Neutral and Polar Lipid Phase Transition of Soybeans with Various Saturated Fatty Acid Contents

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ABSTRACT: Soybeans with modified saturated fatty acid compositions sometimes have lower seed germination rate or other undesirable agronomic traits. To determine if seed germination could be related to the melting transitions of their lipids, triacylglycerols (TAG) and phospholipids (PL) from soybeans with a wide range of saturated fatty acid compositions were examined by differential scanning calorimetry. The melting transition temperatures of both TAG and PL increased with increasing palmitate and stearate percentages. The mean melting points of the various lipids calculated on the basis of the melting points of their fatty acids correlated with the observed transition temperatures. Increased lipid saturation and elevated phase transition temperatures may have contributed to the reduced germination and seedling growth rates of these modified seeds.

Paper no. J9934 in *JAOCS 78*, 1139–1144 (November 2001).

KEY WORDS: Membrane phase transition, neutral lipid, phospholipids, saturated fatty acids, soybean composition modification.

Soybean fatty acid composition has been modified (1) through traditional plant breeding and modern molecular genetics; specifically, soybean oils with reduced and elevated saturates have been obtained. However, we have observed that soybeans with elevated stearate percentages have unpredictable and abnormal field germination and low yield. Wang *et al.* (2) showed that seed with altered saturated fatty acid contents generally did fairly well in viability and vigor tests conducted at various temperatures under controlled laboratory conditions, but in a number of instances, seed vigor was negatively correlated with saturate content, especially stearate content.

The elevation of saturated fatty acids in soybean neutral lipid is reflected in the compositions of phospholipids (PL) (3,4), and this may have physiological consequences. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) are the major classes of PL in soybeans (4,5). They are the major components of cell membranes, and it is important that these membrane PL are in a proper physical state for cells to perform their physiological tasks. The proper physical state requires that the PL have the correct balance of saturated and unsaturated fatty acids (6). The effects of PL composition on membrane physical properties have been studied extensively in microorganisms and animals (7–12), but there have been fewer studies of the effect of fatty acid modification on phase transition of the major classes of plant PL or on their membrane properties (13,14). Nishida and Murata (15) have demonstrated that chilling sensitivity of plants could be manipulated by modulating levels of unsaturated fatty acids of membrane lipids by the action of certain enzymes, and they hypothesized that the changes in membrane fluidity are the initial event of the expression of desaturase genes.

Calculated mean melting point (mmp) of fatty acids from membrane lipid was suggested as a surrogate parameter of membrane fluidity, and it was shown that the composition of saturated fatty acids in plasma PL changed in a way to counteract changes in the mmp during pregnancy; therefore the fluidity of membrane could be maintained (12). But in mmp calculation, the contribution of the polar head groups is not considered. The relationships between calculated mmp and the determined thermal transition temperatures of various PL could be determined to examine how the polar head group may affect the PL thermal properties. These relationships could also be used to predict melting transition for a PL sample with known fatty acid composition.

In this paper, we report the effect of saturated fatty acid contents of soybean seed lipids on the phase transitions of their neutral lipids and PL by differential scanning calorimetry (DSC). The mmp of PL fatty acids were also calculated and correlated with DSC transition temperatures of these PL.

EXPERIMENTAL PROCEDURES

Soybean sample selection. Soybean cultivars and experimental lines (n = 18) with a wide range of fatty acid composition were obtained from the Agronomy Department at Iowa State University (Ames, IA) and Pioneer Hi-Bred International, Inc. (Des Moines, IA). These seeds were classified into five groups based on their saturated fatty acid percentages, as seeds with typical composition (3 lines), with elevated stearic acid (5 lines), with elevated palmitic and stearic acids (2 lines), with elevated palmitic acid (5 lines), and with reduced palmitic acid (3 lines).

