Changes in the Physical State of Membrane Lipids during Senescence of Rose Petals

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ABSTRACT

Changes in the physical state of microsomal membrane lipids during senescence of rose flower petals (Rosa hyb. L. cv Mercedes) were measured by x-ray diffraction analysis. During senescence of cut flowers held at 22°C, lipid in the ordered, gel phase appeared in the otherwise disordered, liquid-crystalline phase lipids of the membranes. This was due to an increase in the phase transition temperature of the lipids. The proportion of gel phase in the membrane lipids of 2-day-old flowers was estimated as about 20% at 22°C. Ethylene may be responsible, at least in part, for the increase in lipid transition temperature during senescence since aminoxyacetic acid and silver thiosulfate inhibited the rise in transition temperature. When flowers were stored at 3°C for 10 to 17 days and then transferred to 22°C, gel phase lipid appeared in membranes earlier than in freshly cut flowers. This advanced senescence was the result of aging at 3°C, indicated by increases in membrane lipid transition temperature and ethylene production rate during the time at 3°C. It is concluded that changes in the physical state of membrane lipids are an integral part of senescence of rose petals, that they are caused, at least in part, by ethylene action and that they are responsible, at least in part, for the increase in membrane permeability which precedes flower death.

Senescence of rose petals involves a climacteric-like rise in ethylene production followed by increased membrane permeability to electrolytes, petal withering, and death (10). Ethylene action is, at least in part, responsible for the increased membrane permeability which presumably leads to death (10, 20). During plant senescence critical changes occur in the physical state of membrane lipids which affect the functional integrity of membranes and lead to cell death (7, 16, 22). Hence we wished to further characterize rose petal senescence in terms of changes in the physical state of membrane lipids.

Two different, but related, changes in physical properties of microsomal membranes have been reported in senescing rose petals: an increase in microviscosity and a corresponding decrease in fluidity (5, 13); and an increase in lipid phase transition (gel to liquid-crystalline) temperature (13). However, the interrelationships between changes in membranes and other senescent processes have not been investigated. For example, it is not known whether the membrane changes precede, or are a result of, the increase in ethylene production. It is known that ethylene increases membrane permeability in rose petals (10) and that changes in the physical state of membrane lipids can increase membrane permeability (2, 8). Therefore, it is reasonable to propose that ethylene may alter the physical properties of rose petal membranes and hence increase their permeability.

In the work reported here we investigated changes in the physical state of microsomal membrane lipids during senescence of rose petals. The physical state of lipids was measured by a direct method, x-ray diffraction analysis, which reveals the phase properties and ordering of the lipid acyl chains (14). The changes in the membrane lipids have been related to other senescence processes (ethylene production and membrane permeability) during senescence, and after treatments which hastened or slowed senescence. In particular, the possible role of ethylene in regulating changes in membrane lipids was investigated.

MATERIALS AND METHODS

Flowers and Treatments. Flowers of the rose (Rosa hyb. L.) cv Mercedes were grown, cut, and treated as described previously (11). For measurements of aging and senescence, flowers were held with stems in water at 22°C, 70% RH, and continuous white fluorescent light of 3 W·m⁻² irradiance. For storage at 3°C, flowers were held without water at 95% RH. Flowers were treated with ethylene at 6 μL·L⁻¹ for 22 h at 22°C by diluting pure ethylene in a stream of air as described by Saltveit (19). Flowers were treated with AOA³ and STS by supplying solutions to the stems at 22°C, AOA at 2 mm for 2 h and STS at 4 mM for 0.5 h.

Preparation of Microsomal Membranes. Microsomal membranes were extracted from petals of 10 flowers as described previously (11). For x-ray diffraction analysis the membrane pellet was equilibrated at 54% RH and 22°C under N₂ for 12 h. This removal of some water from the membranes allowed better resolution of lipid diffraction rings and shorter exposure times (9). The final water content of the membranes was measured by drying the sample in vacuo for 2 h and was at least 20% w/w with respect to final dry weight. Removal of water down to the level did not alter the structures formed by plant membrane lipids in a previous study (15).

