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Lipid Crystallization in Senescent Membranes from Cotyledons

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ABSTRACT

Lipid transition temperatures for rough and smooth microsomal membranes isolated from bean (*Phaseolus vulgaris*) cotyledon tissue at various stages of germination were determined by wide angle x-ray diffraction. The transition temperatures were established by recording diffraction patterns through a temperature series until a sharp x-ray reflection centered at a Bragg spacing of 4.15 Å and denoting the presence of crystalline lipid was discernible. For rough and smooth microsomes from 2-day-old tissue, the transitions occurred at 0 C and 3 C, respectively, indicating that at this early stage in the germination sequence the membrane lipid is entirely liquid-crystalline at physiological temperature. By the 4th day of germination, the transition temperatures had increased to 32 C for smooth microsomes and 35 C for rough microsomes, indicating that at 29 C, which was the growth temperature, portions of the membrane lipid were crystalline. During the later stages of germination, the transition temperature for smooth microsomes continued to rise through 44 C at day 7 to 56 C at day 9, by which time the cotyledons were extensively senescent and beginning to abscise. There was also a dramatic increase in the proportion of membrane lipid in the crystalline phase at 29 C. By contrast, the rough microsomes showed little change in transition temperature and only a slight increase in the proportion of crystalline lipid during this late period in germination. The data indicate that substantial amounts of the lipid is senescing membranes are crystalline even at physiological temperature. Moreover, there is a temporal correlation between the appearance of this crystallinity and loss of membrane function, suggesting that the two may be causally related.

The onset of senescence in cotyledons of germinating *Phaseolus vulgaris* is marked by a general metabolic decline which includes deterioration of membrane function. Plasma membranes, for example, exhibit decreased cation-sensitive ATPase and cholinesterase activities as well as a reduced capability for ATP-dependent cation transport (12, 14). This loss of function correlates temporally with age-dependent alterations in the protein complement of the membrane (15). The enzymic activities of microsomal membranes also decline as senescence intensifies, and the levels of microsomal phospholipid phosphate decrease indicating that there is a structural disassembly of these membranes (18).

There is increasing evidence that the physical state of membrane lipid is an important factor in determining such key membrane functions as enzyme activity and permeability. By means of Arrhenius plots it has been demonstrated for certain membrane-bound enzymes that temperature-induced changes in activation energy correlate with phase transitions in the membrane lipid (5, 6, 9, 25). In addition, the permeability of lipid vesicles and natural membranes can be altered by factors known to influence membrane fluidity (4, 24, 31). We have previously reported that the fluidity of smooth microsomal membranes from bean cotyledons decreases as the tissue becomes senescent (19). In this study, we have examined lipid phase transition temperatures for both rough and smooth microsomes during germination in order to obtain comparative data on membranes of varied function, and thereby evaluate the prospect of a temporal correlation between loss of membrane function and changes in the physical state of the lipid.

MATERIALS AND METHODS

Fractionation Procedures. Untreated seeds of *Phaseolus vulgaris*, variety Kinghorn, were germinated in moist vermiculite under etiolating conditions at 29 C. Cotyledons were harvested at specified intervals after planting. Rough and smooth microsomal fractions were isolated from the cotyledon tissue as previously described (18). For each preparation, the tissue was homogenized in a sufficient volume of 0.05 M $NaHCO_3$-0.3 M sucrose (pH 7) to yield a 33% (w/v) homogenate. The homogenate was filtered through four layers of cheesecloth and centrifuged at 10,000*g* for 20 min. The resulting supernatant was made 15 mM with CsCl and centrifuged through a barrier of 1.3 M sucrose-15 mM CsCl at 150,000*g* for 3 hr in a Beckman type 50 Ti rotor. The smooth microsomes collected at the interface and the rough microsomes together with free ribosomes formed a pellet at the bottom of the tube. The smooth microsomes were removed with a syringe, diluted with 3 volumes of 0.05 M $NaHCO_3$ and pelleted by centrifugation at 150,000*g* for 1 hr.

The rough microsomes were further purified by centrifugation through 1.8 M sucrose as previously described (29). For this purpose the pellets from the previous centrifugation were resuspended in 30 ml of 0.05 M $NaHCO_3$-0.3 M sucrose, pH 7, and 8 ml of this suspension were layered over 4 ml of 1.8 M sucrose in cellulose nitrate tubes for a Beckman type 50-Ti rotor. The tubes were centrifuged for 2 hr at 150,000*g* during which time the rough microsomes collected at the interface and the free ribosomes formed a pellet at the bottom of the tube. The interface region was removed with a syringe, diluted with 3 volumes of 0.05 M $NaHCO_3$ (pH 7), and pelleted by centrifugation at 150,000*g* for 1 hr.

