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PHASE PROPERTIES OF SENESCING PLANT MEMBRANES

ROLE OF THE NEUTRAL LIPIDS

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Summary

Wide-angle X-ray diffraction studies have indicated that rough and smooth microsomal membranes from bean cotyledons acquire increasing proportions of gel phase lipid at physiological temperature as the tissue senesces. In addition, for both types of membrane the lipid phase transition temperature, defined as the highest temperature at which gel phase lipid can be detected, progressively rises with advancing senescence. Liposomes prepared from total lipid extracts of the membranes show a similar increase in transition temperature with age, indicating that separation of the polar lipids into distinct gel and liquid-crystalline domains is not attributable to peculiar protein-lipid interactions. Liposomes prepared from purified phospholipid fractions of the membranes show little change in transition temperature with age, indicating that the altered phase properties of the lipid do not reflect an increase in fatty acid saturation. However, the formation of gel phase lipid that occurs naturally during senescence can be stimulated by preparing liposomes from a mixture of the phospholipid fraction from young membrane and the neutral lipid fraction from old membrane. By adding the separated components of the neutral lipid fraction to purified phospholipid it was found that sterol esters and several unidentified lipids are able to raise the transition temperature of the polar lipids. Sterols have no effect on the phospholipid transition temperature. The data have been interpreted as indicating that several neutral lipids, which presumably increase in abundance with advancing senescence, induce a lateral phase separation of the polar lipids resulting in distinct gel and liquid-crystalline domains of lipid in the senescent membranes.

Introduction

Senescence of plant tissue is accompanied by a general metabolic decline that includes both structural and functional modifications at the level of mem-

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branes. Plasma membranes from senescent cotyledons of *Phaseolus vulgaris* have lower ($Na^+ + K^+$)-stimulated ATPase activity as well as reduced capability for ATP-dependent cation transport compared with corresponding membranes from young tissue [1]. Similarly, the cholinesterase activity of these membranes peaks and then declines with advancing senescence [2]. Rough and smooth microsomal membranes from this tissue also lose function with age. The activities of four microsomal enzymes, NADH-cytochrome *c* reductase, NADPH-cytochrome *c* reductase, glucose-6-phosphatase, and 5'-nucleotidase decline asynchronously to very low levels as the tissue senesces [3].

Senescing membranes from plant tissues have also been shown to contain gel phase lipid at physiological temperatures [4—7]. Furthermore, the appearance of the gel phase can be correlated with several anatomical and biochemical symptoms of senescence [5—7]. For example, the lipid matrix of rough and smooth microsomal membranes from *P. vulgaris* cotyledons is exclusively liquid-crystalline in young tissue, but forms gel-phase domains in older tissue which increase in proportion, relative to liquid-crystalline domains, as senescence intensifies [5]. Chloroplast and microsomal membranes from the senescent primary leaf of *P. vulgaris* also contain regions of gel-phase lipid whose appearance correlates temporally with the initiation of chlorophyll and protein degradation [6]. Gel-phase lipid has also been detected in smooth micorsomal membranes isolated from aged batch cultures of the green alga *Scenedesmus quadricauda* [7].

These altered phase properties of membranes from senescing tissues cannot be accounted for by changes in either fatty acid saturation [5—8] or phospholipid composition [8]. However, it remains possible that as senescence advances there is a lateral rearrangement of individual phospholipid molecules with inherently different fluidities which leads to formation of separate gel and liquid-crystalline domains. In the present study this prospect has been examined by comparing the phase properties of liposomes prepared from lipid fractions of microsomal membranes from cotyledon tissue at various stages of senescence.

Materials and Methods

Untreated seeds of *P. vulgaris*, variety kinghorn, were germinated in moist vermiculite under etiolating conditions at 29°C. Cotyledons were harvested at specified intervals after planting, and smooth and rough microsomes isolated as previously described [5]. Lipids were extracted from the membrane pellets using the procedure of Nichols [9] as outlined previously [8].

