Studies on the Lipid Composition of Developing Soybeans

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ABSTRACT

Studies are reported on changes in fatty acid and lipid class composition in developing soybeans picked at intervals from ca. nine days after flowering to maturity. In the early stages of development of the bean, the lipid was virtually devoid of triglyceride and the major constituents consisted of glycolipids and phospholipids. As the bean developed, there was a rapid synthesis of triglyceride that paralleled the deposition of lipid. Simultaneously, unknown substances which occurred in relatively large amounts in the neutral, as well as the glycolipid and phospholipid, fractions of the immature bean diminished to less than 2% of the total lipid in the mature bean. The glycolipids and phospholipids also increased as the bean developed but at a much slower rate than the triglycerides and became minor components in the mature bean. The major component of the phospholipids in the immature bean was phosphatidic acid. It decreased as the phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl inositol, as well as triglyceride, increased. The major component of the glycolipid fraction in the early stages of the development of the bean had the same migration pattern on two-dimensional thin layer chromatography as phosphatidic acid and gave a positive test for phosphorus; it also gave a positive test for glycolipids and was separated completely from phosphatidic acid and other phospholipids by column chromatography. It also decreased as the bean developed. Changes also occurred in the fatty acid composition of the developing bean. The percentage of saturated fatty acids decreased rapidly in the early stages of the development of the bean; oleic and linoleic increased rapidly as the bean developed. Linolenic acid increased rapidly to a maximum concentration in the early stages of the development of the bean and then gradually decreased as the bean matured.

INTRODUCTION

In recent years there has been considerable interest in the linolenic acid content and biosynthesis of triglycerides (TG) in connection with the development of improved varieties of soybeans (1-6). Studies in Japan (7) and Russia (8), as well as in the U.S. (9-11), indicate that the percentage of linolenic acid in the lipid is highest in the early stages of the development of the bean. As the bean develops, the percentage of linolenic acid decreases concurrently with increases in the percentages of oleic and linoleic acids. Minor changes also have been observed in the percentages of stearic and palmitic acids. In apparent variance with these general observations, Von Grimmer et al. (12) reported that no linoleic or linolenic acid was present in the TG of cotyledons of soybeans until 25 and 50 days, respectively, after pollination. These investigators also reported that in the initial stages of the development of the bean, TG contained a high percentage of myristic acid which disappeared early during maturation. No globules of neutral lipid were detected in the bean by electron microscopic examination until the appearance of linoleic acid ca. 25 days after pollination (13).

Of changes in composition of the lipid classes of soybeans, those of the neutral lipid, particularly TG, have received the most attention (7-9). In the early stages of maturation, the concentration of these compounds is relatively low. As the beans mature, they increase rapidly and change both quantitatively and qualitatively in molecular species composition.

Differences also have been observed in the composition of the polar lipids of immature and mature soybeans (7,10,11,14). Hirayama and Hujii (7) identified phosphatidic acid (PA), phosphatidyl ethanolamine (PE), lecithin, and inositol containing phospholipids (PL) of immature bean and found considerable differences in the composition of PL at different stages of maturation. Singh and Privett (10,14) detected acylphosphatidyl ethanolamine (APE), sulfolipid (SL), esterified sterol glucoside (ESG), sterol glucoside (SG), digalactosyl diglyceride (DGDG), and cerebrosides (CE) in the lipid of immature soybeans. In this article, further studies are reported on the composition of the lipid of soybeans at various intervals during maturation.