X-ray Diffraction. Concentrated membrane samples for x-ray diffraction were prepared by mounting portions of the packed membrane pellets between two jaws of a brass holder (10). The membranes were then allowed to reach equilibrium in N_2 which had been bubbled through distilled H_2O. This treatment removes water from between and around the membranes allowing them to come together, but it does not dehydrate them (10). The holder bearing the membrane specimen was then placed in a temperature-controlled chamber on a wide angle x-ray diffraction camera (Philips type 1030). Diffraction patterns were recorded for 2 to 5 hr at specified temperatures using CμKα radiation from a point focus x-ray tube. Air, which had been bubbled through distilled H_2O, was continuously passed through the chamber while the diffraction patterns were being recorded. The specimen to film distance was calibrated using Teflon. The hydration levels of representative samples were determined gravimetrically at selected stages during the diffraction series.

Densitometer tracings of the diffraction patterns were made with a Clifford densitometer, model 345.

Fatty Acid Analysis. Lipids were extracted from the isolated membranes as described by Nichols (22). Methyl esters of the fatty acids were prepared by the method of Morrison and Smith (20). A portion of the lipid extract containing approximately 1 μmol of phospholipid P was evaporated to dryness under N_2, redissolved in 1 ml of boron trifluoride, and heated at 90 C for 90 min in a water bath. The methyl esters were extracted with pentane-water (2:1, v/v). The presence and purity of the fatty acid methyl esters were confirmed by TLC. The esters were separated and identified by gas chromatography using a Perkin Elmer model 900 chromatograph equipped with a flame ionization detector and a 6-ft stainless steel column packed with 10% EGSS-X in 100/120 Supelcoport and maintained at 180 C. Peak areas were quantified with a mechanical disc integrator.

RESULTS

Wide angle x-ray diffraction patterns and corresponding densitometer tracings depicting the state of membrane lipid at temperatures above and below the transition temperature are illustrated in Figures 1 and 2. These data are for smooth microsomes isolated from 2-day-old cotyledons. Diffraction patterns recorded at the growing temperature (29 C) featured two diffuse bands at Bragg spacings of 4.6 Å and 10 Å (Fig. 1A). This pattern is typical of those obtained for a variety of membranes at physiological temperature (7, 8, 27, 28). The 10 Å reflection is thought to be derived from protein, but is not well characterized (8, 11). The broad ring at 4.6 Å is known to be a lipid reflection (7, 8, 27, 28) and derives from the imprecise arrangement of hydrocarbon side chains that characterizes lipid in the liquid-crystalline phase (28). When diffraction patterns were recorded through a series of decreasing temperatures, a sharp reflection superimposed on the broad 4.6 Å reflection and centered at 4.15

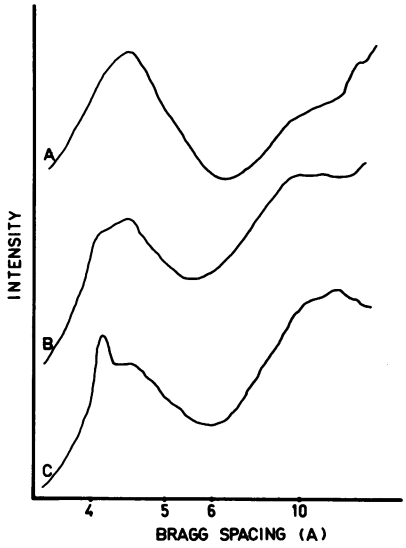

FIG. 2. Densitometer tracings of wide angle x-ray diffraction patterns for smooth microsomal membranes from 2-day-old bean cotyledon tissue. A, B, and C were recorded at 29 C, 0 C and −15 C, respectively. Each tracing represents half of the diffraction pattern recorded in the equatorial plane. Intensity is in relative units.