The total lipid extract was separated into three fractions on a 1.1 × 5 cm silica gel 60 (BDH Chemicals) column, using the method of Rouser et al. [10]. Approximately 50 mg total lipid were applied to the column in chloroform solution and eluted sequentially with 75 ml chloroform, 50 ml acetone and 75 ml methanol, at a flow rate of 1 ml/min, to yield neutral lipid, glycolipid and phospholipid fractions, respectively. Each fraction was evaporated to dryness, resuspended in 1 ml of chloroform/methanol (2 : 1, v/v) and stored under N_2 at −20°C. The purity of each fraction was checked by thin-layer chromatography and by phospholipid-P analysis [8].

The neutral lipid fraction obtained from the Silica gel column was further fractionated by thin-layer chromatography. Initially, a qualitative analysis was carried out using a two-dimensional developing system. Approximately 50 μg of the neutral lipid fraction were spotted onto a heat-activated Baker-flex 1B2-F plate (J.T. Baker) and developed in the first direction with light petroleum/diethyl ether/acetic acid (70 : 30 : 2, v/v) and in the second direction with benzene/diethyl ether/ethyl acetate/acetic acid (80 : 10 : 10 : 0.2, v/v). The plate was air dried and the separated components visualized by spraying with 50% sulphuric acid and charring at 120°C.

Preparative thin layer chromatography was carried out by streaking approximately 10 mg neutral lipid onto a thin layer chromatography plate and developing in one direction with light petroleum/diethyl ether/acetic acid (70 : 30 : 2, v/v). A longitudinal strip was cut from the plate, sprayed with 50% sulphuric acid and charred at 120°C. The separated lipids were located on the remaining, untreated portion of the thin layer chromatography plate by using the sprayed strip as a reference. The appropriate R_F zones were scraped from the plate and the lipids were eluted from the Silica gel with two 4-ml washes of chloroform followed by a 4 ml wash of methanol.

Liposomes for X-ray diffraction analysis were prepared from the total lipid extracts and separated lipid fractions using the basic procedure of Demel et al. [11]. Approximately 5 mg of the lipid sample were dried on to the sides of a conical reaction vial under N_2; residual solvent was removed in a vacuum dessicator. The dried lipid was weighed and an equivalent weight of 40 mM Tris/acetate buffer, pH 7.0, containing 100 mM NaCl was added. The sample was mixed and allowed to equilibrate at room temperature. To confirm that lipid samples prepared in this manner were fully hydrated, liposomes were also prepared by resuspending 5—10 mg lipid in 4 ml Tris buffer and shaking for 1 h at a temperature above the phase transition. The resulting liposomes were then removed from suspension by centrifuging the sample in a Beckman Type 50 Ti rotor at 165 000 $\times g$ for 1 h. The two methods yielded liposomes with essentially similar transition temperatures.

For X-ray diffraction a portion of the hydrated lipid sample, prepared by either method, was transferred to a sealed sample holder and placed in the temperature controlled jacket of the X-ray diffraction camera. Transition temperatures were determined as described previously for membrane samples [5].

Results

It has been previously reported for this tissue that the phase transition temperature of rough and smooth microsomes rises during senescence from a point well below physiological temperature for young membranes to a point well above physiological temperature for senescent membranes [5]. These data have been summarized in Figs. 1 and 2 and compared with corresponding data obtained from liposomes of extracted lipids from the membranes.