Preparation of Lipids. Lipids were extracted from the membranes by a modified Folch method (18). For x-ray diffraction analysis the chloroform extract was dried under N₂ and the lipids (5–10 mg) were hydrated by dispersing them in buffer (1 mL, 20 mM Hepes-Tris, pH 7.5). The lipids were equilibrated under N₂ for 10 min at 40°C and 100% RH for 1 h and then at 22°C and 54%

³ Abbreviations: AOA, aminoxyacetic acid; STS, silver thiosulfate.

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Wide Angle X-ray Diffraction by Membranes. For measurement of the x-ray diffraction pattern the membrane sample was placed in a 1 mm diameter lithium glass capillary and held at 22 ± 1°C, 54% RH, under helium. The x-ray source was an Elliot rotating anode generator GX6 operated at 40 kV and 30 mamp producing Cu radiation. Two Franks mirrors monochromated and focused the beam to a point, nominal diameter 200 μm. The diffraction pattern was recorded on two layers of Agfa Structurix x-ray film. The specimen to film distance was 30 mm and was calibrated with NaCl. This allowed measurement of diffraction rings with equivalent Bragg spacings of 2.6 to 20 Å. Exposure times were 18 to 24 h. Densitometer tracings were recorded with a Joyce-Loebl Mark II densitometer. Three or four replicate membrane samples, from different flowers, were measured.

Measurement of Lipid Phase Transition Temperature. Wide angle x-ray diffraction patterns showed the presence of two phases, due to different degrees of ordering of the lipid acyl chains: the disordered liquid-crystalline phase and the ordered gel phase (9, 14). The phase transition temperature is defined here as the highest temperature at which gel phase lipid could be detected. To record diffraction patterns, lipid samples were placed in a 1 mm quartz glass capillary in a temperature controlled sample holder (±0.5°C) in air. The x-ray source was a Philips sealed-tube, fine-focus, generator operated at 40 kV and 30 mamp producing Cu radiation. Monochromatization was provided by a Ni filter and one Franks mirror and the beam was collimated to 4 mm height, 300 μm width, in the plane of the specimen. The specimen to detector distance was 100 mm, calibrated with CaSO₄, and this allowed measurement of diffraction peaks equivalent to Bragg spacings of 2.5 to 29 Å. The diffraction pattern was recorded by a one-dimensional, position-sensitive electronic detector. Exposure times were 1 h and approximately 40,000 counts per channel were recorded in the region of the lipid diffraction peaks. During acquisition, the data was stored in a Z-80 based microprocessor unit and displayed on an oscilloscope. Following completion of the experiment the data were transferred to an IBM 3081 computer for further analysis. The oscilloscope display of the diffraction pattern was photographed with a Tektronix C27 oscilloscope camera and Polaroid 667 film. To determine the transition temperature the diffraction pattern of each sample was measured at several temperatures, at 4°C intervals. Three or four replicates, extracted from different flowers, were measured.

Measurement of Phase Changes in Lipids by Low-Angle X-Ray Diffraction. The lipid samples, x-ray source, and detector system were the same as those described above for measurement of transition temperature. A specimen to detector distance of 465 mm was used, allowing measurement of diffraction peaks equivalent to Bragg spacings of 18 to 124 Å. Diffraction patterns were recorded at temperatures between 6 and 50°C. Exposure times were for 0.5 h and approximately 18,000 counts per channel were measured in the region of the lipid diffraction peaks. The diffraction patterns showed two peaks, interpreted as arising from the packing of lipid lamellae (bilayers) of two different phases in the lipid acyl chains (liquid-crystalline and gel). The proportion of the lipid in each phase was estimated by calculating the integrated intensity of each diffraction peak and expressing values as a percentage of the peak at 50°C which was assumed to be from lipid of 100% liquid-crystalline phase. The integrated intensities were calculated from the diffraction data by the CURFIT computer programme (H Jaros, Weizmann Institute Computer Center) which attempts to fit either Gaussian or Lorentzian curves to the data according to a modified Marquardt procedure.

Measurement of Ethylene Production and Membrane Permeability. The ethylene production rate by petals and the membrane permeability, measured as ion leakage from petals, were measured as described previously (10).