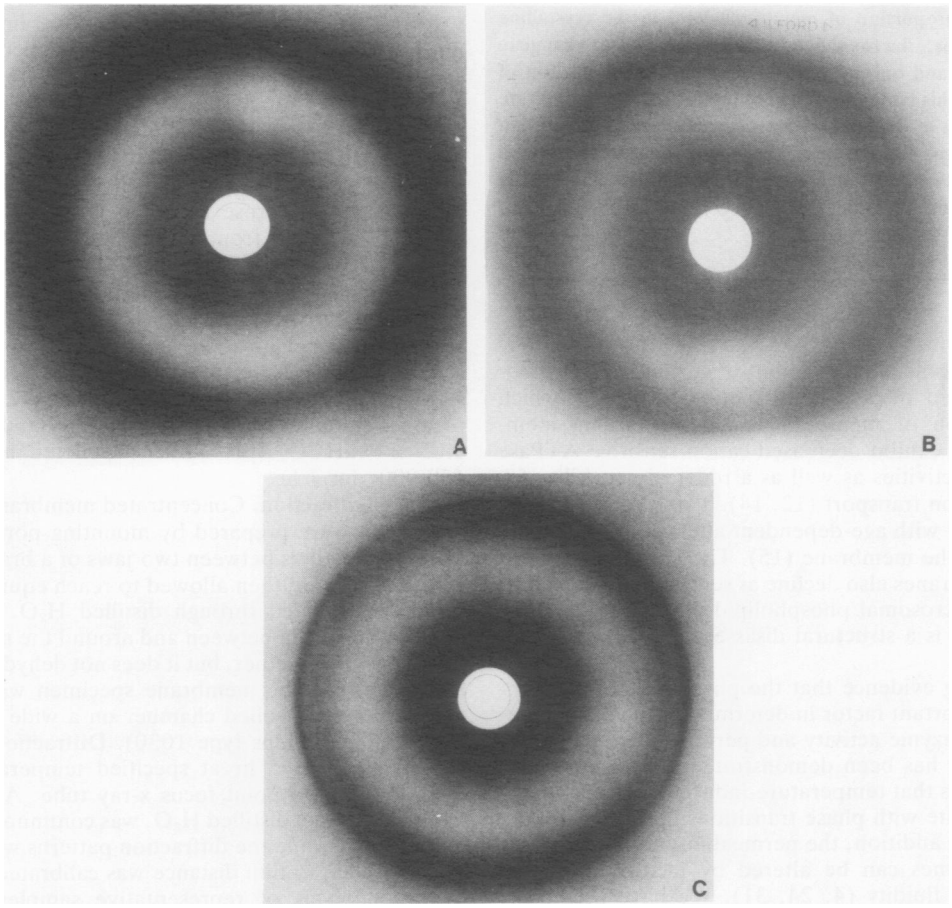

FIG. 1. Wide angle x-ray diffraction patterns recorded from smooth microsomal membranes isolated from 2-day-old bean cotyledon tissue. A: pattern recorded at 29 C showing (from outside to inside) diffuse bands at 4.6 Å and about 10 Å; B and C: patterns recorded at 0 C and −15 C, respectively, showing (from outside to inside) a sharp band at 4.15 Å and diffuse bands at 4.6 Å and about 10 Å.

Å became discernible at 3 C and increased in intensity at lower temperatures (Fig. 1, B and C). This sharp 4.15 Å reflection is also well characterized. For example, wide angle diffraction patterns of long chain paraffins in a crystalline hexagonal phase feature a sharp ring at 4.15 Å (13, 16, 21). Its appearance in wide angle patterns from membranes also reflects an ordered crystalline or gel phase of the lipid in which the hydrocarbon chains form a rigid hexagonal lattice (7, 8, 27, 28). The temperature at which this sharp reflection first appears, in this case 3 C, is the lipid phase transition temperature, and it marks the beginning of the transition from the liquid-crystalline phase to the gel phase (7, 8). Moreover, the transition is reversible; when the temperature of the cotyledon membrane sample was subsequently raised, the 4.15 Å band decreased in intensity and completely disappeared above 3 C.

The enhanced intensity of the 4.15 Å band with decreasing temperature is more clearly portrayed in densitometer tracings of the diffraction patterns (Fig. 2). At temperatures below the transition, the 4.15 Å peak progressively intensified, and the 4.6 Å peak diminished (Fig. 2, B and C). This reflects conversion of an increasing proportion of the membrane lipid to the gel phase. However, the 4.6 Å peak did not disappear until the temperature had been dropped about 40 degrees below the transition temperature. This broad temperature range over which the transition occurs is typical for other biological membranes (7, 27), and presumably reflects the heterogeneous nature of the membrane lipid. The lower limit of the transition cannot be determined as precisely as the upper limit by x-ray diffraction because of the diffuse nature of the 4.6 Å band.