The rise during senescence in the transition temperature for liposomes of total lipid extracts from smooth microsomes was essentially similar to that for the intact membranes (Fig. 1). At 2 days of age a liquid-crystalline to gel-phase

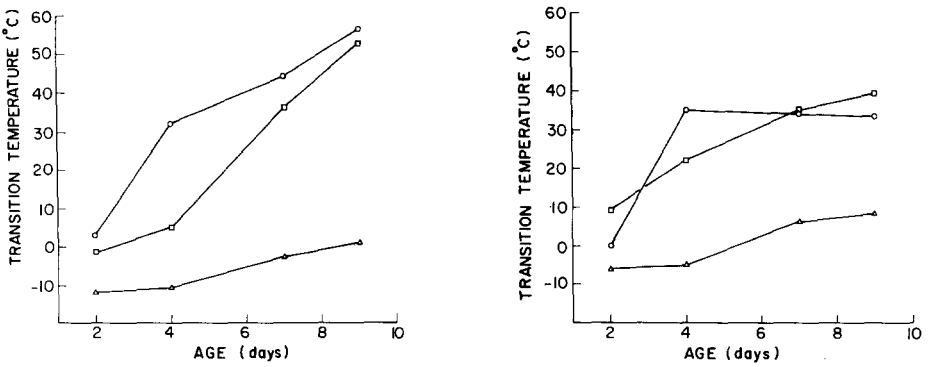

Fig. 1. Changes during senescence in the lipid phase transition temperature for smooth microsomal membranes from senescing cotyledons of *P. vulgaris* and for liposomes of their extracted lipids. ○, intact membranes; □, liposomes of total lipid extracts from the membranes; △, liposomes of purified phospholipid from the membranes. The phase transition temperature is defined as the highest temperature at which gel phase lipid can be detected by wide-angle X-ray diffraction. Values for the membranes are summarized from ref. 5. Values for total lipid extracts are means of 3—5 determinations; standard errors are less than 3.0. Values for purified phospholipid are means of duplicate determinations; duplicates had a range of less than 4°C.

Fig. 2. Changes during senescence in the lipid phase transition temperature for rough microsomal membranes from senescing cotyledons of *P. vulgaris* and for liposomes of their extracted lipid. ○, intact membranes; □, liposomes of total lipid extracts from the membranes; △, liposomes of purified phospholipid from the membranes. The phase transition temperature is defined as the highest temperature at which gel phase lipid can be detected by wide-angle X-ray diffraction. Values for the membranes are summarized from ref. 5. Values for total lipid extracts are means of 3—5 determinations; standard errors were less than 3.2. Values for purified phospholipid are means of duplicate determinations; duplicates had a range of less than 4°C.

transition was initiated at −2°C for total lipid and at 3°C for the smooth membranes, and at 9 days of age the transitions occurred at 52°C for total lipid and 56°C for the membranes. Only at day 4 of the ages tested did the patterns of change in transition temperature diverge; the liquid-crystalline to gel-phase transition was initiated at 32°C for 4-day-old membrane and at only 5°C for corresponding total lipid extracts (Fig. 1).

Similar data were obtained for the rough microsomal membranes. In this case the transition temperature for the intact membranes rose from a low of 0°C at day 2 to 35°C by day 4, and thereafter remained essentially constant. By contrast, the transition temperature for liposomes of corresponding total lipid extracts rose in approximately linear fashion from 9°C at day 2 to 38°C at day 9. Nonetheless, the transition temperatures for membrane and corresponding total lipid were closely similar at all ages tested apart from day 4, where the transition was initiated at 35°C for membrane and at only 22°C for total lipid (Fig. 2).

It was apparent, therefore that for both rough and smooth microsomes the rise in transition temperature with age is largely attributable to changes within the membrane lipid rather than to an influence of protein on the lipid. To investigate this further, the total lipid extract was separated into three fractions using a Silica gel column, and each of the fractions was hydrated and examined by X-ray diffraction. The first fraction, which was eluted from the column with chloroform, contained the neutral lipids [10] and pigments. When this sample

was hydrated liposomes did not form, as evidenced by the failure of the sample to form a stable suspension in buffer when shaken, and by the absence of both the 4.6 and 4.15 Å bands in the X-ray diffraction patterns. Instead, the patterns displayed a series of sharp reflections at 4.0, 4.7, 5.9, 8.2 and 26 Å.