EXPERIMENTAL PROCEDURES

Anoka soybeans (*G. max*) were grown at the University of Minnesota Experimental Station, Waseca, Minn. Beans were picked from the second and third nodes of plants at

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TABLE I

Analyses of Soybeans

Days after flowering[a]	Average wt/bean (mg)	Average length/bean (mm)	Average width/bean (mm)	Total lipid (%)	Nonlipid solid (%)	Moisture[b] (%)
9	5.4	3.0	1.8	1.2	12.3	86.5
11	14	4.5	3.2	1.0	10.7	88.3
18	59	7.1	5.3	1.5	11.3	87.2
32	130	9.5	6.7	2.3	14.3	83.4
40	193	9.0	6.9	4.0	18.7	77.3
50	347	11.8	8.5	7.5	25.7	66.8
55	421	13.0	8.9	8.0	28.5	63.5
60	452	11.3	8.3	9.0	32.2	58.8
67	374	11.3	8.3	10.3	32.5	57.2
80	261	8.4	7.9	13.6	43.1	43.3
97	220	7.5	7.0	20.8	66.7	12.5

[a]Based upon date of picking and average wt of bean.

[b]Determined by difference between wt of beans and sum of lipid and solid residue.

intervals from ca. 9-97 days after flowering, quick frozen on dry ice in the field, and stored in moisture-tight bags at -20 C in the laboratory.

Extraction of the lipid: The beans were separated from the pods and screened into groups based upon their size and wt. The lipid was extracted in a Virtis Model 45 homogenizer three times with 20 volumes of chloroform-methanol (2:1) under an atmosphere of nitrogen. The combined extract was evaporated under reduced pressure to ca. one-third to one-quarter of its original volume precipitating proteolipid (15) which was separated by filtration through a Whatman No. 1 paper. The precipitate was washed with a few ml chloroform-methanol (2:1) to recover occluded lipid, and the filtrate was evaporated to a thin slurry. Chloroform was added to the slurry and the solution evaporated to ca. 10 ml. The process was repeated twice more to remove most of the methanol by coevaporation. The chloroform solution was diluted with ca. 10 volumes of petroleum ether, washed first three times with 0.5% sodium chloride. These washings were combined and re-extracted with petroleum ether. Finally all the petroleum ether extracts were combined, dried over anhydrous sodium sulfate, and filtered. The amount of lipid in the original sample of beans was determined from the wt of material in an aliquot of this solution.

Lipid class analysis: Total lipid extracts of ca. 50-100 mg (depending upon the amount of TG) were fractionated into neutral lipid, glycolipid (GL) and PL fractions by silicic acid column chromatography using a 50 x 1.25 cm column of 100-200 mesh Mallinckrodt silicic acid (SilicAr CC-7) treated with ammonium hydroxide (16). The neutral lipids were separated first by elution with chloroform-carbon tetrachloride (2:1), GL next with acetone, and PL with methanol.

Identification of the lipids was carried out by thin layer chromatography (TLC) using chromatoplates coated with Silica Gel G or H (Merck & Co., Darmstadt, Germany) by one- and two-dimensional solvent systems that have more or less become standard procedures (17-21). Neutral lipids were separated with mixtures of petroleum ether-ethyl ether-acetic acid (90:10:1) or ethyl ether-isooctane (80:20). GL fraction was fractionated by two-dimensional TLC using chloroform-methanol-concentrated ammonium hydroxide (80:20:2) in the first dimension; after allowing the plates to dry for 10 min in an atmosphere of nitrogen, they were developed in the second dimension with chloroform-acetone-methanol-acetic acid-water (6.5:2:1:1:0.4). PL were separated by the same procedure using chloroform-methanol-ammonium hydroxide (65:35:5) in the first dimension and chloroform-acetone-methanol-acetic acid-water (5:2:1:1:0.5). Spots were visualized by charring them as a universal method of detection (17,18). Phosphorus-containing compounds were identified by the use of a phosphomolybdic acid spray reagent and GL with an orcinol reagent (19) in separate analyses. Ninhydrin was used to detect compounds containing free amino groups (19).