The diffraction analyses revealed a general increase in the transition temperatures of both rough and smooth microsomes with advancing senescence. As early as 16 hr after planting there was a slight protrusion of the radicle through the seed coat, indicating that the seeds had imbibed sufficient water to begin germination. At this stage, the transition temperatures were −4 C and −2 C for rough and smooth membranes, respectively (Fig. 3). At 2 days, the transitions occurred at 0 C for the rough surfaced membrane and at 3 C for smooth membranes. Between the 2nd and 4th days of germination, the transition temperatures increased dramatically to 32 C for smooth microsomes and 35 C for rough microsomes (Fig. 3). By this stage, the food reserves of the cotyledons are partially depleted and there are regions of autolysis in the tissue (23, 30). During the later stages of germination, the transition temperature for the smooth microsomes continued to increase through 44 C at day 7 to 56 C at day 9, by which time the cotyledons were extensively senescent and beginning to abscise. By contrast, the rough microsomes showed little change in transition temperature during this period (Fig. 3). For each membrane sample, the transition temperature was established by recording diffraction patterns at various temperatures until the appearance or disappearance of the 4.15 Å band was defined to within 1 C. To ensure that the specimen had not become modified in the interim, the first diffraction pattern was repeated at the end of the series.

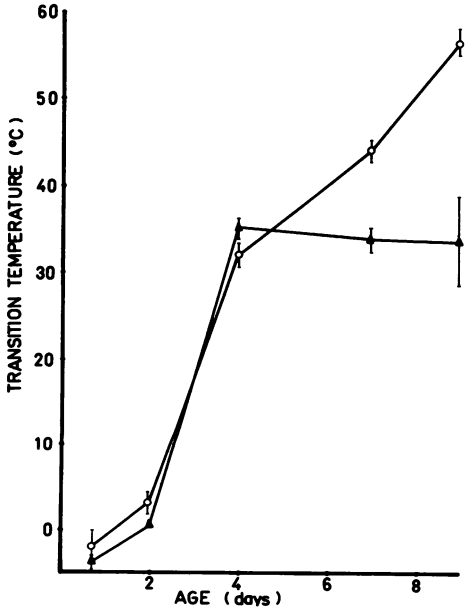

FIG. 3. Changes during germination in the lipid transition temperatures for microsomal membranes from bean cotyledon tissue. (○): smooth microsomes; (▲): rough microsomes. Standard errors of the means are indicated; N = 3 to 6.

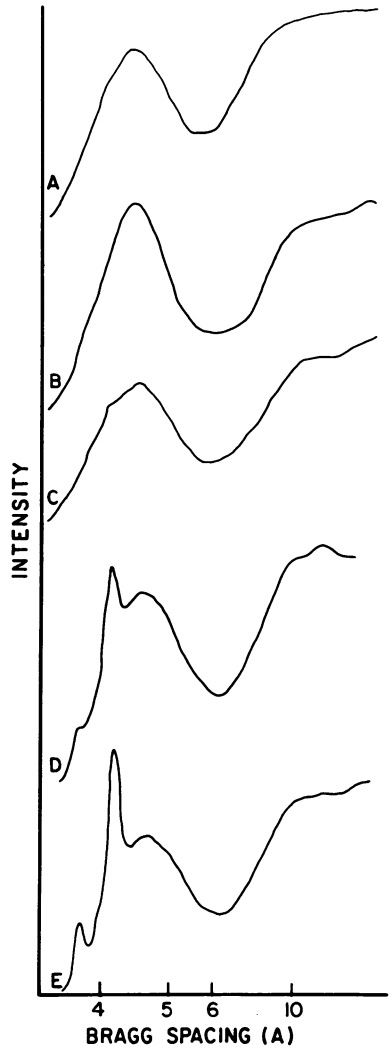

FIG. 4. Densitometer tracings of wide angle x-ray diffraction patterns recorded at 29 C from smooth microsomal membranes of cotyledon tissue at various stages of germination. A,B,C,D, and E are tracings for membrane from tissue 16 hr, 2 days, 4 days, 7 days, and 9 days of age, respectively. Each tracing represents half of the diffraction pattern recorded in the equatorial plane. Intensity is in relative units.