The second fraction, which was eluted with acetone, normally contains glycolipids [10], but when examined by thin layer chromatography was found to be contaminated with pigments and a few neutral lipids. Consequently, the acetone elution step was used primarily as a wash to ensure complete removal of all neutral lipids and pigments from the column prior to elution of the phospholipids. This glycolipid fraction did, however, produce liposomes when hydrated, and diffraction patterns for this fraction displayed the typical 4.6 Å and 4.15 Å reflections attributable to a lamellar structure. However, additional sharp rings were detected for some samples at Bragg spacings of 4.7, 5.3, 6.3 and 15 Å. Furthermore, the transition temperatures for this fraction proved to be variable, ranging in a random fashion between 10 and 50°C for all ages of tissue.

The third fraction, which was eluted from the column with methanol, contained the phospholipids [10] as evidenced by thin layer chromatography and phospholipid-P analysis. This fraction readily formed liposomes when hydrated and consistently yielded only the 4.6 and 4.15 Å reflections in X-ray diffraction patterns. Moreover, for the purified phospholipid from both rough and smooth microsomes there was relatively little change in transition temperature with advancing senescence (Figs. 1 and 2). For purified phospholipid from 2-day-old smooth microsomes a liquid-crystalline to gel-phase transition was initiated at −12°C, approximately 10°C below the temperature at which the same transition was observed for corresponding total lipid extract, and then rose only slightly to reach a high of 1°C by day 9 (Fig. 1). By comparison, the transition temperature for the total lipid extract from 9-day-old smooth microsomes was 52°C (Fig. 1). Similarly, the transition temperature for phospholipid from rough microsomes rose only slightly with advancing senescence from −6°C at day 2 to 8°C by day 9, a temperature 31°C lower than that at which the same transition was initiated for the total lipid extract from 9-day-old rough microsomes.

In another series of experiments liposomes prepared from a mixture of purified phospholipid from 2-day-old smooth microsomes and purified neutral lipid from 9-day-old smooth microsomes were examined by X-ray diffraction. At 29°C, the temperature at which the seeds were germinated, liposomes from the purified phospholipid yielded only a diffuse lipid reflection centered at a Bragg spacing of 4.6 Å (Fig. 3A), indicating that the hydrocarbon side chains are in a liquid-crystalline phase [5]. However, when neutral lipid from 9-day-old membrane was mixed in at a concentration of 50% by weight, portions of the phospholipid became crystalline as evidenced by the sharp 4.15 Å ring superimposed on the outer edge of the broad 4.6 Å band (Fig. 3B). In fact this diffraction pattern is essentially indistinguishable from that obtained for intact smooth microsomal membranes from 9-day-old cotyledons (Fig. 3C). Both patterns feature a sharp 4.15 Å band representing an ordered crystalline phase of the lipid in which there is hexagonal packing of the fatty acid chains, and a less intense band at a Bragg spacing of 3.75 Å representing an alternative

