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Senescence and the Fluidity of Rose Petal Membranes[1]

RELATIONSHIP TO PHOSPHOLIPID METABOLISM

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ABSTRACT

In previous work, senescence of rose petal cells has been shown to be accompanied by a gradual decrease of membrane fluidity, as measured by a fluorescence polarization technique. Concomitantly, an increase in the free sterol-to-phospholipid ratio was found. Both observations were verified in this study. Further, experiments carried out on whole tissue and isolated protoplasts during senescence revealed that there was no quantitative change in the level of free sterols. The content of phospholipids decreased without any significant change in their composition. Results from experiments measuring the incorporation of [^{32}P]orthophosphate indicated a reduced capacity for phospholipid synthesis in senescent cells. Both young and old tissue showed phospholipase A and D activity, the former increasing with age.

It was concluded that the fluidity of rose petal membranes decreases with age as a result of a decrease in phospholipid content, brought about by both reduced synthesis and enhanced degradation. Evidence supporting the view that the phenomena observed are related specifically to changes in the plasmalemma is discussed.

Senescence of flower petals is accompanied by many visual and physiological changes (13). Recently, attention has been directed to changes in plasma membrane characteristics with age, assuming that they play a primary role in the senescence process (2, 21, 28).

In previous reports (3, 4), we showed that senescence of rose petals is accompanied by a decrease in fluidity of the lipid core of its membranes, as deduced from microviscosity values calculated from fluorescence polarization measurements of protoplasts. This phenomenon occurs in both intact and cut flowers as well as in isolated protoplasts (3, 4), and it was attributed to an increase in the ratio of the free sterols to phospholipids in the plasmalemma with increasing age (4).

The present report summarizes experiments attempting to elucidate the metabolic processes leading to the change in the ratio of sterols to phospholipids, which influences the fluidity of rose petal membranes upon aging.

MATERIALS AND METHODS

Plant Material and Environmental Conditions. Roses ([*Rosa* hyb.] Golden Wave [Syn. Dr. Verhage]) were grown in a greenhouse under standard cultural conditions. Flowers were picked and treated as described previously (4). The procedures and the techniques for protoplast isolation and handling and for viability evaluation were as described previously (4).

Evaluation of Fluidity. Membrane lipid fluidity was determined by calculating the microviscosity (which is the reciprocal value of fluidity) from fluorescence polarization measurements of 1,6 diphenylhexatriene, as described previously (4). Microviscosity measurements for liposomes and isolated membranes were carried out under the same conditions as those for protoplasts.

Membrane Isolation. The procedure for isolation of the plasmamembrane enriched fraction from protoplasts was based on two recent works (6, 12), which described isolation and purification of plasmalemmas from protoplasts. A 5-ml aliquot of 2×10^6 protoplasts $\times$ ml^{-1} was spun down at 100*g* for 5 min, and the pellet was resuspended in 10 ml buffer containing 300 mM sucrose (fluorescence-free; Merck), 10 mM Tris-HCl (pH 7.0), and 1 mM $MgCl_2$. The protoplasts were disintegrated, using a glass homogenizer, until no intact protoplasts could be observed under a microscope ($\times$ 1000). The suspension was centrifuged for 5 min at 480*g*. The supernatant was diluted with 10 ml of the same buffer and loaded onto 10 ml buffer containing 1.8 M sucrose, 10 mM Tris HCl (pH 7.0), and 1 mM $MgCl_2$; then it was centrifuged for 60 min at 130,000*g* in Beckman ultracentrifuge L5-65, SW27 rotor. Six ml of the interface were collected, diluted with the same volume of homogenizing buffer, and loaded onto the top of a sucrose step gradient constructed from 12 ml of 35% (w/w) sucrose (lower layer) and 30% (w/w) sucrose (upper layer). Both layers contained 10 mM Tris-HCl (pH 7.0) and 1 mM $MgCl_2$. The samples were centrifuged for 3 h at 100,000*g* as before, at the end of which the interface above the 35% sucrose fraction was collected, diluted with 0.6 M mannitol solution to yield 0.5 mg lipid $\times$ ml^{-1}, and used immediately.

Chemical Analysis. Lipid extraction and quantitation of total phospholipids and free sterols were carried out as previously described (4). Phospholipid analysis was carried out by TLC. Samples were loaded on preactivated TLC plates (Kieselgel 60; Merck) and developed with chloroform:methanol:acetic acid:H_2O (32:4:19:2, v/v). The different phospholipids were located using authentic markers (Sigma) and specific stains (27). Quantitation of the different phospholipids was carried out in the presence of the silica, according to the method of Rouser *et al.* (22). Protein determination was carried out according to Bramhall *et al.* (7). Carotenoid content was estimated spectroscopically by the method of Jensen (15).

