Acceleration of Membrane Senescence in Cut Carnation Flowers by Treatment with Ethylene

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ABSTRACT
The lipid microviscosity of microsomal membranes from senescing cut carnation (Dianthus caryophyllus L. cv. White Sim) flowers rises with advancing senescence. The increase in membrane microviscosity is initiated within 3 to 4 days of cutting the flowers and coincides temporally with petal-inrolling denoting the climacteric-like rise in ethylene production. Treatment of young cut flowers with aminoethoxyvinylglycine prevented the appearance of petal-inrolling and delayed the rise in membrane microviscosity until day 9 after cutting. When freshly cut flowers or aminoethoxyvinylglycine-treated flowers were exposed to exogenous ethylene (1 microliter per liter), the microviscosity of microsomal membranes rose sharply within 24 hours, and inrolling of petals was clearly evident. Thus, treatment with ethylene accelerates membrane rigidification. Silver thiosulphate, a potent anti-ethylene agent, delayed the rise in microsomal microviscosity even when the flowers were exposed to exogenous ethylene. Membrane rigidification in both naturally senescing and ethylene-treated flowers was accompanied by an increased sterol:phospholipid ratio reflecting the selective loss of membrane phospholipid that accompanies senescence. The results collectively indicate that the climacteric-like surge in ethylene production during senescence of carnation flowers facilitates physical changes in membrane lipids that presumably lead to loss of membrane function.

Cut flowers held in water senesce within 2 to 14 d depending upon the species (10). Aging of the flower petals is accompanied by morphological, biochemical, and biophysical deterioration. In carnations, for example, the earliest morphological indication of advancing senescence is a striking inrolling of the petals, a phenomenon that has been termed sleepiness (24), and in Ipomoea flowers, there is also an inrolling of the corolla (12). Invaginations of the tonoplast, thought to be a manifestation of autophagic activity by the vacuole, have also been observed during the early stages of morning glory senescence (16). In addition, flower senescence entails enhanced respiration and autolysis of the cell cytoplasm (18). Increased activities of such enzymes as RNase, DNase, and cell wall polysaccharide hydrolases are paralleled by a corresponding drop in the macromolecular constituents of cells (18).

Evidence for an increase in membrane permeability during senescence of several flower species (11, 19, 24) suggests that there is disruption of membrane integrity and loss of intracellular compartmentation. Kende et al. (3, 28) have noted a strong correlation between membrane leakage and phospholipid breakdown in senescing flowers. Moreover, treatment of Tradescantia with ethylene accelerates the onset of membrane leakiness and phospholipid deterioration in petals, but the ethylene effect is dependent upon synthesis of new protein (28). Borochov et al. (6, 7) have reported that the microviscosity of plasma membranes from rose petals rises with advancing senescence in a manner that correlates with an increase in sterol:phospholipid ratio reflecting phospholipid breakdown.

Senescing carnation flowers exhibit a climacteric-like rise in ethylene production (5, 17, 20). In addition, exposure of carnation flowers to exogenous ethylene induces inrolling of the petals and results in increased ethylene synthesis (10, 24, 26). In the present study, we have used cut carnation flowers to examine the ability of ethylene to induce chemical and physical changes in microsomal membrane lipids of senescing petals.

MATERIALS AND METHODS
Plant Material. Carnation flowers (Dianthus caryophyllus L. cv. White Sim) were grown in raised beds in a greenhouse according to established culturing procedures. Mature flowers were cut at the commercial stage of development (fully open with a yellowish tinted center) and either used directly for membrane isolation or trimmed to an 8 cm stem length and placed individually in 20-ml vials containing either deionized water or test solutions. Flowers held in water or test solutions were maintained at 22°C, and the levels of water or test solutions were adjusted as necessary to 1 cm below the calyx.

Treatments. For treatment with ethylene, flowers were placed in deionized H2O in specially constructed Plexiglas chambers (135 L capacity) equipped with an internal fan to promote circulation, two ports for gas flow, and a removable front panel. Exposure to ethylene was achieved by injecting ethylene into the chambers to a final concentration of 1 μL/L. Throughout the exposure, the chambers were connected to an air stream containing 1 μL/L ethylene that was flowing at 20 μL/min. Chambers containing control flowers were flushed at the same rate with air that had been rendered ethylene-free by passage through potassium permanganate coated with aluminum silicate (Purafil, Chamblee, GA). Ethylene treatments were terminated by removing the flowers from the chambers to a well-ventilated room.