Lipid sample preparation. PL isolation, class separation, and fatty acid composition analysis were performed as described by Wang *et al.* (3). Briefly, total lipid was extracted from 10 g of ground seed with chloroform/methanol (2:1, vol/vol). Neutral and polar lipid class separation was achieved by solid phase

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extraction by using a 900-mg silica cartridge (Alltech Associate, Inc., Deerfield, IL.). Neutral lipid (triacylglycerol—TAG) was eluted with chloroform and collected for fatty acid composition and melting transition analysis. Polar lipids were sequentially eluted with chloroform/methanol (1:1, vol/vol), methanol, and chloroform/methanol/water (1:2:0.8, vol/vol). To separate the major PL classes, thin-layer chromatography (TLC), with a 20 × 20 cm, 500-μ Adsorbosil-plus1 preparative plate (Alltech) and chloroform/methanol/acetic acid/water (100:45:5:2, by vol) as developing solvent, was used. PC, PE, and PI bands were scraped from the plate and extracted five times with 15 mL chloroform/methanol/water (1:2:0.8, by vol).

For TAG and PL fatty acid composition determination, fatty acid methyl esters (FAME) were prepared with 1.0 M methanolic sodium methoxide, and they were analyzed with a Hewlett-Packard (HP) (Avondale, PA) 5890A gas chromatograph equipped with a flame-ionization detector and capillary DB-23 (15-m length, 0.25-mm i.d., and 0.25-μm film thickness) column from J&W Scientific (Deerfield, IL). Oven temperature was 220°C, inlet and detector temperatures were 250°C; and split ratio was 10:1. Theoretical correction factors were calculated and applied to correct the FAME weight percentages. Mole percentages were calculated and reported.

Membrane lipid and neutral lipid thermal phase transition by DSC. PL thermal phase transitions were measured according to Singh *et al.* (13). TLC-purified PC, PE, and PI (1–5 mg) in chloroform were transferred to aluminum DSC pans, the solvent was evaporated, and remaining traces of solvent were removed by placing the pans in a vacuum desiccator for 2 h. About 8 mg of 50% ethylene glycol in water was added to the pan to hydrate the PL, and the pan was sealed and equilibrated at ambient temperature for 4 h. Silica cartridge-purified TAG (about 6 mg) was transferred to the DSC pan for melting transition determinations. The PL samples were scanned at a 6ºC/min rate from –70 to 30°C in a PerkinElmer DSC 7 instrument (PerkinElmer Corp., Norwalk, CT). The scan rate for TAG was 5°C/min from –60 to 30°C. The melting temperature of cyclohexane was used for standardization. Duplicate DSC analyses were performed for all samples.

The mmp calculation. The mole fraction of each of the five fatty acids in a purified lipid sample was multiplied by its melting temperature (63, 71, 16, –5, and –11°C for palmitic, stearic, oleic, linoleic, and linolenic acids, respectively) (16), and mmp was calculated as the sum of the multiplication products for all fatty acids of a particular type of lipid (12).

Statistical analysis. General linear model of SAS program (17) was used for the analysis of variance. The least significant differences were calculated ($P = 0.05$) to compare treatment means. The five groups of seeds were considered as treatments with a various number of replications due to availability of the seed lines.

RESULTS AND DISCUSSION

Fatty acid compositions. The average fatty acid compositions of TAG and PL of the five groups soybean lines classified based on the amounts of saturated acids in their neutral lipid are shown in Table 1.

For soybean lines with typical compositions, the saturate percentages are TAG < PC < PE < PI. When one of the saturated acyl percentages (palmitate or stearate) increased in the TAG relative to the amount in typical soybeans, its percentage in PL also increased, although to a smaller extent, and the percentage of the other saturate usually decreased relative to the typical group. When palmitate percentage was reduced in TAG, its percentage also was reduced in the PL, but to a lesser extent than in TAG. These observations suggest that saturate levels in PL are regulated more closely than in TAG.

Neutral lipid phase transition. The phase transition temperatures of the neutral and polar soybean lipids are summarized in Table 2. The onset, peak, and terminal temperatures are presented in the table. The shallowness of the peaks made estimation of the extremes approximate. An example of a melting transition of TAG from each composition category is shown in Figure 1. TAG of lines with elevated stearate or with both elevated palmitate and stearate had melting transitions at significantly higher peak temperatures than lines with elevated palmitate. The melting transitions of lines with elevated palmitate were at significantly higher temperatures than those with typical compositions. Lines with reduced palmitate tend to have even lower TAG melting transitions than those of typical lines, although these differences were not statistically significant.