Quantitative analysis of GL and PL, as well as neutral lipids, were carried out by TLC using the charring densitometry technique (17,18) with one-dimensional systems. TG and free fatty acids were determined with petroleum ether-ethyl ether-acetic acid (90:10:1), sterol, and 1,2-diglycerides with ethyl ether-isooctane (80:20). The GL were separated using chloroform-methanol-ammonium hydroxide (80:20:2), and the PL with chloroform-methanol-ammonium hydroxide-water (65:37:4:2). Standards for the analyses were purchased from the Lipids Preparation Laboratory, The Hormel Institute, Austin, Minn., or were isolated by TLC from samples of soybean lipid. Some material remained at the origin of the plate; and, in some cases, material migrated with the solvent front in these analyses. These materials, which included pigments, alcohols, and tocopherols, as well as unknown compounds, were designated unanalyzed material and measured collectively by the difference between the amount of sample applied to the plate and the sum of the known components.

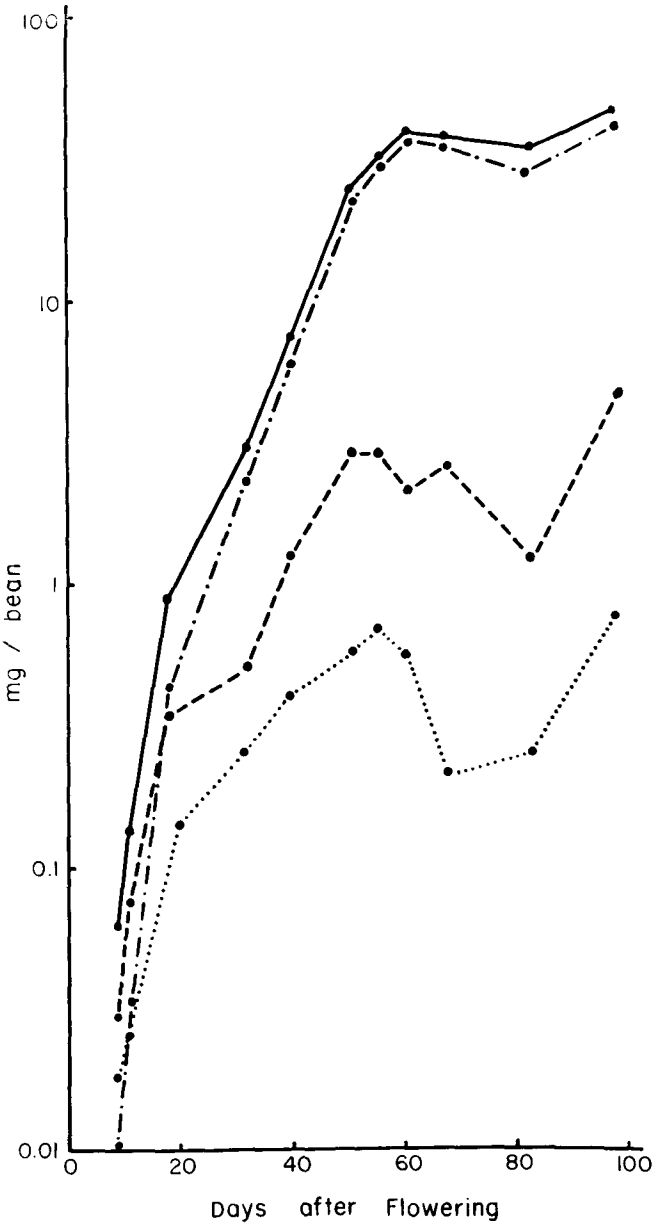

FIG. 1. Analysis of major lipid groups of the lipids of soybeans during maturation. —— total oil, —·— neutral lipid, - - - phospholipid, and · · · glycolipid.

Analytical liquid chromatography (ALC): These analyses were made with a 1 m x 2.8 mm inside diameter column of Corasil II (Waters Associates, Inc., Framingham, Mass.) operated at 20-60 lb pressure with a gradient solvent composition, as described by Stolyhwo and Privett (16), using a flame ionization detector of new design (22). The signal was recorded with a conventional strip chart recorder (5 mV fsd) in conjunction with an electrometric amplifier containing a modified input circuit to give a time constant of ca. 15 sec. Identification of peaks was made based upon retention times of authentic reference compounds and by TLC analysis of separated components.