RESULTS

Lipid Phases in Membranes. Typical wide-angle x-ray diffraction patterns from microsomal membranes are shown in photographs of the film (Fig. 1) and in densitometer traces of the film (Fig. 2). The effect of sample temperature was examined to see whether the lipids changed phase with temperature as expected. At 50°C only a broad, diffuse diffraction band at a Bragg spacing of about 4.7 Å was present (Figs. 1A and 2A). This is indicative of lipid acyl chains in the disordered, liquid-crystalline phase (9, 14). At 6°C an additional, narrow, sharp diffraction ring at 4.2 Å was present (Figs. 1B and 2B). This is indicative of lipid acyl chains in the ordered, rigid, gel phase (9, 14).

Changes in Lipid Phases in Membranes during Flower Senescence. The presence of each lipid phase in microsomal membranes was measured as flowers aged at 22°C. In freshly cut young flowers (Fig. 2C) and during the next 3 d only liquid-crystalline phase was present in membrane samples measured at 22°C (Fig. 3A). On the fourth and later days gel phase lipid was also present (Fig. 3A). This is also depicted in the densitometer tracing by the appearance of a peak reflecting a diffraction ring at a Bragg spacing of 4.2 Å (Fig. 2D). The appearance of gel phase lipid followed a rise in petal ethylene production and preceded the increase in ion leakage from petals (Fig. 3A). Rose flower senescence at 22°C is advanced by a period of prior cold storage of 3°C (10). In flowers which had been stored for 10 or 17 d at 3°C and then transferred to 22°C, gel phase lipid was present in the microsomal membranes 2 or 4 d earlier than in freshly cut flowers (Fig. 3, B and C). Gel phase lipid was present immediately after 17 d at 3°C (Fig. 3C). In all flowers, the appearance of gel phase lipid coincided with a rise in ethylene production and preceded the increase in ion leakage (Fig. 3, B and C).

Changes in Lipid Phase Transition Temperature during Flower Senescence. A phase transition temperature, defined as the highest temperature at which gel phase lipid could be detected, was measured in lipids isolated from membranes sampled during senescence of the flowers. The diffraction patterns of lipids dispersed in water were measured at 4°C intervals, with a one-dimensional electronic detector and displayed on an oscilloscope.

Fig. 1. Wide-angle x-ray diffraction patterns from microsomal membranes of rose petals. Diffraction patterns were measured with membrane sample held at 50°C (A) and 6°C (B) and were recorded on film. The general characteristics are a broad, diffuse band centered at a Bragg spacing of about 4.7 Å (50°C) and an additional sharp, intense ring on the outside of the diffuse band, at about 4.2 Å (6°C). Print magnification x1.5.
which smaller (4, 9).

In the example illustrated in Figure 4 the transition temperature, as defined in "Materials and Methods," was 26°C. When flowers were aged at 22°C or stored at 3°C, the phase transition temperature of microsomal membrane lipids increased with time, but more slowly at 3°C (Fig. 5A). At both temperatures the increase in transition temperature clearly preceded the rise in petal ethylene production (Fig. 5).

Role of Ethylene in Changes in Membrane Lipids during Senescence. In untreated (control) flowers held at 22°C the membrane lipid transition temperature increased with age for 4 d (Fig. 6). Applied ethylene, at a concentration of 6 μL·L⁻¹, had little effect on the transition temperature. However, both AOA, the inhibitor of ethylene synthesis (1), and STS, the inhibitor of ethylene action (23), inhibited the rise in transition temperature.

Determination of Relative Proportions of Lipid Phases in Membranes. It was of interest to determine what proportions of the membrane lipids were in each of the liquid crystalline and gel phases, both at the physiological temperature of 22°C and at the measured transition temperature. This could not be calculated readily from the wide-angle diffraction peaks, since the structure factors for the two peaks do not have the same form and the peaks overlap so extensively that the integrated intensity of each peak could not be determined. An alternative approach, using low-angle x-ray diffraction analysis, was taken. This was used to measure the interbilayer spacing of isolated membrane lipids which were believed to be in a multilamellar arrangement (4, 9). The interbilayer spacing depends on bilayer thickness, which in turn depends on the phase of the lipid acyl chains. A smaller interbilayer spacing results from a thin bilayer when the

FIG. 2. Densitometer scans of wide angle diffraction patterns from membranes. Diffraction patterns were measured as described in Figure 1 and one-half of the film was scanned by densitometer. A, Membranes measured at 50°C; B, membranes at 6°C; C, membranes of freshly cut (young) flowers measured at 22°C; D, membranes of flowers aged 6 d at 22°C (old) measured at 22°C; E, as D, but lipids isolated from membranes.