Most TAG are polymorphic and can exist in at least three crystalline forms, designated α, β′, and β. When TAG samples were cooled rapidly in the DSC pans, they may have solidified in the lower-melting α or β′ forms. During the gradual increase in temperature, the less stable forms may have melted first and recrystallized in higher melting forms to give the melting profiles illustrated in Figure 1. Such transitions would account for the endotherm observed in the reduced saturate sample (Fig. 1e). The melting transitions in Figure 1 resembled the profile described by Roos (18).

Neutral lipid serves as seed energy storage and should be biologically available during seed germination and seedling growth. If field germination temperature is lower than the melting temperature of TAG, TAG will be in a plastic, partly crystalline state and may be less accessible for metabolism, thus limiting the energy supply for seed germination and growth. The lines with elevated stearate would be more subject to this danger than lines with elevated palmitate because of their higher melting points. Our previous results on physiological test (2) showed that germination and seedling growth rate (SGR) at 15 and 25°C were negatively correlated with palmitate and stearate percentages in various lipid classes, and a number of these were statistically significant. At 35°C, SGR became positively correlated with palmitate, but it still remained negatively correlated with stearate percentage. Therefore, elevation in stearate content seemed more detrimental to seed vigor than elevation in palmitate, and the difference in their neutral lipid melting points may be partially responsible for this observation.

TABLE 1
Average Fatty Acid Compositions (%) of the Neutral TAG and Phospholipids (PL) of Soybeans Grouped According to Their Saturate Content[a]

			Mean ± SD				
Lipid	Classification	# of lines	16:0	18:0	18:1	18:2	18:3
TAG	Typical	3	11.4 ± 1.6	4.2 ± 0.1	26.1 ± 4.3	50.3 ± 3.3	7.9 ± 0.8
	High 18:0	5	10.1 ± 1.3	22.8 ± 1.7	17.3 ± 0.7	42.2 ± 2.0	7.7 ± 1.1
	High 16:0 and 18:0	2	24.6 ± 1.4	18.7 ± 2.0	8.6 ± 1.1	37.5 ± 1.1	10.7 ± 1.2
	High 16:0	5	28.0 ± 4.5	4.7 ± 1.5	13.8 ± 2.5	42.1 ± 5.0	11.4 ± 3.1
	Low 16:0	3	3.4 ± 0.3	2.6 ± 0.2	18.0 ± 2.8	64.8 ± 3.5	11.2 ± 5.8
PC	Typical	3	13.8 ± 1.7	4.1 ± 0.4	10.1 ± 1.4	63.4 ± 2.3	7.7 ± 0.3
	High 18:0	5	10.6 ± 0.9	13.0 ± 1.5	9.2 ± 1.7	59.4 ± 1.2	7.9 ± 1.4
	High 16:0 and 18:0	2	16.3 ± 0.4	11.3 ± 0.6	5.4 ± 2.8	55.8 ± 3.5	11.6 ± 0.1
	High 16:0	5	22.1 ± 2.0	3.1 ± 0.1	7.5 ± 1.1	58.2 ± 3.2	9.2 ± 1.6
	Low 16:0	3	8.2 ± 1.1	6.1 ± 0.6	15.4 ± 4.9	63.3 ± 8.6	6.9 ± 2.6
PE	Typical	3	19.8 ± 2.7	3.2 ± 0.3	9.4 ± 0.3	59.3 ± 2.2	7.7 ± 0.9
	High 18:0	5	14.6 ± 1.0	9.0 ± 1.0	8.3 ± 1.8	59.8 ± 1.8	8.4 ± 2.0
	High 16:0 and 18:0	2	21.6 ± 0.4	6.4 ± 0.6	4.1 ± 2.0	57.3 ± 2.7	10.7 ± 0.2
	High 16:0	5	26.0 ± 1.6	1.9 ± 0.2	5.9 ± 0.9	57.6 ± 2.6	8.7 ± 1.2
	Low 16:0	3	12.6 ± 0.6	5.8 ± 1.4	14.1 ± 4.6	61.3 ± 6.7	6.3 ± 2.5
PI	Typical	2	31.5 ± 2.2	8.3 ± 1.1	8.6 ± 1.3	45.1 ± 2.7	6.6 ± 0.6
	High 18:0	4	18.6 ± 2.3	21.7 ± 3.2	8.2 ± 1.6	44.1 ± 3.4	7.5 ± 0.4
	High 16:0 and 18:0	2	9.2 ± 1.7	16.8 ± 1.2	2.8 ± 0.5	40.2 ± 1.3	11.1 ± 1.3
	High 16:0	4	38.5 ± 1.9	5.1 ± 1.2	4.3 ± 0.9	43.0 ± 2.7	9.2 ± 2.0
	Low 16:0	2	16.8 ± 0.7	17.3 ± 2.0	10.5 ± 1.2	49.5 ± 2.5	6.1 ± 2.5
Total PL	Typical	3	18.3 ± 2.5	4.7 ± 0.4	9.7 ± 0.6	58.9 ± 1.9	7.6 ± 0.5
	High 18:0	5	13.3 ± 0.9	13.5 ± 1.5	8.7 ± 1.6	56.5 ± 1.2	8.0 ± 1.4
	High 16:0 and 18:0	2	0.0 ± 0.1	10.9 ± 0.7	4.6 ± 2.1	53.5 ± 2.9	11.2 ± 0.1
	High 16:0	5	25.9 ± 1.0	3.1 ± 0.3	6.5 ± 0.8	55.4 ± 2.6	9.1 ± 1.6
	Low 16:0	3	10.9 ± 1.0	8.0 ± 1.0	14.3 ± 4.2	60.0 ± 7.5	6.9 ± 2.6

