots of lipid extracts (6 mg) were dried under N_2 and digested in 72% perchloric acid prior to lipid phosphorus determinations and phospholipid calculations (14).

Glycolipids. Silicic acid column chromatography was employed to isolate glycolipids (15). Approximately 40 mg of lipid extract in diethyl ether was loaded on the column and non-polar lipids were removed with 40 ml of diethyl ether. Glycolipids were eluted with 40 ml of acetone; the solvent was removed under N_2, and the sugar content of the glycolipid residue was determine (16). Glycolipid content was calculated by multiplying the sugar content (from a galactose standard curve) by 4.77. This factor was derived by dividing the average molecular weight of monogalactosyl diglyceride and digalactosyl diglyceride by the molecular weight of galactose.

TABLE II

Composition (g/100 g lipid) of Lipid Extract of Seeds of Cacao at Various Stages of Fruit Growth and Ripening[a,b]

Growth stage	Glycerides			Free fatty acids	Sterols	Polar lipid	
	Tri-	Di-	Mono-			Phospo-	Glyco-
				Experiment 1			
1	68.87	2.48	0.45	5.22	1.79	10.24	2.75
2	83.27	1.41	0.15	1.69	0.61	4.12	0.73
3	91.38	1.04	0.07	0.73	0.28	2.43	0.46
4	93.29	1.29	0.08	0.72	0.23	1.66	0.41
5	92.52	1.06	0.04	0.50	0.19	1.37	0.28
7.5	94.45	0.56	0.07	0.43	0.17	0.68	0.31
				Experiment 2			
1	80.42	2.40	0.38	5.17	1.26	6.08	1.71
2	88.74	0.95	0.13	1.10	0.48	3.66	0.62
3	93.73	0.86	0.07	0.64	0.29	2.38	0.45
4	94.05	0.79	0.07	0.60	0.27	1.73	0.32
5	93.92	0.68	0.05	0.52	0.19	1.58	0.28
5.5	97.50	0.66	0.04	0.41	0.19	1.44	0.23
6	97.27	0.64	0.05	0.40	0.19	1.33	0.25
6.5	95.77	0.66	0.05	0.35	0.19	1.20	0.19
7	95.61	0.84	0.05	0.46	0.21	1.02	0.32
7.5	96.30	0.57	0.04	0.47	0.18	1.01	0.26

[a]Solvent-chloroform/methanol, 2:1; mean of 6 determinations.

Fatty Acid Analysis. Benzene solutions of the total lipid extracts (20 mg in 0.5 ml) were saponified with 0.5% sodium hydroxide in methanol (2.0 ml), and the soaps were converted to fatty acid methyl esters (FAME) by refluxing with 2.5 ml of 14% Boron trifluoride in methanol. FAME were extracted into petroleum ether and chromatographed (Hewlett Packard Model 8130 Gas Chromatograph) on a 1.8 m x 3.2 mm stainless steel column packed with 15% diethylene glycol succinate on acid-washed chromosorb W (Supelco Inc.). Fatty acids were quantified by internal standardization using pentadecanoate.

Nonpolar and Polar Fatty Acid Composition

Utilizing small-scale silicic acid column chromatography in a Pasteur pipette, 50 mg of lipid extract was separated into nonpolar and polar lipid fractions by elution with 15 ml diethyl ether and 10 ml of methanol, respectively. FAME were prepared as above and separated by gas chromatography, but without the addition of an internal standard. A standard curve, prepared by chromatographing various amounts of a quantitative standard FAME mixture (Applied Science Laboratories), was used for quantification purposes. A plot of peak area versus component weight allowed estimation of sample component weights from peak area.

RESULTS AND DISCUSSION

Visual Changes during Development of Seeds and Fruit

In the earliest stages of development, fruit (pods) of *Theobroma cacao* appeared as tiny cucumber-like projections emerging from small white and pink flowers. The small pods grew rapidly, often becoming one inch long in the first week. The seed ovules differentiated early in pod development. Initially, they were tiny, liquid-filled vacuoles surrounded by a thin white testa embedded in white pith. Each ovule was connected to a central vascular bundle or placenta. During the next two months, the pods increased in size and began to broaden in the midsection; the ovules continued to develop, enlarging and filling with a gel-like material which was clear and free of solid inclusions. The outer covering of the pod became thicker and differentiated into two distinct layers.