The observation that the transition temperature is below the growing temperature at day 2 for both types of membrane indicates that in young cotyledon tissue the membrane lipid is exclusively liquid-crystalline at physiological temperature. The rise in transition temperature to a point above the growing temperature later in the germination sequence signifies that portions of the lipid in senescing membranes become crystalline under physiological conditions. An indication of the relative

proportions of gel phase and liquid-crystalline phase at various stages of senescence can be gleaned from densitometer tracings of the diffraction patterns recorded at 29 C. Tracings for both rough and smooth microsomes isolated at intervals of 16 hr and 2 days after planting portray a single lipid peak centered at 4.6 Å, and thus confirm that at these early stages in the germination sequence the lipid is exclusively liquid-crystalline (Figs. 4 and 5). By day 4, a shoulder centered at 4.15 Å, which signifies crystalline lipid, can be discerned for both membrane types (Figs. 4 and 5). For smooth microsomes, the intensity of the 4.15 Å peak increases with advancing germination such that by day 9 the gel phase is predominant (Fig. 4, D and E). By contrast there is only a slight increase in the intensity of the 4.15 Å peak beyond day 4 for rough microsomes (Fig. 5, D and E).

The densitometer scans for 7- and 9-day-old smooth microsomes also display a peak at 3.75 Å that is not present in scans for membrane from younger tissue (Fig. 4). In the temperature series this 3.75 Å reflection disappeared at 34 C for 7-day-old membrane and 46 C for 9-day-old membrane, temperatures which are about 11 degrees below the transition points for these preparations. The 3.75 Å band was not discernible in densitometer scans for rough microsomes.

To confirm that detection of the gel phase in microsomes from

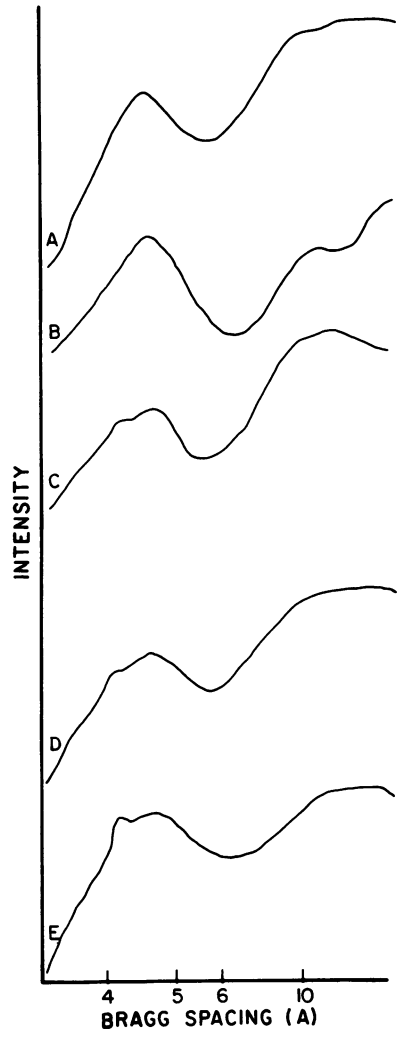

FIG. 5. Densitometer tracings of wide angle x-ray diffraction patterns recorded at 29 C from rough microsomal membranes of cotyledon tissue at various stages of germination. A,B,C,D, and E are tracings for membrane from tissue 16 hr, 2 days, 4 days, 7 days, and 9 days of age, respectively. Each tracing represents half of the diffraction pattern recorded in the equatorial plane. Intensity is in relative units.

Table I. Changes in the Unsaturated to Saturated Fatty Acid Ratio in Microsomal Membranes from Senescing Cotyledons

Tissue Age (days)	Ratio[1] Smooth Microsomes	Rough Microsomes
2	2.06 a	1.98 a
4	1.43 b	1.54 b
7	1.64 b	1.66 b
9	1.54 b	1.45 b

[1] The values are means of three separate experiments. Any two means not followed by the same letter are significantly different at the 5% level.

older senescing cotyledons did not simply reflect lipid crystallization brought on by dehydration, membrane specimens used for diffraction were analyzed gravimetrically to determine their water content. Smooth microsomal membranes containing 50 to 75% moisture with respect to final dried weight still gave rise to diffraction patterns featuring sharp reflections at 4.15 and 3.75 Å. In another series of experiments, membrane samples from young tissue were air-dried over a period of several days to induce crystallization attributable to dehydration. Diffraction patterns for dried membranes from 2-day-old tissue featured sharp rings at 3.5, 5, and 4.8 Å as well as the diffuse 4.6 Å band. However, these samples could not be induced to display reflections at either 4.15 Å or 3.75 Å as a result of dehydration.