For measurements of ethylene, individual flowers were enclosed in round glass chambers (300 ml capacity) ventilated continuously with ethylene-free air (20 μL/min). Twice a day the air stream was disconnected, the inlet and outlet ports were plugged for 1 h, and a 4-ml sample was withdrawn from the chambers and introduced into a Varian gas chromatograph through a 2-ml sampling valve (17). The gas chromatograph was equipped with an alumina column (0.32 cm x 1.82 m) and operated at 40°C.
Flowers were treated with AVG and STS by transpirational uptake. Cut flowers were placed in test solutions containing 6.8 × 10⁻⁵ M AVG or 2 mM STS prepared by mixing equal volumes of 100 mM sodium nitrite (NaNO₂) and sodium thiosulphate (Na₂S₂O₃·5H₂O) solutions of concentrations in the molar ratio 1:4, respectively. The flowers were treated for 20 min and then held in deionized H₂O for the duration of the experiment.

Membrane Isolation and Fluorescence Depolarization Measurements. For isolation of microsomal membranes, 20 g of flower petals were homogenized at 4°C in 75 ml of 0.01 M Hepes buffer, pH 7.5, in a Sorvall omni mixer for 5 periods of 10-s duration with 30-s intervening cooling periods in ice. The resulting slurry was filtered through four layers of cheesecloth and homogenized again with 20 strokes of a tight-fitting Potter Elvehjem homogenizer. This final homogenate was centrifuged at 10,000g for 20 min, and the supernatant was centrifuged again at 133,000g for 1 h to yield a pellet of microsomal membranes. The membranes were washed by resuspension in an equal volume of buffer, centrifuged again at 133,000g for 1 h, and then resuspended in 2 to 5 ml of Hepes buffer for fluorescence depolarization measurements. In some experiments, unwashed microsomal membranes were used directly for physical measurements, for there proved to be no significant difference in the fluorescence depolarization values for washed and unwashed membranes.

The membranes were labeled with DPH for fluorescence depolarization measurements. For this purpose, membranes (100–200 µg membrane protein/ml) were suspended in a 10⁻⁶ M dispersion of DPH and incubated with shaking for 15 min (6, 7). The degree of fluorescence depolarization was measured at 22°C in an Elscint fluorescence spectrophotometer under steady-state conditions (27). Microviscosity (η̅) was calculated according to the empirical relation:

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\eta = \frac{2P}{0.46 - P}
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where P is defined as the degree of fluorescence depolarization (27).

Lipid Analysis. Extraction of lipids from the membranes and determinations of total phospholipid and free sterols were carried out as described previously (21), except that the trimethyl silyl derivatives of the sterols were analyzed on a Hewlett-Packard fused silica capillary column (0.31 mm × 12.5 m) coated with OV-101, which was operated on a temperature program initiated at 200°C and increased at the rate of 8°C/min to a maximum of 270°C. The peaks were integrated using a Hewlett-Packard model 3385A microprocessor. Cholestane was used as an internal standard.

RESULTS

The pattern of ethylene production by carnations cut as young fully emerged flowers and held in water at 25°C is illustrated in Figure 1. Ethylene production by freshly cut flowers was relatively low, but 4 d after cutting, production of the volatile began to increase and reached a peak by day 5 before beginning to decline again. Moreover, the peak in ethylene production at day 5 coincided temporally with the onset of petal-inrolling, which is the first morphological symptom of carnation senescence. This pattern of ethylene production has been noted previously for senescing carnations and, because it marks the onset of visual symptoms of flower senescence, has been likened to the climacteric rise in ethylene production by ripening fruits (10, 18). The temporal coincidence of petal-inrolling and the rise in ethylene production is inviolate for carnations, and thus, petal-inrolling can be used to
denote the timing of the climacteric-like rise in ethylene production during senescence of the flower. Treatment of cut flowers with ethylene accelerates the onset of a rise in endogenous ethylene production as well as petal-inrolling (Fig. 1).

Fluorescence depolarization of microsomal membranes isolated from flowers at various stages of senescence and labeled with the fluorescent lipid-soluble probe DPH revealed a marked decrease in inherent rotational mobility of the probe with advancing senescence. Changes in mobility were scored as a change in the degree of fluorescence depolarization (P), and converted to units of membrane lipid microviscosity (7, 27). During the first 2 d after cutting, there was little change in microviscosity for microsomal membranes from the carnations, but by 3 or 4 d after cutting a significant increase was detectable (Fig. 2). Moreover, the rise in microviscosity coincided temporally with the onset of petal-inrolling reflecting a rise in ethylene production (Fig. 2). Two of four separate experiments performed are illustrated in Figure 2, and it is clear that petal-inrolling and a rise in membrane lipid microviscosity were temporally coupled whether they occurred on day 3 or day 4 after cutting. In fact, in most cases the initiation of a rise in microviscosity preceded the onset of petal-inrolling (Figs. 2–5). Microviscosity of the microsomal membranes continued to increase beyond the point at which petal-inrolling was first observed, reaching values as high as 5.0 poise by day 5 (Fig. 2).