When the pods were 3.5 to 4 months old, several changes occurred during the next three weeks. The ovules began to solidify and pass through several stages during which the amount of unsolidified gel-like material continuously decreased until the seeds visually appeared to be solid. Seeds were unpigmented when first solidified, but gradually changed from white or light tan to lavender. As the pods developed over the next two months, the seeds enlarged and deepened in color until they were violet. Typically, color varies with genetic origin from deep purple of Amelonado to almost white seeds of Catongo and Criollo varieties. The Catongo X Amelonado hybrids used in this investigation were lighter in color than Amelonado seeds, reflecting an averaging of pigment intensity.

Just before and during ripening noticeable changes occurred. The pods changed color from green to a mixture of green and pale yellow, denoting the onset of the ripening phase. This normally occurs 5 to 5.5 months after pollination, the timing having been related to mean daily temperature (C) in the equation $N = \frac{2500}{T-9}$ where N = number of days required to ripen and T = mean daily temperature (17). During ripening, which lasted 10 to 14 days, the pods turned golden yellow, and the pulp was transformed from a dry, pithy mass to a wet, pleasantly sweet and tart tasting mucilage. Alterations in pulp texture began just prior to the color change and continued until the pods were fully yellow.

Fat Deposition in Developing Seeds

Fruit from field trials carried out over the two Brazilian growing seasons were collected at intervals displayed in Table I, which also contains data characterizing the samples. In both trials, approximately the same number of days were required for full growth and ripening of cacao fruit, and for the development of final seed characteristics relative to seed weight, and dry matter and fat contents. However, the most active periods of seed development were different. In Experiment 1, fat synthesis was greatest after 140 days, about 20% of it being produced during the final

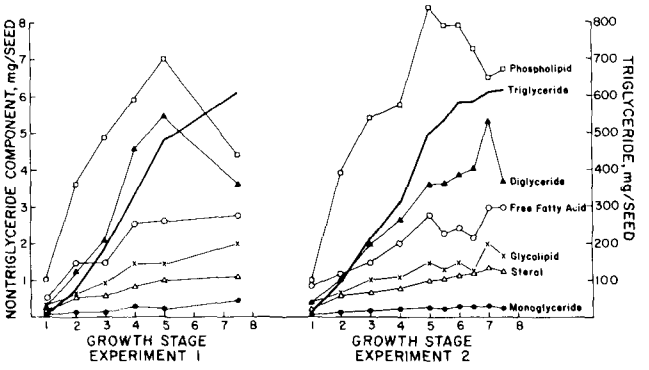

FIG. 1. Comparison of two cropping seasons relative to lipid components in individual seeds (mg/seed) at various stages during the growth and ripening of cacao fruit.

10 days of ripening (Table I). In Experiment 2, fat accumulation started 3 weeks earlier (120 days) and was nearing completion by the onset of ripening (160 days). Only 4% of the fat was synthesized over the final 10 days of ripening. Probably, seasonal temperature accounts for the differences observed. In Bahia, the October-November and May-June periods have similar average daily temperatures of 22-23 C (17). These months were the periods of most active fat synthesis in Experiment 1 and Experiment 2, respectively.

Seed Solids and Pod Age. Since environmental and, perhaps, other seasonal factors caused differences in the rate of seed development between the two experiments, comparisons based strictly on age would be misleading relative to metabolic activity and fat synthesis. A more meaningful index was judged to be the solids content of individual seeds (dry matter/g seed, Table I). Therefore, each sample was assigned to a growth stage (GS), GS-1 through GS-7.5, according to dry matter content. Further discussion of analytical data are based on assigned growth stages rather than on days since pollination.

Fat Accumulation by Growth Stage. The contribution of fat to total dry matter content reached a level near that of mature seed by GS-4 (Table I). However, weight of fat per seed continued to increase until ripened pods were harvested. Prior to GS-5, fat was produced at a faster rate than other solids. Although fat continued to accumulate thereafter in seeds until pods were harvested, rate of production was about the same as for nonfat solids.