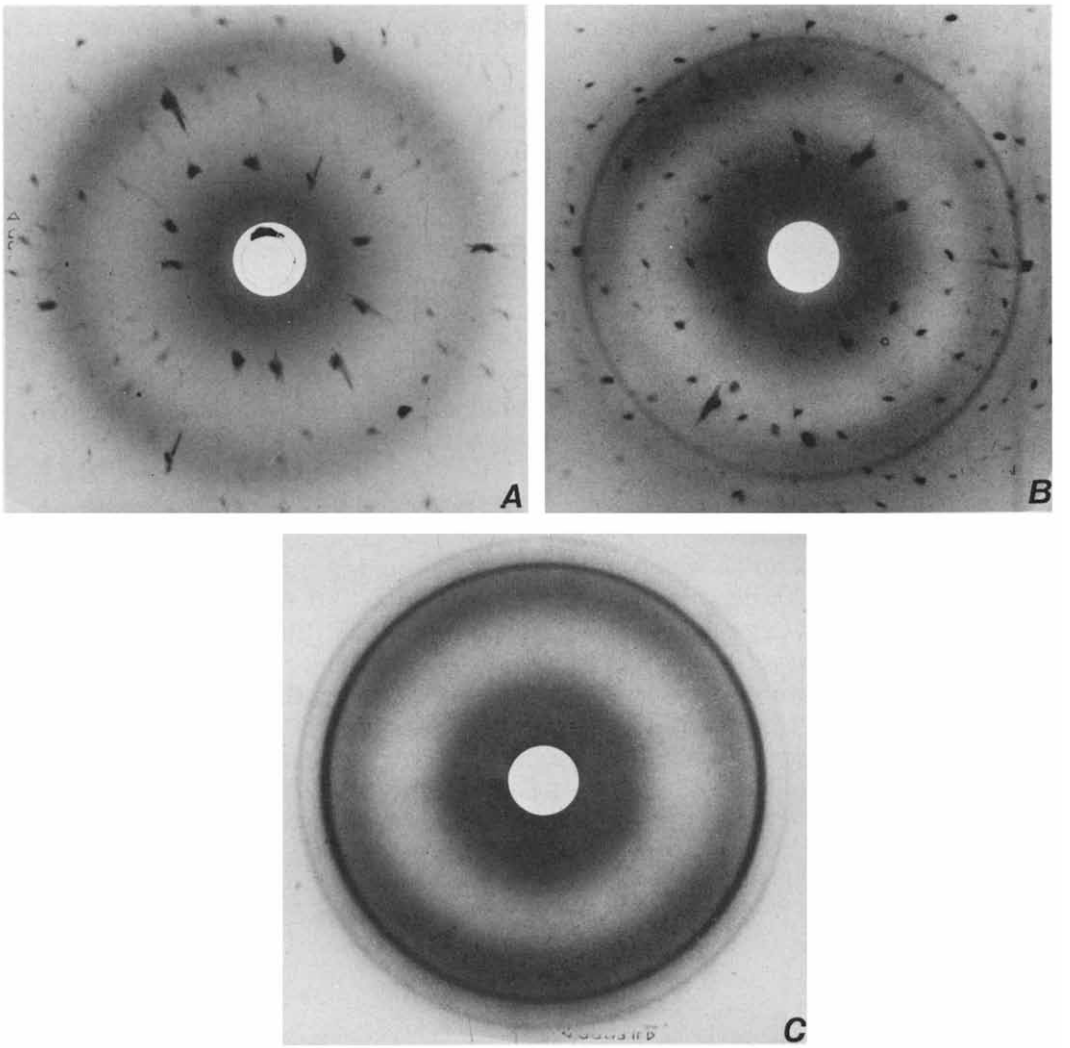

Fig. 3. Wide-angle X-ray diffraction patterns recorded at 29°C for smooth microsomal membranes and for liposomes prepared from lipid fractions of smooth microsomal membranes. A, pattern for liposomes of purified phospholipid from 2-day-old membrane showing (from outside to inside) diffuse bands at Bragg spacings of 4.6 and about 10 Å; B, pattern for liposomes of purified phospholipid from 2-day-old membrane containing 50% (by weight) neutral lipid from 9-day-old membrane which shows (from outside to inside) sharp bands at Bragg spacings of 3.75 and 4.15 Å and diffuse bands at Bragg spacings of 4.6 and about 10 Å; C, pattern for smooth microsomal membranes from 9-day-old tissue from ref. 4 included for comparison. The spots in patterns A and B derive from the mica window of the sample holder used for liposomes.

crystalline state of the lipid in which there is an orthorhombic packing of the hydrocarbon chains [4,5].

To further examine this effect of neutral lipid on the phase properties of phospholipid, transition temperatures were determined for liposomes containing phospholipid from 2-day-old smooth microsomes and varying proportions of neutral lipid from either 2- or 9-day-old smooth microsomes. With no

neutral lipid present the transition from the liquid-crystalline to gel phase was initiated at −12°C (Fig. 4). As the proportion of 9-day-old neutral lipid was increased, the transition temperature rose such that for liposomes containing 50% neutral lipid and 50% phospholipid by weight, the transition temperature was 42°C. The addition of neutral lipid from 2-day-old smooth microsomes also raised the transition temperature but to a lower extent (Fig. 4). Similar results were obtained using neutral lipid from rough microsomes.