This close temporal correlation between membrane rigidification and ethylene production during natural senescence raises the prospect that ethylene induces physical changes in membrane lipids either directly or indirectly. To test this further, freshly cut flowers were exposed to exogenous ethylene. Within 24 h of the exposure, the microviscosity of isolated microsomal membranes had risen significantly and inrolling of petals was evident (Fig. 3).

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3 Abbreviations: AVG, aminoethoxyvinylglycine; STS, silver thiosulphate; DPH, diphenyl hexatriene.

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FIG. 1. Pattern of ethylene evolution from senescing cut carnation flowers. (©), control flowers; (●), ethylene-treated flowers. Arrows denote inrolling of petals that accompanies the climacteric-like rise in ethylene production. The decline in ethylene for the treated flowers at day 2 reflects gradual removal of treatment-ethylene by an ethylene-free air stream flowing continuously through the chamber.
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FIG. 2. Changes in the lipid microviscosity of microsomal membranes isolated from senescing cut carnation flowers at various stages of senescence and labeled with diphenyl hexatriene. Two separate experiments are shown. Arrows denote inrolling of petals that accompanies the climacteric-like rise in ethylene production.

FIG. 3. Effect of exposing cut carnation flowers to ethylene on the lipid microviscosity of microsomal membranes. (C), control flowers; ( ), flowers exposed to 1.0 \( \mu l/l \) ethylene immediately after cutting. Arrows denote inrolling of petals that accompanies the climacteric-like rise in ethylene production. SE of the means are shown; \( n = 3 \) to 4.

Membrane microviscosity continued to increase for flowers exposed to ethylene, reaching a value of 5.2 poise by day 3 (Fig. 3). A large rise in microviscosity for membranes from untreated flowers was not observed until after day 3. Thus, it is clear that treatment of the flowers with ethylene induces a premature rise in membrane microviscosity.

AVG is known to inhibit ethylene biosynthesis (15) and delay carnation senescence (1). For carnations not showing inrolling of petals by reason of being held in a solution of AVG \( (6.8 \times 10^{-2} \text{ mm}) \), the rise in membrane microviscosity did not occur until after 9 d (Fig. 4). By day 12, the microviscosity of membrane lipid for these flowers had risen to a value of 4.3 poise, and the flowers appeared wilted, but there was still no indication of petal-inrolling (Fig. 4). This inrolling of petals is thought to reflect a turgor differential between the adaxial and abaxial sides of the petals resulting from permeability changes induced by ethylene (11). In

the absence of a surge in ethylene production, permeability changes occur more slowly such that a turgor differential is not realized, and the flowers wilt without showing petal-inrolling symptoms. Measurements of ethylene production throughout the 12-d period during which the carnations were held in AVG confirmed that, in the absence of petal-inrolling, there was no climacteric-like surge in production of the volatile. However, when flowers that had been in AVG solution for 1 d were exposed to 1 \( \mu l/l \) ethylene, the microviscosity of microsomal membranes rose sharply within 24 h, and inrolling of petals was clearly evident (Fig. 4).

The longevity of cut carnations can also be increased by pretreatment with silver salts (9). Indeed, silver ion has been shown to be a potent anti-ethylene agent in various plants (4), and in carnation achieves its effects both by inhibiting production of the volatile and by blocking its senescence-promoting action (30). It is clear that silver applied as STS prevents the rise in microsomal membrane microviscosity for carnations even when the flowers are exposed to exogenous ethylene (Fig. 5). Corresponding control flowers displayed petal-inrolling and a coincident rise in mem-

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brane microviscosity by day 3 and within 24 h of being exposed to exogenous ethylene (Fig. 5).

Microsomal membranes from young, freshly cut flowers contained two major sterols (Fig. 6). One of these (peak 4 in the chromatograph scan) is sitosterol; the other (peak 7 in the chromatograph scan) could not be identified inasmuch as it did not co-chromatograph with any of a range of standards available from commercial sources including stigmasterol, sitosterol, campesterol, ergosterol, fucosterol, demosterol, and cholesterol. The membranes also contained trace amounts of cholesterol and three other unidentified sterols (Fig. 6). This pattern of sterol composition did not change significantly during natural senescence or as a result of ethylene treatment (Fig. 6). However, the sterol:phospholipid ratio in the membranes increased with advancing senescence from 115 μmol sterol per mmol phospholipid for freshly cut flowers to 355 within 4 d of cutting, by which time petal-inrolling was evident (Table 1). This ratio also increased to 228 μmol sterol per mmol phospholipid within 24 h of treating freshly cut flowers with 1 μl/l ethylene (Table 1). Phospholipid levels expressed on a per mg total membrane lipid basis decreased by about 50% within 4 d of cutting and by 25% within 24 h as a result of ethylene treatment (Table 1). There was also an apparent rise in the ratio of free sterols to total membrane lipid that was due in part to the decline in phospholipid (Table 1).