[a]Abbreviations: TAG, triacylglycerols; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid.

Thompson and Li (19) reported the effect of stearate content on seed germination of canola. When canola seeds were genetically modified to produce oils with greater than 30% stearate, their germination rates were lower, and seedlings were less vigorous. One of the suggested causes was that the more saturated molecular species of canola TAG may be less preferred substrates for rapeseed lipase, or that the TAG may have crystallized into a physical state less accessible to lipases. The other suggested cause was the potential accumulation of stearate in membrane lipids, making membranes less able to adapt to changes in temperature and moisture content.

Polar lipid phase transition. A series of DSC PL phase transition profiles is shown in Figure 2. The peaks were small and very broad, which is typical for biological samples (20). The shapes of the transition peaks were similar for all PL samples. Generally, the low-temperature inflection tended to be quite gradual, which made the determination of the onset temperature difficult, but the high-temperature terminals were more abrupt. For the PL mixtures obtained from the soybean lines, there were significant differences in the temperatures of melting onset among the fatty acid composition classes, but there was no difference in peak or terminal melting point. For lines with elevated stearate percentages and those with typical fatty acid compositions, melting onset occurred at higher temperatures. When the PL was fractionated, PC, which is the major fraction, showed significant differences in peak and terminal melting points among composition class. The lines with elevated stearate or elevated stearate and palmitate melted at higher temperatures. The PE fractions showed significant differences with composition class only for the terminal melting point where samples with both elevated palmitate and stearate percentages melted higher. The PI fractions had significantly lower onset and peak temperatures in samples with reduced palmitate percentages than the other composition classes. Overall, transition temperatures of the more saturated PL were higher than those of more unsaturated ones.

Melting enthalpy was estimated to be 2 to 12 J/g PL with an average of 6 J/g, which was comparable to previously reported values (9).

PC had a significantly lower transition temperature compared to PE and PI isolated from the same soybean source, as shown in Figure 2. The average transition temperatures of PE and PI were not very different (Table 2). This trend in melting transitions corresponds to the percentage of saturated fatty acid in these lipid classes. PL phase transition temperature is believed to depend primarily on acyl chain structure and only secondarily on their polar head groups (21). In model PL systems, for every two-carbon increase in the chain length, the transition temperature increased 14°C, and for each double-bond addition, the transition temperature decreased 70°C (21). In another model system (22), PE with saturated acyl chains underwent phase transition at temperature about 20–30°C higher than the corresponding PC, because the smaller head group of PE allowed very close molecular pack-