Changes in Lipid Components

Triglycerides. The predominant class of lipid at all stages of development was triglyceride accounting for 69-96% of the total lipid fraction (Table II). As shown in Fig. 1, triglycerides were synthesized between GS-2 and GS-5 at nearly a linear rate. During the final period of seed development synthesis proceeded more slowly.

Minor Lipid Components. Since triglyceride is the primary energy store for germination of the seed, other lipid constituents are logically present at much lower concentrations. Some components are precursors in triglyceride synthesis, while other function mainly as structural elements in cellular inclusions.

Table II shows that the percentage of total lipid contributed by each minor component was higher in seeds at immature growth stages than in more mature seeds. As pods developed, triglyceride accumulation had a diluting effect until GS-5 when lipid composition tended to stabilize. However, on a mass basis (mg/seed), the amount of monoglyceride, sterol, free fatty acid (FFA) and glycolipid increased throughout growth and ripening (Fig. 1).

The share of total lipid attributable to FFA was quite high 5%) at GS-1, but, between GS-1 and GS-3, FFA level decreased to near that found at maturity. Being an intermediate in lipid ester synthesis, accumulation of FFA would not be expected. A similar trend in FFA content was reported for maturing rape seeds (18).

Lipid analysis of seed material collected prior to GS-1 had revealed phospholipids as the major component (48%). As the seeds solidified, phospholipid concentration in fat of cacao decreased steadily (Table II). High levels prior to seed

TABLE III

Fatty Acid Composition of Nonpolar and Polar Lipid Fractions of the Lipid Extract of Seeds from Cacao at Various Stages of Fruit Growth and Ripening

	Total lipid				Nonpolar lipids				Polar lipids			
	% Fatty acid				% Fatty acid				% Fatty acid			
Growth stage	16:0	18:0	18:1	18:2	16:0	18:0	18:1	18:2	16:0	18:0	18:1	18:0
					Experiment 1							
1	30.2	12.6	31.4	25.8	28.3	17.7	34.8	19.1	33.1	8.6	21.6	37.0
2	27.7	21.3	39.7	11.3	24.9	24.3	39.8	10.9	28.3	12.1	34.6	24.9
3	28.0	31.3	35.5	5.0	25.8	32.2	35.3	6.7	28.1	14.9	42.3	14.7
4	28.7	33.4	34.0	3.9	26.6	34.2	33.1	5.9	29.3	14.3	44.1	12.2
5	27.9	33.8	34.5	3.6	26.7	34.2	33.9	5.3	26.1	18.3	43.1	12.4
7.5	27.8	34.6	34.0	3.6	26.3	34.9	33.4	5.4	26.8	17.8	37.4	18.1
					Experiment 2							
1	32.3	13.3	31.8	22.5	28.6	20.1	36.1	15.2	37.0	9.2	24.7	29.1
2	28.4	25.4	37.2	9.0	26.1	27.3	37.9	8.7	29.2	10.3	38.2	22.3
3	26.8	32.8	34.8	5.6	25.5	32.4	36.1	6.0	26.9	10.3	38.2	22.3
4	27.7	34.3	34.0	4.1	26.4	32.9	35.5	5.2	27.2	13.7	46.2	12.9
5	27.4	35.3	33.5	3.8	25.5	34.3	34.8	5.3	23.1	11.0	49.6	16.2
5.5	26.4	35.8	34.1	3.6	24.6	34.6	35.0	5.6	24.1	13.1	47.6	15.2
6	25.4	35.9	35.1	3.6	25.1	35.4	34.3	5.2	21.8	11.4	47.6	19.2
6.5	25.4	35.8	35.1	3.7	26.5	34.3	34.1	5.3	24.2	12.8	42.7	20.2
7	25.0	35.0	35.9	4.0	25.5	34.5	34.2	5.7	23.9	13.4	40.2	22.5
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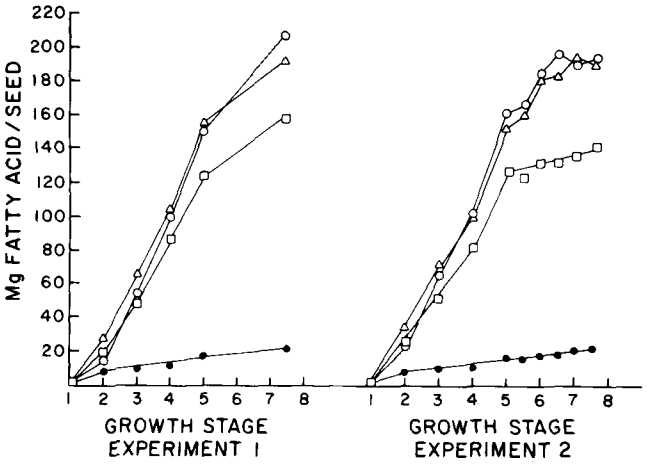