Liposome Preparation. Sonicated liposomes were prepared from cholesterol, stigmasterol (Sigma), or sitosterol (Aldrich), with egg lecithin (Sigma; grade I) in different ratios, as described previously (26).

[^{32}P]Orthophosphate Incorporation. The degree of incorporation of [^{32}P]orthophosphate into protoplast lipids was measured by incubating 1.5 ml protoplast suspension of 5×10^6 protoplasts $\times$ ml^{-1} with 10 μCi (0.3–1.0 Ci $\times$ M^{-1}) of [^{32}P]orthophosphate

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(Negev Nuclear Center, Israel) for 60 min at 20°C. Preliminary experiments demonstrated no difference in the accumulation of the [^{32}P]orthophosphate for protoplasts of different ages. This incorporation was terminated by extracting the lipids with chloroform:methanol (5:8, v/v) from the suspension. The chloroform fraction was dried under N_2 in scintillation vials, and the dried lipid film was dissolved in scintillation cocktail composed of 150 mg POPOP (Packard) and 7.5 g PPO (Packard) in 1 L of toluene. The samples were counted in liquid scintillation counter (Searle; Isocap 300) using programs 3 and 6.

Assays. Cyt *c* oxidase and K^+-stimulated ATPase activities, as mitochondrial and plasma-membrane marker enzymes, respectively, were measured according to Hodges and Leonard (14). NADPH-Cyt *c* reductase activity, as a marker enzyme for ER, was measured by the method of Lord *et al.* (18).

Phospholipase Activity. Determination of phospholipase activity was done on the crude extract of petals of differing ages. All of the different steps were conducted at 0 to 4°C. A 15-g sample of petals was homogenized in 30 ml 200 mM sucrose, 3 mM 2-mercaptoethanol, 2 mM EDTA, 0.1% Triton X-100 (Packard) (pH 7.0), using ultra turrax for 60 s. The homogenate was filtered through two layers of cheese cloth, and the filtrate was dialyzed for 1 h against 35 volumes of the homogenization buffer and then against another 30 volumes of the same buffer for an additional 2 h. After dialysis, the samples were diluted to 2 mg protein × ml^{-1}. Phospholipase activity of the extract was determined by measuring the amounts of phosphatidic acid and lysophosphatidylcholine liberated from phosphatidylcholine by the action of the petal extract. For this, 1 ml of extract was suspended with 100 ml of sonicated liposomes prepared from egg lecithin at 20 mg × μl^{-1}, 100 μl of 375 mM $CaCl_2$, and 300 μl of 200 mM citrate buffer at pH 4.5 or 6.5. The reaction was carried out for 60 min at 30°C and was terminated by the addition of a solution of chloroform: methanol (5:8, v/v) to the sample for lipid extraction. The lipid extract was concentrated under N_2 and analyzed on TLC, as described above. The spots corresponding to phosphatidic acid and lysophosphatidylcholine were scraped off the plate, and their phospholipid contents were measured.

All measurements reported in this work were repeated at least three times, and the results are means of four replications.

RESULTS AND DISCUSSION

The fluidity of the lipid core of biomembranes has been shown to play a significant role in the control of many physiological processes, including permeability, enzyme activity, and protein accessibility (25). We have used fluorescence polarization technique (24) to measure actual values of the reciprocal parameter of fluidity, namely the microviscosity of rose petal membranes. In previous reports (3, 4), we demonstrated an increase of membrane microviscosity of rose petal cells with age which was affected by temperature, pH, and Ca^{2+}. The results summarized in Table I confirm our previous observations (3, 4) that plasmalemma microviscosity in protoplasts isolated from rose petals is higher in old flowers than it is in younger ones. Protoplasts isolated from petals of young flowers which were held for 24 h in acidic solution (pH 4.5) gave values similar to those of protoplasts from old flowers (Table I).

An isolated membrane fraction showed essentially the same microviscosity as did the original protoplasts (Table I). These membrane fractions were isolated according to two recent detailed reports (6, 12). Measurements of enzyme activities generally considered as markers of the mitochondrial, plasma-membrane, and ER membranes (14, 18) were carried out with the crude membrane fraction (interphased at 1.8 M sucrose) and the purified membrane fraction (interphased at 35% sucrose). These assays revealed a recovery of 40% and almost 3-fold increase in K^+-ATPase activity in the purified fraction as compared to the crude membrane fraction (1.70 *versus* 0.59 μmol Pi/mg protein · h^{-1}, respectively) and only one-fifth of the carotenoid content (10 *versus* 51 μg carotenoids/mg protein, respectively). No activity of either Cyt *c* oxidase or NADPH-Cyt *c* reductase was detected in the purified membrane fraction.