FIG. 3. Changes in lipid phases in membranes during flower aging at 22°C. Flowers were either freshly cut (A), or had been held at 3°C for 10 d (B) or 17 d (C). The presence (+) or absence (−) of gel phase lipid in membranes at 22°C was measured by wide-angle x-ray diffraction, in four replicate membrane extracts and the result (+ or −) found in the majority of extracts (always 75 or 100%) is shown. Ethylene production rate of petals (O) and ion leakage from petals (C) were measured. Values are means of 7 replicates ± se; se of ethylene values are only shown where they exceeded the size of the symbols.

FIG. 4. Determination of lipid phase transition temperature. The diffraction patterns of hydrated membrane lipids were measured at several temperatures, recorded by a one-dimensional position-sensitive detector and the signal displayed on an oscilloscope. Photographs of the oscilloscope display are shown. The flowers used had been held at 22°C for 2 d.
Fig. 5. Changes in membrane lipid phase transition temperature and petal ethylene production at 3 and 22°C. Values for transition temperature and ethylene are means of 4 and 10 replicates, respectively, ± SE.

Fig. 6. Effects of ethylene and inhibitors of ethylene synthesis and action on changes in membrane lipid transition temperature, during flower aging at 22°C. Flowers were treated as described below and then held at 22°C. (O), Untreated control (CONT); Ethylene, 6 μL·L⁻¹, applied for 24 h at 22°C (C₂H₄). Aminooxyacetic acid, 2 mM, supplied to the stems for 2 h, at 22°C (AOA); Silver thiosulfate, 4 mM, supplied to the stems for 0.5 h at 22°C (STS). Values are means of 4 replicate extractions ± se. Where there are no se bars the se was zero.

Acyl chains are in the disordered, liquid-crystalline phase and a wider spacing, from thicker bilayers, when the acyl chains are ordered and extended in the gel phase (9, 14).

The low-angle diffraction patterns of lipids from flowers aged for 2 d at 22°C were measured. At 50°C there was a single strong peak at a Bragg spacing of 43.4 Å (Fig. 7). At 22°C and lower temperatures there was an additional peak at a greater spacing and this was interpreted as resulting from the presence of gel phase lipid in the bilayer. To estimate the proportion of lipid in each phase the integrated intensities of each peak were calculated and the proportion in the liquid-crystalline phase was calculated as described in “Materials and Methods.” Representative data of an experiment repeated three times are shown in Figure 8. At 50°C the lipid was assumed to be 100% in the liquid-crystalline phase. At the first measurable observation of two phases, at 26°C, 20% of the lipid was in the gel phase. As the temperature decreased less lipid was in the liquid crystalline phase and more in the gel phase. Phase separation, the coexistence of two phases, occurred over a wide temperature range from 26°C or higher to less than 6°C. At the physiological temperature of 22°C about 20% of the lipids were in the gel phase (Fig. 8). The proportion of gel phase varied between 16 and 22% in the various experiments conducted.

**DISCUSSION**

During aging and senescence of rose petals, gel phase lipid forms in the microsomal membranes as a result of some process which leads to increased lipid phase transition temperature. This is likely to lead to malfunction of the membranes and subsequent senescence and death. Such formation of gel phase lipid in cell membranes is characteristic of senescence in several leaves, cotyledons, and fruits (22). It also occurs in petal microsomal membranes of another rose cultivar (13). In that work with intact membranes it was possible that gel phase lipid was influenced by other membrane components such as protein. In the present...
work with isolated lipids the increase in lipid phase transition temperature was shown to be due to changes in the lipids themselves.

The appearance of gel phase lipid in membranes at 22°C was evident from the appearance and the increasing intensity of the gel phase diffraction ring during aging of flowers. Legge et al. (13) also observed this increase in gel lipid with flower age. An increase in gel phase in the heterogenous membrane lipids at 22°C can also be inferred from the increase in the phase transition temperature of the isolated membrane lipids with flower age (14, 16, 22). An apparent discrepancy as to the time at which gel phase lipid at 22°C was observed (compare data in Fig. 3 with data in Figs. 7 and 8). We suggest this may be best explained by the variation of methodology used; wide angle x-ray coupled with detection by film (Fig. 3) versus low angle x-ray coupled with detection by one-dimensional electronic detector (Figs. 7 and 8). Also better resolved diffraction patterns were achieved with lipid preparation compared with membrane preparations.