TABLE 2
Average Neutral and Polar Lipid Phase Transition Temperatures (°C) of Modified Soybeans Grouped According to Their Saturate Content

Lipid	Classification	# of sample	Onset T[a,b]	Peak T[a,c]	Terminal T[a,d]
TAG	Typical	3	–39.6d*	–9.4c	–0.6c
	High 18:0	5	–13.7a	18.3a	20.7a
	High 16:0 and 18:0	2	–17.1b	16.8a	18.9a
	High 16:0	5	–21.8c	8.4b	11.6b
	Low 16:0	3	–46.1e	–13.8c	–8.1d
	$LSD_{.05}$		3.3	4.6	4.1
PC	Typical	3	–51.3a	–38.9b	–27.4b
	High 18:0	5	–51.1a	–32.3a	–20.1a
	High 16:0 and 18:0	2	–50.2a	–32.8a	–21.6a
	High 16:0	5	–51.7a	–37.8b	–24.3a,b
	Low 16:0	3	–51.9a	–40.2b	–27.5b
	$LSD_{.05}$		2.3	3.4	4.9
PE	Typical	3	–31.2a	–10.5a	–0.9b
	High 18:0	5	–29.8a	–10.7a	0.7a,b
	High 16:0 and 18:0	2	–32.6a	–10.0a	8.4a
	High 16:0	5	–32.1a	–9.4a	–2.5b
	Low 16:0	3	–31.5a	–9.9a	–2.2b
	$LSD_{.05}$		6.0	3.0	9.1
PI	Typical	2	–21.3a	–11.0a	–1.2a,b
	High 18:0	4	–21.0a	–9.2a	2.3a
	High 16:0 and 18:0	2	–18.9a	–9.2a	0.9a
	High 16:0	4	–22.4a	–10.9a	0.5a,b
	Low 16:0	2	–33.3b	–15.0b	–3.6b
	$LSD_{.05}$		6.8	2.6	4.1
Total PL	Typical	3	–37.1a	–19.3a	–2.4a
	High 18:0	5	–38.5a	15.0a	1.5a
	High 16:0 and 18:0	2	–43.9b	–17.6a	–5.2a
	High 16:0	5	–38.8a,b	–19.1a	–2.8a
	Low 16:0	3	–41.8a,b	–20.7a	–2.7a
	$LSD_{.05}$		5.3	6.2	6.7

[a]Different letters in the same column and lipid category indicate statistical differences, at 5%.
[b]Temperature of the start of the transition peak.
[c]Temperature of the peak temperature.
[d]Temperature of the end of the transition peak. For abbreviations, see Table 1.

ing. But the transition of unsaturated PE fell in the same region as the corresponding PC, probably because *cis* double bonds no longer allowed close molecular packing.

The PL transition temperatures obtained in this experiment were all below 0°C, as expected. DSC peak transition temperatures of isolated PC rehydrated with ethylene glycol/water (1:1, vol/vol) were –13, –23, and 21°C for brain, liver, and lung of chick embryo, respectively, with enthalpies ranging from 4.0 to 10.2 J/g (9). Ladbrook and Chapman (23) reported that the transition region for egg-yolk PC, which had over 70% of its molecules containing both 16:0 and 18:1, was –15 to –5°C. Silvius (24) reported that the peak transition temperatures of PC with acyl chain combinations of 18:1*cis* Δ9/18:1*cis* Δ9, 16:0/18:1*cis* Δ9, and 18:0/18:1*cis* Δ9 were –22, –5, and 13°C, respectively, but the transition temperatures for corresponding PE were much higher (–16, 20, and 30.4°C, respectively). Thus, isolated PL from many tissues or organisms melt at temperatures well below those at which the