On the basis of these results, we are inclined to believe that this fraction, though containing some contaminations, is at least enriched with plasmalemmas. Their measured microviscosity values support the view that the microviscosity measurements carried out on the intact protoplast give information on the plasmalemma microviscosity. Additional support for this view comes indirectly from two observations. First, using fluorescence microscopy, we observed fluorescence only from the outer surface of DPH-labeled protoplasts (4). Inasmuch as fluorescence polarization measures only the visible fluorescence (24), it is likely that measurements made on intact protoplasts represent the microviscosity of the outer surface of the protoplasts. Further, single-cell measurements, using a different instrumentation, revealed similar observations (5). Second, sterol depletion and enrichment of intact protoplasts, a treatment which affects only the outer membrane (8), resulted in corresponding changes in the protoplast membrane microviscosity (Table III of Ref. 4). As the outer surface, the outer membrane, and the plasmalemma of the protoplast are actually the same, we concluded that results obtained from DPH-labeled intact protoplasts represent plasmalemma microviscosity. These considerations do not rule out the possibility of similar fluidity changes in other cell membranes. In fact, age-related fluidity changes have been reported for microsomal and chloroplast membranes from *Phaseolus* leaves (20). Reduced fluidity of plasma membranes with age appears to be a general phenomenon and has also been demonstrated for both bacterial (1) and animal cell membranes (23).

The presence of cholesterol in membranes is known to increase their microviscosity (9, 24, 25) in temperature above the phase transition of the phospholipids, which is the normal situation for active organisms (25). Since cholesterol in only a minor component of the total plant sterols (10), we have studied the effect of increasing content of several typical plant sterols on the microviscosity of sonicated egg lecithin liposomes (Fig. 1). As shown, the elevation of the ratios of sitosterol and stigmasterol to egg lecithin results in an increased lipid microviscosity in a similar way as it does with cholesterol. Though it would be much more desirable to conduct the lipid analysis on the isolated membranes, the yield obtained was too low for reliable quantitative analysis. Therefore, we decided to make the different measurements on a whole cell basis.

Table I also shows that there is no change in the free sterol level in the protoplasts with age. We purposefully examined the free sterols alone, as it is widely accepted now that sterol derivatives (*e.g.* sterol esters) do not participate in the functional composition of biomembranes (9). In contrast to sterols, the phospholipid level of the protoplasts decreased with senescence to about one-half of its original level. The results were similar both for protoplasts isolated from petals of old flowers and for protoplasts aged for 24 h after isolation from young petals. The amount of total phospholipids decreased with increasing age, but the relative amount of each phospholipid class remained about the same (Table II). Similar results were found for *Ipomea* and *Tradescantia* flowers of different ages (2, 28).

As a result of the reduction in the phospholipid content relative to the free sterol content, there was an increase of the free sterol to phospholipid ratio (Table I), similar to our previous results (4). Liposomes made from sitosterol and egg lecithin of the same mole ratio found in the protoplasts exhibited similar microviscosities to the corresponding protoplasts and membranes (Table I).

The above results suggest that the main reason for the changes observed in the rose petal membrane microviscosity with increas-

Table I. *Microviscosity of Liposomes and Protoplast Membranes of Different Ages and Lipid Composition of Protoplasts Aged in Situ and in Vitro*

Protoplast Age[a]	Membrane Microviscosity		Free Sterols	Phospholipids	Free Sterols/Phospholipids (M:M)	Liposome Microviscosity[b]
	Intact protoplasts	Isolated membranes				
	poise at 22°C		*nmol·mg^{-1} lipid*		*ratio*	
A	1.97 c[c]	1.90 b	70 a	315 a	0.24 c	2.0 c
A, aged[d]	3.70 a		70 a	110 b	0.64 a	3.8 a
D	2.82 b	2.85 a	71 a	145 b	0.49 b	3.0 b

[a] A, Protoplasts isolated from young flower petals; D, protoplasts isolated from old flower petals (see Ref. 3).
[b] Liposomes were made from sitosterol and egg lecithin of the same mole ratio as that found in the protoplasts.
[c] Values followed by different letters are significant at the 5% level (multiple range test).
[d] Protoplasts isolated from young flower petals were aged in dark for 24 h at 37°C (pH 4.5).