The composition of the neutral lipid fraction was examined by two-dimensional thin layer chromatography. Neutral lipid from 2-day-old smooth microsomes separated into at least 16 distinguishable components (Fig. 5), and that from 2-day-old rough microsomes was found to have a comparable degree of complexity. Sterol esters, triglycerides, 1,2-diglycerides and free sterols were identified by co-chromatography with authentic standards. The other separated components were not identified. Patterns of separation for neutral lipid fractions from microsomes of older tissue were even more complex, and sterol esters, triglycerides, 1,2-diglycerides and free sterols were again among the components separated.

In order to determine which of these neutral lipids contribute to induction of the gel phase when mixed with phospholipid, 10 mg of neutral lipid obtained from 2-day-old smooth microsomes were fractionated by one-dimensional thin layer chromatography into 10 distinct R_F bands as detailed in Fig. 5. (Young tissue rather than old tissue was used as the source of neutral lipid in these experiments because of the lower yield of microsomal membranes from the older tissue.) The lipid from each R_F band was eluted and mixed with 4 mg of purified phospholipid from 2-day-old smooth microsomes. Liposomes were then prepared from these mixtures and examined by wide-angle X-ray

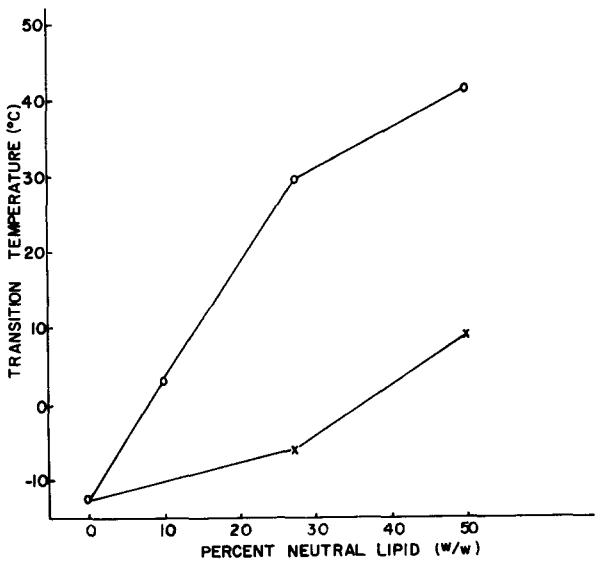

Fig. 4. Influence of 2-day-old (X) and 9-day-old (○) smooth microsomal neutral lipid fraction on the lipid phase transition temperature of 2-day-old smooth microsomal phospholipid. Each value is the mean of duplicate determinations; duplicates had a range less than 3°C.

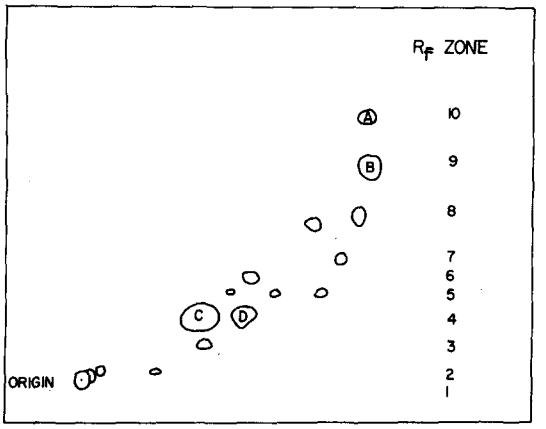

Fig. 5. Two-dimensional thin-layer chromatographic separation of 2-day-old smooth microsomal neutral lipids. Spots were visualized by sulphuric acid charring and identified by co-chromatography with lipid standards. A, sterol esters; B, triglyceride; C, free sterols; D, 1,2-diglyceride. R_F zones numbered 1—10 represent the bands obtained by preparative thin layer chromatography that were used in the phase transition temperature experiments described in Table I.