Fatty acid analysis: Analysis was carried out by gas liquid chromatography (GLC) of methyl esters, prepared by interesterification with methanol-HCl, on a 6 ft x 1/4 in. glass column of 10% EGSS-X on 100-120 mesh Gas Chrom P (Applied Science, State College, Pa.) at 180 C with a Barber Coleman series 5000 instrument equipped with a 5040 electrometer and a flame ionization detector. Nitro-

TABLE II

Analyses of Soybean Lipid by Preparative Column Chromatography[a]

Days after flowering[b]	Neutral lipid (% wt)	Glycolipid (% wt)	Phospholipid (% wt)	High polar lipid (% wt)	Recovery (% wt)
9	17.0	29.2	49.0	4.8	95.2
11	24.3	21.5	54.2	0	96.8
18	46.4	14.7	36.5	2.4	95.8
32	75.0	8.3	16.6	0	99.6
40	78.1	5.3	16.5	0.1	103.9
50	86.2	2.2	11.0	0.6	100.2
55	88.6	2.1	8.6	0.7	105.6
60	90.0	2.3	5.5	1.2	101.9
67	92.2	0.6	6.9	0.3	104.5
80	92.4	1.6	3.8	2.2	102.6
97	88.1	1.6	9.8	.5	96.3

[a]See text for details.

[b]Based upon date of picking and average wt of beans (Table I).

gen was used as the carrier gas at a flow rate of 30 ml/min.

RESULTS

The pattern of changes in the general composition of the soybeans during maturation are indicated by the results in Table I. The last picking was taken after frost had stopped further growth. As the beans developed, the total lipid increased from ca. 1% to ca. 20% wet wt. The percentage of nonlipid solids increased, and the moisture content decreased concurrently. These changes agreed with those generally observed (7,23-25) and, together with the size and wt, served to define the stages at which the beans were picked.

The general changes in the relative amounts of the major lipid groups during maturation of the bean are shown in Figure 1. These results show that the neutral lipid increased rapidly and paralleled the deposition of lipid. The PL and GL fractions also increased, but at a much slower rate, and became minor components in the mature bean. These results were obtained by column chromatography. The separation of each fraction was complete as indicated by TLC and gravimetric analysis. The complete analyses of the column fractionations are summarized in Table II. A chloroform-carbon tetrachloride solvent system was used to separate the neutral lipids, because chloroform alone eluted the esterified sterol glucoside slowly. All of the compounds in the PL fraction gave a positive test for phosphorus, and all of the compounds in the GL fraction gave a positive orcinol test, including the component that had a migration pattern similar to PA in the PL fraction. This compound also gave a positive test for phosphorus, but was eluted completely with the GL fraction. Because it contained phosphorus and gave a positive test with orcinol, it was designated as a glyco-phospholipid (GPL). To ensure against overlapping of the GL and PL fractions, elution with acetone was continued until the eluate was free of solute. Even after exhaustive elution with methanol, a small amount of material remained on the column. This fraction was eluted with 80% aqueous methanol and assured virtually complete recovery of the sample within experimental error of the gravimetric analysis. Although this fraction charred, showing that it was organic, its composition was not studied. Because of its high polarity, it was designated as highly polar lipid (HPL). The complete separation of GL and PL fractions may be attributed to the method of treating the column with ammonium hydroxide (16).

TABLE III

Analysis of Lipid Classes of Neutral Lipids, Glycolipids, and Phospholipids (% wt)