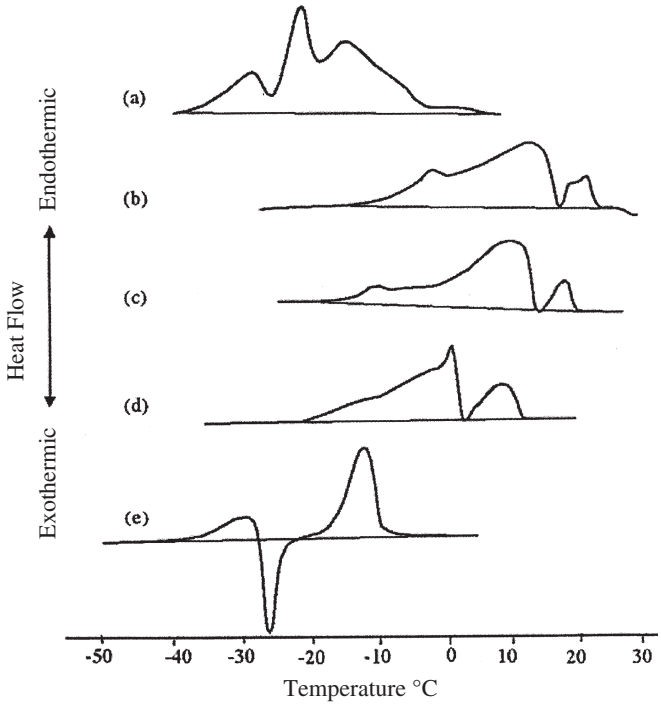

FIG. 1. A schematic representation of differential scanning calorimetry (DSC) thermographs of selected soybean oils with modified fatty acid compositions. Oils were from soybeans with (a) typical composition, (b) elevated stearate, (c) elevated stearate and palmitate, (d) elevated palmitate, and (e) reduced palmitate. Scan rate was 5°C/min from –60 to 30°C.

tissues function or organisms flourish, but the lack of bulk transition at growth temperatures does not imply that phase behavior is unimportant. Specialized microcrystalline regions may exist that regulate membrane function (25) and require a variety of membrane lipids for proper physiological function

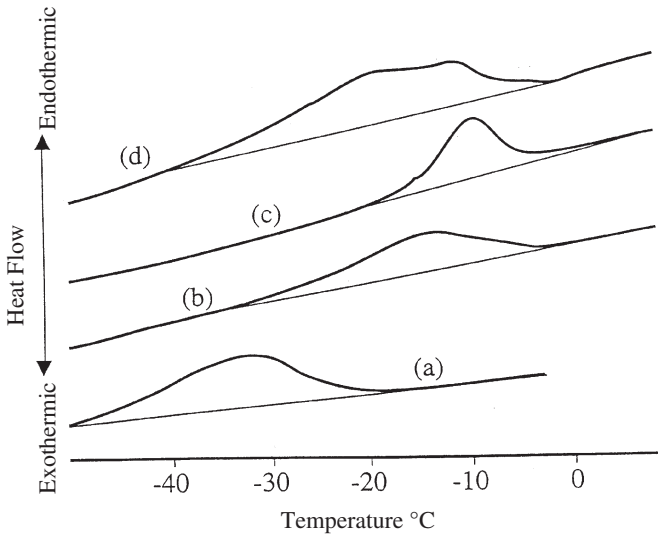

FIG. 2. DSC scan of soybean phospholipids isolated from a sample with elevated palmitate percentage (33% C16:0). (a) Phosphatidylcholine (PC), (b) phosphatidylethanolamine (PE), (c) phosphatidylinositol (PI), and (d) total phospholipid (PL). Samples were hydrated in 50% ethylene glycol in water and scanned at 6°C/min from –70 to 30°C.

(26). Lateral phase separations of PL species in membranes have been noted (20,22), and these may affect the distribution of membrane proteins (27).

Numerous studies have shown that organisms adapt to changes in ambient temperature by altering the acyl composition of the PL in their membranes (11,28–32), and this is believed to affect the fluidities and permeabilities of their membranes. But other investigators have failed to find a direct relation between acyl composition and PL thermal properties (33) or have found only small shifts in melting properties from great changes in acyl composition (11).

It is worth mentioning that extracting lipids from their native environment and separating them into various groups may significantly alter their physical properties. Co-crystallization and mutual solubility of various PL and other minor membrane components may occur in biological membranes, and their melting transition may be different from that estimated by isolated PL. Nevertheless, studying the purified lipids can provide information on how fatty acid saturation affects PL physical property. Although it is suggested that DSC is one of the methods of choice for studies of lipid phase transition (20,34), many other physical techniques can be used to monitor the membrane phase transition and lipid fluidity (34). X-ray diffraction and nuclear magnetic resonance are nonperturbing techniques, whereas electron-spin resonance is a perturbing technique. Fluorescence polarization is a popular technique for measuring fluidity by using an extrinsic probe. The nonperturbing method could be used to accurately determine membrane transition and fluidity.