diffraction. Diffraction patterns were recorded at −5°C, a temperature just above the phase transition temperature of the pure phospholipid, in order to score for the presence or absence of the sharp ring at 4.15 Å which indicates the presence of gel-phase lipid. When the separated neutral lipids from 2-day-old smooth microsomes were tested, it was found that the components of R_F regions 1, 2, 8 and 10 induced a gel phase in the phospholipid (Table I). The identity of all the components in regions 1, 2 and 8 has not been established, although region 1 contains pigment and region 8 contains free fatty acids.

TABLE I

PHASE PROPERTIES OF LIPOSOMES PREPARED FROM PURIFIED PHOSPHOLIPID AND SEPARATED NEUTRAL LIPIDS FROM 2-DAY-OLD SMOOTH MICROSOMES

Liposomes were prepared from 2-day-old smooth microsomal phospholipid and separated 2-day-old smooth microsomal neutral lipids as detailed in the text. The bands numbered 1 to 10 are those obtained by one-dimensional thin layer chromatography as specified in Fig. 5. R_F values of the separated bands are indicated in parentheses. Lipid phases were determined by wide-angle X-ray diffraction.

Neutral lipids added to the phospholipid	Phases detected at −5° C
None	Liquid-crystalline
Band 1 (0)	Liquid-crystalline and gel
Band 2 (0.04)	Liquid-crystalline and gel
Band 3 (0.08)	Liquid-crystalline
Band 4 (0.18)	Liquid-crystalline
Band 5 (0.24)	Liquid-crystalline
Band 6 (0.31)	Liquid-crystalline
Band 7 (0.37)	Liquid-crystalline
Band 8 (0.49)	Liquid-crystalline and gel
Band 9 (0.63)	Liquid-crystalline
Band 10 (0.76)	Liquid-crystalline and gel
10 mol% stigmasterol * +10 mol% β-sitosterol *	Liquid-crystalline

* From Applied Science Laboratories.

Region 10 contains sterol esters (Fig. 5). Region 4, which contains the free sterols and 1,2-diglycerides had no effect; nor did the collective addition of 10 mol% β-sitosterol and 10 mol% stigmasterol (from Applied Science Laboratories) (Table I). These relative proportions of sterol and phospholipid are comparable to those occurring naturally in senescent microsomal membranes [8]. The components of the other R_F regions were also incapable of inducing a gel phase (Table I). Similar results were obtained when the separated neutral lipids from 2-day-old rough microsomes were tested.

Discussion

Wide-angle X-ray diffraction studies of rough and smooth microsomal membranes from senescing cotyledons of *P. vulgaris* have provided evidence for a lipid phase separation in senescent membranes leading to formation of separate gel and liquid-crystalline domains at physiological temperature [4,5]. Several factors are known to influence the phase behaviour of phospholipids, including associated proteins [12], divalent cations [13], fatty acid saturation and phospholipid head group [14,15] and sterol concentration [16]. To ascertain which of these factors might be responsible for inducing a phase separation in senescing membranes, it was first necessary to establish whether the changes in phase behaviour resulted from alteration of the membrane lipid itself or from its interaction with protein or some other extrinsic factor, such as ions. This was accomplished by preparing liposomes from total extracts of rough and smooth microsomes, thereby removing any effects attributable to the presence of protein or ions.

The rise with age in the transition temperature for such liposomes from both rough and smooth microsomes was comparable in magnitude to that observed for the intact membranes. Thus, it is clear that the senescence-related phase separations within these membranes are largely attributable to changes in the lipid moiety itself. A slight effect of protein on the phase behaviour of the lipid is evident from a detailed comparison of the transition temperatures for liposomes and corresponding microsomal membranes. For example, the transition temperatures of smooth microsomes were about 5°C higher than those for corresponding lipid extracts for most ages of tissue, indicating a slight ordering effect by the protein. The transition temperatures for rough microsomes from 2- and 9-day-old tissue were lower than those for corresponding liposome preparations suggesting that the proteins can also perturb the phospholipid bilayer.