FIG. 2. Changes in individual fatty acids in the lipid extract of individual seeds (mg/seed) during growth and ripening of cacao fruit. Comparison between two cropping seasons. Palmitic acid □——□, stearic acid ○——○, oleic acid △——△ and linoleic acid ●——●.

solidification signals the involvement of phospholipids with membranes, and as precursors for lipid synthesis which proceeded at a rapid rate after GS-1.

Per individual seed, phospholipids and diglycerides were present in higher concentrations than FFA, monoglycerides, sterols, and glycolipids. Phospholipids and diglycerides declined in mass per seed during the later stages of seed maturation, and this coincided with a slowdown in the rate of triglyceride accumulation (Fig. 1). This synchronous change suggests that that classical Kennedy pathway (19) is active in the synthesis of cacao triglycerides. Results conform with those obtained when Abyssinian Kale seed was incubated with labelled glycerol (20). Labelled intermediates of triglyceride synthesis were found in the following order over time: glycerol phosphate, phospholipid, diglyceride, and finally triglyceride. Assuming that a slowing of triglyceride synthesis would follow a decrease in the rate of production of intermediate, one would expect a loss of phospholipid and diglyceride in cacao lipid as the synthesis mechanism consumed existing intermediates. Reduction of phospholipid content concurrent with a decreased rate of triglyceride synthesis has also been observed in maturing soybeans and rapeseed (21,22).

Changes in Individual Fatty Acids during Maturation

Total Fat Fraction. The fatty acid composition of cocoa butter of cacao (lipid extract) changed markedly between GS-1 and GS-4 (Table III). In both experiments the major alterations noted were an increase in stearic acid concentration and a decrease in linoleic acid content. Fatty acid composition stabilized by GS-5. Logically, the higher linoleate content relative to other fatty acids in the early stages of seed development reflect the dominance of lipids associated with membranes, such as glycolipids and phospholipids, which contain high levels of unsaturated fatty acids (Table III).

The actual amount (mass/seed) of each fatty acid increased throughout seed development, but the rate of linoleate accumulation was less than that for other fatty acids (Fig. 2). The rate for all fatty acids decreased at GS-5. Ratios among the three major acids (palmitic, stearic, oleic) did not change appreciably during seed development. This suggest that once storage fat is produced it is not subject to further modification.

The fatty acid composition of the seed fat of cacao at various stages of growth and ripening followed trends which were quite similar to those reported for oil seeds, notably soybeans (23) and castor seeds (24). The composition of these seed fats stabilized before ripening began, which indicated that storage lipids, produced during the second and third phases of oil seed development, are not metabolized once deposited. This conclusion was supported by the results of studies on the matabolic activity of ^{14}C-labeled lipid components of maturing rapeseed (22). Essentially no change in the neutral lipids occurred, whereas glycolipids and phospholipids showed considerable activity.

Polar Lipids. The fatty acid composition of the polar lipid fraction of fat from cacao exhibited greater variation during growth and ripening than was the case for neutral lipids. Although alterations were slight between total saturated and total unsaturated acids, differences were quite pronounced between palmitate and stearate and between oleate and linoleate (Table III). The cause and significance of these shifts warrant further investigation.

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