Relationship between thermal transitions and mmp of various lipid classes. The relationship between measured DSC melting transition and calculated mmp of all lipid classes is shown in Figure 3. For TAG, the measured and calculated melting points showed a good correlation ($R^2 = 0.914$, P, probability of no correlation = 0.011). The relationships for PL are also present ($R^2 =$ 0.721 and 0.853, $P = 0.070$ and 0.026 for PC and PI, respectively), except for PE and total PL ($R^2 = 0.107$ and 0.457, $P =$ 0.587 and 0.205, respectively). PC and PE, with similar average calculated mmp (11.9 and 13.1°C, respectively), had very different measured transition peak temperatures (average of –36.4 and –10.1°C, respectively). This observation is in partial agreement with Van Dijck *et al.* (22), as noted before. In addition, PI and PE, with very different average calculated mmp (24.8 and 13.1°C, respectively), had very similar measured transition temperatures (average of –11.1 and –10.1°C, respectively). Therefore, the head groups of PL play a very important role in membrane phase transition and fluidity. The ethanolamine group of PE may have caused tight packing that resulted in higher melting transition temperature than that of PC, and the effect of fatty acid unsaturation on PE phase transition may be minimal. The inositol group of PI may have prevented ordered packing, thus giving a lower transition temperature than expected. Thus, when using mmp as an indicator of membrane fluidity, the type and composition of its PL need to be considered. These relationships could be refined by examining more PL samples with wide range of fatty acid composition. It is possible that mathematical models can be developed to predict the PL and membrane phase transition temperature by using the calculated mmp of fatty acids of particular lipid class.

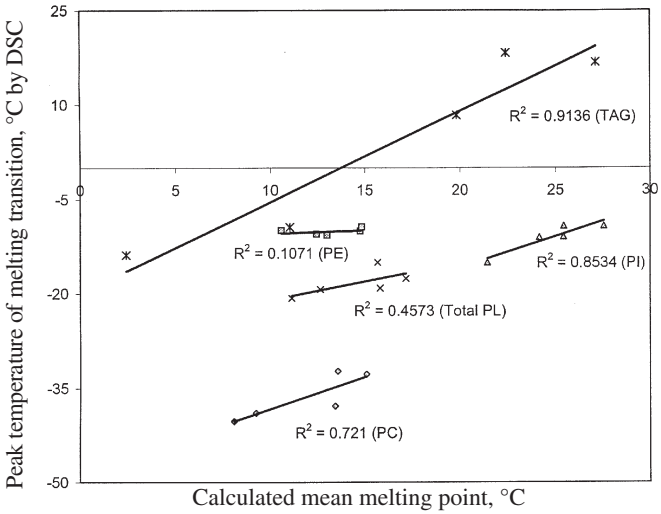

FIG. 3. Relationship of measured phase transition temperature (peak) by DSC and calculated mean melting point of various lipids of modified soybeans. See Figures 1 and 2 for other abbreviations.

It is interesting to note that for PC, the mmp of the elevated 18:0 and elevated 16:0 samples are very similar, but their measured phase transition temperatures are quite different, as shown in Figure 3. An elevated level of stearic acid in seed lipids tends to contribute more to the increase in phase transition.

In summary, the changes of melting transition documented here may account for the modest reduction in germination and SGR observed for soybean lines with elevated saturate content and for the very poor field performance sometimes exhibited by soybean lines with elevated stearate percentages. But a better correlation of composition with physiological tests and a better understanding of the conditions responsible for the poor field performance are needed before a definite association can be made.

ACKNOWLEDGMENTS

This work was supported by a research grant from Pioneer Hi-Bred International, Inc. (Des Moines, IA). Journal Paper No. J19258 of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA. Project No. 0178 and supported by Hatch Act and State of Iowa funds.

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[Received March 19, 2001; accepted August 24, 2001]