At day 4, however, for both rough and smooth microsomes there is a pronounced drop in the transition temperature for liposomes relative to intact membrane. This could reflect a specific effect of protein on the lipid which is lost during the latter stages of germination, but there is an alternative explanation. Anatomical studies indicate that senescence proceeds asynchronously in the storage cells of the cotyledon [17,18]. Consequently, at day 4 a few cells are in the late stage of senescence, but most are structurally intact. Granted the assumption that the gel phase originates from these few senescent cells, the total amount of gel phase present in a microsomal preparation, although detectable by X-ray diffraction, would be relatively small. When the lipid is extracted,

this small amount of the gel-phase lipid is greatly diluted by the liquid-crystalline lipid from non-senescent cells. By reason of this dilution, gel-phase domains may not reaggregate when liposomes are formed. This could readily account for the lower transition temperature of liposomes compared to intact membranes at this age, and would preclude a major protein effect.

The changes in saturation of total lipid and individual phospholipids are of insufficient magnitude to account for the altered phase behaviour of these membranes [8]. There are, however, pronounced differences in degree of saturation among the different classes of phospholipids in these membranes which are maintained during senescence [8]. This suggests that the formation of crystalline lipid in the older membranes could reflect a rearrangement of phospholipids within the membrane matrix whereby those of least fluidity are induced to cluster forming a gel-phase domain. This could occur by one of two possible mechanisms. One or more neutral lipid components, which increase in abundance as senescence advances, could selectively interact with the phospholipids and induce a lateral phase separation. Alternatively, the liquid-crystalline phase may be imposed in the membranes of young tissue by the presence of perturbing agents which prevent phospholipid interaction. As the tissue senesces, these agents may be selectively removed from the membrane allowing the more saturated phospholipids to interact and form the gel phase.

The data obtained in the present study support the first mechanism. Of particular significance is the observation that the purified phospholipids from both rough and smooth microsomes of young tissue have a very low phase transition temperature which rises only slightly with advancing senescence. This implies that the dramatic rise in transition temperature with age for total lipid extracts and intact membranes reflects the character of the non-phospholipid components of the membranes. The gel phase does originate from the phospholipid molecules, however, as evidenced by the failure of the neutral lipid fraction to form a stable lamellar structure. Finally, by means of an in vitro system, in which phospholipid from young membrane is mixed with neutral lipid from old membrane, it has been possible to simulate the formation of crystalline lipid that occurs naturally in membranes as they age. It seems likely, therefore, that one or more components of the neutral lipid fraction, which increase in abundance with advancing senescence, induce a rearrangement of the phospholipids resulting in a mixture of gel and liquid-crystalline domains.

It has been previously reported for this tissue that the sterol : phospholipid ratio rises during senescence by about 50% for rough microsomes and by more than 400% for smooth microsomes [8]. However, it is clear from the in vitro experiments of the present study, in which separated neutral lipids were added back to purified phospholipid from young membrane, that the sterols do not increase the phase transition temperature of the polar lipids. Even the addition of purified stigmasterol and β-sitosterol, the two sterols largely accounting for the increase in sterol : phospholipid ratio [8], at concentrations approximating those known to occur naturally in the senescent membranes did not increase the transition temperature of purified phospholipid from 2-day-old tissue. Indeed, it would appear that the formation of gel phase lipid in senescing membranes is not attributable to any single neutral lipid interacting with the phospholipid since, in the in vitro experiments, several of the separated

neutral lipids including sterol esters and some unidentified lipids were able to raise the transition temperature of phospholipid. Thus, the formation of separate gel and liquid-crystalline domains in the senescent membranes must reflect the cumulative interactions of several neutral lipids with the polar phospholipids.

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