**DISCUSSION**

Inrolling of petals in carnation flowers, also termed sleepiness (10), is the first morphological manifestation of senescence, and inasmuch as it coincides temporally with the climacteric-like rise in ethylene production, appears to be induced either directly or indirectly by ethylene. This temporal coincidence has been noted previously (10, 24), and in the present study was observed under conditions in which cut flowers were allowed to senesce naturally as well as when the rate of senescence was experimentally altered. For example, exposure of freshly cut flowers to exogenous ethylene for as little as 5 h caused both inrolling of petals and a rise in ethylene production within 48 h. During natural senescence, the two phenomena were also temporally coupled but did not become manifest until 4 to 6 d after cutting.

A unique and potentially important feature of the present study is the observation that ethylene induces an abrupt increase in $P_e$, the degree of fluorescence depolarization, for microsomal membranes labeled with DPH. Microsomes are a heterogenous mixture of small membrane vesicles derived in varying proportions from

![Capillary gas chromatograph tracings of microsomal membrane sterols from carnation flowers. A, freshly cut flowers; B, flowers exposed to 1 μl/l ethylene for 24 h after cutting; C, senescent flowers (9 d after cutting). Peak 1, cholestan (internal standard); peak 2, cholesterol; peak 3, unknown; peak 4, sitosterol; peaks 5 to 7, unknowns.](image-url)
the plasma membrane and membranous organelles. DPH is strongly hydrophobic and partitions almost exclusively into membranes, assuming an essentially random distribution throughout the hydrophobic core of the membrane lipid bilayer (27). Accordingly, the increase in P reflects an average decrease in mobility of the probe, and for membranes can be interpreted as reflecting an increase in lipid microviscosity (27), although there are indications that, for lipid bilayers, changes in the degree of fluorescence depolarization measured under steady-state conditions provide only an estimate of microviscosity (13, 27). A close temporal correlation between this increase in membrane microviscosity and the climacteric-like rise in ethylene production denoted by the onset of sleepiness symptoms was evident both for flowers senescing naturally and for those in which the rate of senescence was altered. During natural senescence, petal-inrolling and a corresponding increase in membrane microviscosity occurred between 3 and 4 d after cutting. The involvement of ethylene in mediating these membrane changes is supported by the fact that treatment of flowers with the volatile either in the presence or absence of AVG, an inhibitor of ethylene biosynthesis from methionine, caused an increase in microviscosity within 24 h, well in advance of when it normally would have occurred, which again coincided temporally with petal-inrolling. AVG in the absence of exogenously applied ethylene prevented the onset of petal-inrolling but only deferred the rise in membrane microviscosity. Similarly, STS, a putative inhibitor of the action of ethylene, delayed the rise in membrane microviscosity but did not prevent it. These observations suggest that ethylene simply facilitates the chemical changes in lipid that lead to a rise in membrane microviscosity rather than acting as the sole cause of such changes.

Changes in the phase properties and microviscosity of plant membrane lipids have been noted previously for a variety of senescing tissues (6, 7, 14, 21–23). However, the present study is the first demonstration of membrane viscosity being affected by ethylene. In some senescing systems, these physical changes have been correlated with loss of membrane enzyme activity (22) and large increases in membrane permeability resulting in loss of osmotic integrity (25, 29). The viscosity and phase changes of senescing plant membranes have also been correlated with an increase in sterol:phospholipid ratio reflecting loss of membrane phospholipid (7, 8, 21). From the present study, it is clear that the rise in membrane microviscosity associated with the climacteric-like rise in ethylene production in senescing carnations is also accompanied by a rise in sterol:phospholipid ratio that can be attributed, at least in part, to loss of phospholipid from the membranes. Suttle and Kende (28) have noted that the ethylene-mediated increase in membrane permeability in senescing *Tradescantia* correlates temporally with a reduction in tissue levels of phospholipid. Barber and Thompson (2) have reported that the physical changes incurred in plant membrane lipids during senescence, which parallel this loss of phospholipid, greatly increase the permeability of lipid bilayers. Thus, it seems that ethylene can facilitate both chemical and physical changes in the membrane lipids of senescing tissues that presumably lead to loss of intracellular compartmentalization.

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LITERATURE CITED