Both rough and smooth microsomal membranes were found to contain palmitic, stearic, arachidic, oleic, linoleic, and linolenic acids throughout the germination period. However the unsaturated fatty acid ratio decreased significantly from a mean value of 2.06 at day 2 to 1.43 at day 4 for the smooth microsomes and from 1.98 to 1.54 over the same period for rough microsomes. Thereafter, the ratio remained essentially unchanged for both types of membrane (Table I).

DISCUSSION

For most biological membranes, the phase transition temperature of the lipid is below physiological temperature, meaning that the lipid matrix of the membrane is in a liquid-crystalline phase (2, 28). Formation of the gel phase, during which the hydrocarbon chains lose entropy and mobility to form an ordered array, can be induced by lowering the temperature, and this is often accompanied by a loss of membrane function (9, 26, 27). It may be significant in the context of membrane deterioration during senescence that both rough and smooth microsomes from senescing cotyledon tissue display a gel or crystalline phase at physiological temperature.

By the 4th day of germination, the transition temperatures for both rough and smooth microsomes have risen synchronously to a point well above the growing temperature. Beyond day 4, the transition temperature remained relatively constant for the rough microsomal fraction but rose steadily for the smooth microsomes to a high of 56 C by day 9 (Fig. 3). This suggests that as senescence intensifies the nature of the crystalline phase changes in smooth microsomes but not in rough microsomes. This interpretation is further borne out by the observation that subsequent to day 4 diffraction patterns for smooth microsomes recorded at 29 C featured two sharp reflections at 4.15 and 3.75 Å, whereas corresponding patterns for rough microsomes displayed only the 4.15 Å reflection. These two sharp bands reflect different modes of hydrocarbon chain packing in the crystalline phase. The 4.15 Å reflection has been widely observed below the transition temperature in both membranes and pure triglycerides, and derives from a hexagonal packing of the fatty acid side chains (7, 8, 28). The 3.75 Å ring reflects an alternative crystalline state of the lipid in which there is an orthorhombic rather than hexagonal packing of the hydrocarbon chains. This reflection has been observed and characterized for purified triglycerides (13, 16), but to our knowledge has not been previ-

ously observed in hydrated biological membranes. The smooth microsomal fraction is heterogeneous, comprising vesicles of membrane derived from several organelles. Accordingly, the rising transition temperature of the smooth fraction as well as the incidence of orthorhombic packing may well reflect temporal contributions to the crystalline phase by additional species of membrane, or perhaps distinct regions of the same membrane, as they in turn become altered in the face of intensified senescence.

The distinctive crystalline characters of the rough and smooth microsomes suggest that the different types of membrane comprising these fractions are differentially sensitive to senescence. The rough microsomal fraction is largely comprised of rough endoplasmic reticulum (3, 18). Fine structural studies have revealed that this organelle retains its structural integrity even after other organelles display morphological symptoms of deterioration (23). This suggests that the rough endoplasmic reticulum is less sensitive to senescence than are other organelles. The x-ray diffraction data for rough microsomes are consistent with this interpretation for they indicate that even by day 9 in the germination sequence only a small proportion of the lipid in these membranes is crystalline. By contrast, much more of the smooth microsomal lipid is crystalline by this stage, suggesting that the membranes comprising this fraction are more susceptible to the pressures of senescence.

The progressive transition to a crystalline phase during germination presumably reflects chemical changes in the membrane brought on by senescence. The increase in fatty acid saturation doubtless contributes to this, but the absence of a close correlation between changes in the unsaturated to saturated fatty acid ratios and changes in the transition temperature of the rough and smooth microsomes suggest that it is not the sole cause (compare Table I and Fig. 3). Furthermore, these changes in fatty acid saturation are of a much lower magnitude than those known to cause lipid changes in bacterial membranes (17).

It has been previously reported that microsomal membranes from this tissue lose enzyme activity as germination advances (18). The present study has revealed that portions of the lipid in these membrane systems become crystalline during the same period. Regions of the membrane in which the lipid is crystalline could be expected to be essentially devoid of enzyme activity since it is known that during formation of the gel phase protein is displaced into adjacent more fluid regions (27). It has been established in other systems that a decrease in membrane lipid fluidity as measured by electron spin resonance coincides with a decline in membrane enzyme activity (9, 25). It is conceivable that the changed physical state of the lipid in senescing membranes contributes to their loss of function. The presence of crystalline lipid could also account for changes in permeability, for it has been previously demonstrated that the permeability of liposomes to various ions increases at the phase transition temperature (1, 24, 31). In addition, agents such as Ca^{2+} and Mg^{2+}, which influence the phase behavior of lipid bilayers, also alter their permeability properties (4, 31).

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