It is of interest to know the proportion of the membrane lipids which are in the gel phase. For example, as Legge et al. (13) suggested, there may be only a small amount of boundary lipid around proteins present in the gel phase. However, our measurements by low-angle x-ray diffraction techniques have shown that even in relatively young flowers (2 d at 22°C) there was approximately 20% of the lipid in the gel phase at the physiological temperature of 22°C. In membranes the proportion of lipid in the gel phase is likely to be a few percent greater than the 20% measured in isolated lipids, since the phase transition temperature is likely to be a few degrees higher (22). To the best of our knowledge this is the first estimate of the relative amounts of different lipid phases in membranes of higher plants at physiological temperatures. In Vigna radiata it was estimated by scanning calorimetry that about 7% of the leaf polar lipids were in the gel phase at the transition temperature of −7°C (17). For comparison, E. coli can live normally with 20% of membrane lipids in the gel phase but growth stopped when there was 55% (12).

The increase in lipid phase transition temperature, the presence of gel phase lipid, and the increase in lipid microviscosity (11) are the first observed changes during aging and senescence of rose petals. The presence of gel phase lipid and phase separation in a membrane is likely to cause increased permeability of the lipid bilayer to ions and small molecules (2, 9). This could be the cause of the observed increase in membrane permeability in rose petals if the observed phase separation occurred primarily in either the plasma membrane or tonoplast. At the same time as gel phase forms in the microsomal membranes there is a parallel change in the related, but different, physical property of the membrane lipids: lipid microviscosity increases (11) and conversely lipid fluidity decreases (13). Increased microviscosity was also suggested to lead to membrane malfunction and death (7). There might also be a possibility, not addressed by our experiments, according to which the content and composition of microsomal membranes, included in our preparations, varied with the age of the flower petals. Hence, our data may not reflect the proportion of lipid in gel phase in young petals.

Although the changes in the physical parameters measured in rose petal membranes precede the onset of the climacteric rise in ethylene production, ethylene nevertheless appears to influence their development. This proposition is supported by the observation that inhibitors of ethylene synthesis or action delayed the rise in lipid transition temperature. A similar observation has been reported in carnation petals where ethylene advanced the increase in membrane microviscosity (22). This may suggest that a general mechanism of ethylene action may be to alter the physical state of membrane lipids. This would be likely to involve changes in membrane lipid composition; for example, a decrease in phospholipids (3, 6, 21), or lipid peroxidation and an increase in long-chain neutral lipids (23). These changes are in effect reflecting the progression of senescence, of which the climacteric-like rise in ethylene production is characteristic of advanced irreversible senescence.

Cold storage is used as a horticultural technique and is assumed to prevent, or at least severely inhibit, metabolism and aging. During storage at 3°C there were increases in lipid phase transition temperature (Fig. 5A) and microviscosity (12) and in ethylene production rate (Fig. 5B) which were parallel to, but slower than, changes during aging at 22°C. Thus, it can be concluded that important physical and physiological events normally associated with aging occur during cold storage of rose flowers. Similar evolution of gel phase in thylakoid membranes of wheat leaves exposed to low temperature were reported previously (24). Since aging during cold storage is very similar to that at 22°C it seems likely that ethylene also influences changes in membranes at 3°C, but this has not yet been tested experimentally. After cold storage, senescence at 22°C was advanced relative to that in freshly cut flowers, as reflected by earlier appearance of gel phase lipid in membranes and earlier increases in ethylene production and membrane permeability.

In conclusion, the sequence of events which occur during aging and senescence of rose petals appears to involve the following: changes in lipid composition leading to increased lipid phase transition temperature and microviscosity, lipid phase separation, a climacteric rise in ethylene production, increased membrane permeability, and probably other malfunctions in membranes and ultimately cell death.

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LITERATURE CITED

11. FARAGHER JD, S MAYAK, T TIRIOH 1986 Physiological responses of cut rose flowers to cold storage. Physiol Plant. In press