	Days after flowering					
Lipid class	9	18	40	55	67	97
Neutral lipid[a]						
TG	Trace	25.9	57.0	82.4	97.0	98.0
FFA	3.7	4.6	Trace	Trace	Trace	Trace
ST	8.5	3.3	0.4	0.3	0.7	0.5
UN	87.6	66.2	42.6	17.3	2.3	1.4
Glycolipid[a]						
ESG	9.8	6.6	5.6	6.1	9.4	33.3
MGDG	1.5	1.4	9.1	6.6	6.8	4.6
SG	3.0	2.9	5.3	3.9	7.3	17.3
CE	11.0	21.3	11.6	16.9	10.4	20.2
DGDG	16.7	20.4	7.5	16.6	15.1	16.8
GPL	26.3	18.7	7.5	10.0	10.8	14.4
UN	30.9	28.8	35.5	39.4	40.4	6.8
Phospholipid[a]						
PG + DPG	3.3	3.1	3.5	2.7	6.7	3.3
PE	3.5	3.1	5.0	1.4	3.5	26.3
PC	2.0	1.2	4.4	3.6	10.2	45.0
PI	6.3	8.9	19.9	15.3	12.5	14.1
PA	64.2	75.9	51.0	51.8	39.2	5.0
UN	20.6	7.8	25.4	25.4	27.9	6.3

[a]TG = triglycerides, FFA = free fatty acid, ST = sterols, ESG = esterified sterol glucoside, MGDG = monogalactosyl diglyceride, SG = sterol glucoside, CE = cerebroside, DGDG = digalactosyl diglyceride, GPL = glycophospholipid, PG = phosphatidyl glycerol, DPG = diphosphatidyl glycerol, PE = phosphatidyl ethanolamine, PC = phosphatidyl choline, PI = phosphatidyl inositol, PA = phosphatidic acid, UN = unidentified material.

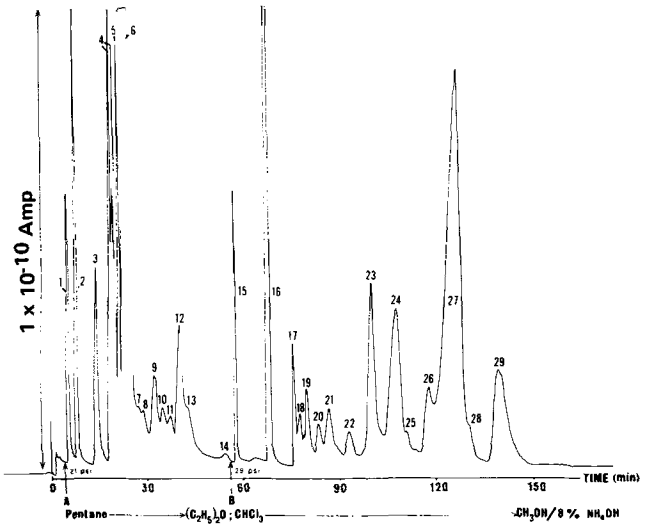

FIG. 2. Analytical liquid chromatography (ALC) of lipids of immature soybeans. Column = 1 m x 2.8 mm diameter, Bio Rad minus 325 mesh silicic acid, flow rate = 0.1 ml/min. Identification of peaks: 1, 2, 3 = hydrocarbons, waxes, pigments, and unknowns; 4, 5 = STE; 6 = TG; 7-11 = unidentified (terpenes, tocopherols, alcohols); 12 = ST; 13 = FFA; 14 = X; 15 = ESG; 16 = MGDG; 17 = SG; 18 = X; 19 = CE; 20 = X; 21 = PG; 22 = DGDG; 23 = PE; 24 = PI; 25 = X; 26 = PC; 27 = PA; 28 = X; 29 = LPC and other very polar lipids. X = unidentified components, STE = sterol esters, and LPC = lyso PC (see Table III for other abbreviations).

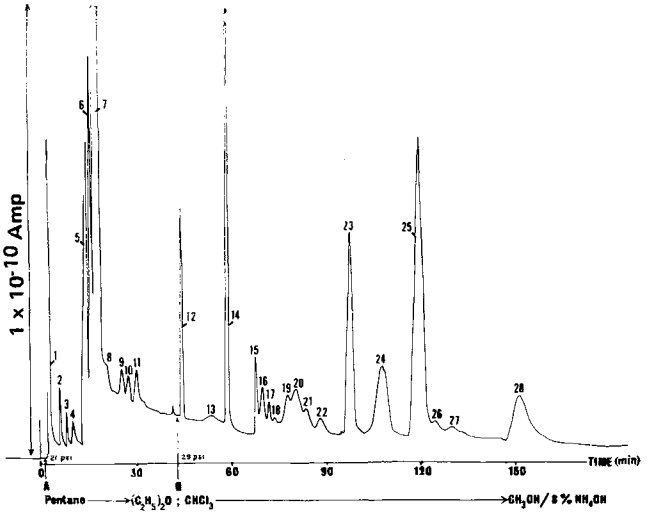

FIG. 3. Analytical liquid chromatography (ALC) of lipids of mature soybeans. Column = 1 m x 2.8 mm diameter, Bio Rad minus 325 mesh silicic acid; flow rate = 0.1 ml/min. Identification of peaks: 1-4 = hydrocarbons, waxes, esters, pigments; 5,6 = STE; 7 = TG; 8-10 = terpenes, tocopherols, alcohols; 11 = ST; 12 = ESG; 13 = Y; 14 = MDGD; 15 = SG; 16-19 = CE and glycolipids; 20 = PG; 21 = Y; 22 = DGDG; 23 = PE; 24 = PI; 25 = PC; 26 = PA; 27 = LPC; 28 = very polar lipids. Y = unidentified, STE = sterol esters, and LPC = lyso PC (see Table III for other abbreviations).

Examination of the analyses of the individual lipid classes (Table III) showed that the increase in neutral lipid, which consisted largely of unknown components (pigments, alcohols) and sterols in the initial stages of the development of the bean was due to the syntheses of TG. However, the initial sample did not appear to contain any TG. The initial samples contained traces of 1,2-diglycerides, free acid, and sterols which diminished rapidly as the bean developed. The formation of large amounts of free fatty acids and monoglycerides reported by Hirayama and Hujii could not be confirmed.

The major component of the PL fraction during the early stages of the development of the bean was PA. This compound decreased rapidly as phosphatidyl choline (PC) and PE, as well as TG, increased. However, because of the large increase in TG (neutral lipid, Table II), the major PL of the mature bean, PE, PC, and phosphatidyl inositol (PI), never became major components of the total lipid at any time during the development of the bean.

The major component of the GL fraction in the early stages of the development of the bean that resembled phosphatidic acid (GPL) also decreased as the bean matured. It was obviously an intermediate of lipid synthesis like PA, but its structure was not elucidated. Both GL and PL fractions, as well as the neutral lipids, contained appreciable amounts of unidentified material in the early stages of the development of the bean. These fractions consisted of a widely diverse group of substances with varying degrees of polarities, inasmuch as they were obtained in all three of the major lipid groups. However, they illustrated the complexity of the lipid, especially in the initial stages of the development of the bean. It was also apparent that the isolation of these compounds by TLC would be difficult. Therefore, studies were undertaken to apply high resolution liquid chromatography to the analysis of the lipid classes, not only to obtain information on these components, but to develop a method for the complete analysis of the lipid. Preliminary results of the application of this technique are shown in Figures 2 and 3. Only two samples were analyzed, one at ca. 40 days after flowering (Fig. 2) and the other at maturity (Fig. 3). However, they show large quantitative differences between the lipid classes of immature and mature bean, especially in the peak for PA among the PL. Many of the peaks in the chromatogram could not be associated with specific compounds as indicated in the legends of the chromatograms. However, some indication of the composition was obtained based upon the composition of the eluting solvent, inasmuch as only the neutral lipids are elutable with hexane or chloroform, for example. In future work, the major lipid groups will be examined separately at all stages in the development of the bean from just a few days after its formation to full maturity to provide more extensive information of the composition of the unanalyzed fractions, as well as the known components.

Large changes occurred in the fatty acid composition of the bean during maturation, as illustrated in Figure 4. The percentages of stearic and palmitic acid were relatively high in the initial stages of the development of the bean and then decreased rapidly as the bean developed. The percentages of oleic and linoleic acids were low initially, but continued to increase throughout the development of the bean. In contrast, linolenic acid increased rapidly initially and then decreased.

DISCUSSION

The complexity of the metabolic processes in the developing soybean is well demonstrated by the compo-

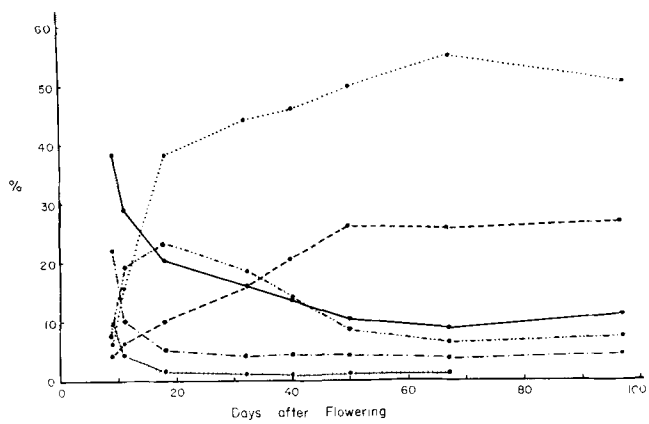

FIG. 4. Changes in fatty acid composition of the lipids of soybeans during maturation. —— 16:0, ▪▪▪▪ 16:1, —•— 18:0, - - - 18:1, • • • 18:2, and —·—·— 18:3.

sitional analysis of the lipid reported in this work. The most dominant change in the developing bean is the large increase in net synthesis of TG, which is associated with the large net synthesis of oleic and linoleic acids. There was no evidence for the formation of large amounts of free fatty acids or monoglycerides during development of the bean, as reported by Hirayama and Hujii (7). This led these investigators to propose an alternative to the Kennedy pathway (26) for TG synthesis. In fact, the presence of diglycerides and PA would seem to indicate that the major route of synthesis of TG in soybeans is by this pathway. Large quantitative changes occur in the molecular species composition of TG during maturation of the bean (7-9). Whether these changes occur exclusively as a result of an increase in net synthesis of oleic and linoleic acid or via a mechanism that involves transformation of fatty acids among host molecules is not known. Recent evidence (27,28) indicates that interconversion of fatty acids in the plant, particularly desaturation, can occur in the host molecule. Changes in molecular species composition of TG, as well as that of the other lipid classes, could occur via such a mechanism, as well as by de novo synthesis. Although the net synthesis of GL and PL is much slower than TG, it appears that the compounds of these groups are also in a dynamic state of metabolic activity, because of the change in fatty acid composition as the bean matures.

It seems that the enzymes involved in fatty acid synthesis in soybeans are influenced by both environmental and inheritance characteristics (1-5,29,30). Many of the compositional changes observed may be related to morphological changes as the bean matures, but no work appears to have been done on this aspect of lipid metabolism during maturation of the bean. The studies of von Düvel et al. (13), are of interest in this connection, because they did not detect globules of neutral fat in the early stages of the development of the bean. The present work also indicates little, or no, TG is present in the initial stage of the development of the bean. However, our work varies from their findings regarding fatty acid composition, inasmuch as linoleic and linolenic acid were detected in the early stages of the development of the bean. The observation that linolenic acid increases to a maximum in the early stage of the development of the bean before it decreases appears to be novel. The relatively large percentages of palmitic and stearic acid in the early stages of the development of the bean also appear to be a new observation, and agrees with the observation of Grimmer et al. (12) of the high content of myristic acid in the early stages of the development of the bean, because it also is a saturated fatty acid. This pattern of changes between saturated and linolenic acid may be related, because recent evidence indicates that linolenic acid may not arise by desaturation of linoleic acid as generally believed (31) but by an alternative pathway involving short chain acids (32). A detailed examination of the changes in the composition of the lipid of the bean in the early stages of its development is indicated by these observations. In the past these studies have been limited by the small concentration of lipid and problems in methods of lipid analysis, but now, hopefully, with the development of ALC, investigations at this stage of the development of the bean eventually can be undertaken.

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