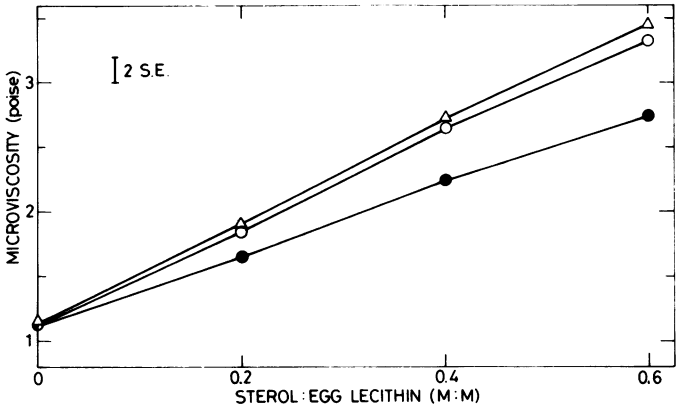

FIG. 1. The effect of different mole ratios of sitosterol (△), cholesterol (○), and stigmasterol (●) to egg lecithin on microviscosity of sonicated liposomes. Measurements were done at 22°C.

Table II. *Relative Content of Phospholipid Classes of Protoplasts Obtained from Young (Stage A) and Old (Stage D) Petals*

Phospholipid Class	Relative Content	
	Young	Old
	% of total phospholipids	
Phosphatidylcholine	45 a[a]	43 a
Phosphatidylethanolamine	30 b	31 b
Phosphatidylserine	10 c	9 c
Phosphatidylinositol	9 c	8 c
Phosphatidic acid	6 c	9 c

[a] Values followed by different letters are significant at the 5% level (multiple range test).

ing age is the decrease in phospholipid level. The decrease in phospholipid level with age has been found also in other plant tissues (2, 11, 28), and it may result from a reduction in the phospholipid synthesis, an increase in degradation, or both.

To examine the reason for the decrease in phospholipid level with age, we first measured the ability of protoplasts of different ages to synthesize new phospholipids by following the incorporation of [^{32}P]orthophosphate into lipids (Table III). Reduced counts in the protoplast lipid fraction with senescence were found both for protoplasts isolated from old petals and for protoplasts held for 24 h at pH 4.5 after isolation from young petals. Similar protoplasts, incubated for 24 h at higher pH (6.5), did not show reduction of orthophosphate incorporation (Table III). A similar age-related reduction in phospholipid synthesis capacity has been demonstrated in castor bean endosperm (16) and cucumber endosperm (19).

Table III. *Effect of the Protoplast Age on [^{32}P]Orthophosphate Incorporation into Lipid Fraction*

Protoplast Age[a]	[^{32}P]Incorporation into Lipid Fraction
	cpm/10^6 protoplasts·h^{-1}
A	8,295 a[b]
A, aged[c], at pH 6.5	8,000 a
A, aged, at pH 4.5	3,507 b
D	1,915 b

[a] A, Protoplasts isolated from young flower petals; D, protoplasts isolated from old flower petals (see Ref. 3).
[b] Values followed by different letters are significant at the 5% level (multiple range test).
[c] Protoplasts isolated from young flower petals were aged in dark for 24 h at 37°C.

Table IV. *Effects of Age and pH on the Phospholipase Activity of Rose Petal Crude Extract*

Petal Stage of Development[a]	Phospholipase A Activity[b]		Phospholipase D Activity[c]	
	pH 4.5	pH 6.5	pH 4.5	pH 6.5
A	0.12 b[d]	0.09 b	0.29 a	0.26 a
D	0.32 a	0.15 b	0.32 a	0.30 a

[a] A, Young flower petals; D, old flower petals (see Ref. 3).
[b] Phospholipase A activity defined as nmol lysophosphatidylcholine production from phosphatidylcholine per mg protein·h^{-1}.
[c] Phospholipase D activity defined as nmol phosphatidic acid production from phosphatidylcholine per mg protein·h^{-1}.
[d] Values for each enzyme followed by different letters are different at the 5% level (multiple range test).

Evaluation of the phospholipase activity in petals of different ages was carried out by measuring the degradation products of egg lecithin liberated by the action of petal extracts, which yields phosphatidic acid and lysophosphatidylcholine (Table IV). The production of phosphatidic acid from lecithin was referred to phospholipase D activity, while the activity leading to formation of lysophosphatidylcholine was referred to phospholipase A (17). Results shown in Table IV demonstrate the presence of both phospholipase A and phospholipase D activities in rose petals. The activity of phospholipase D did not change with age, while that of phospholipase A more than doubled in senescent petals. A similar phenomenon was demonstrated recently (28) in *Tradescantia*, in which the phospholipase activity which results in age-related phospholipid reduction was attributed to acyl hydrolases. In rose petals, there was no detected activity of both phospholipases in the absence of Ca^{2+}. However, only phospholipase A

activity is pH-dependent, *i.e.* increased at acidic pH (4.5 compared to 6.5) (Table IV). Indeed, it was demonstrated earlier (4) that the change in rose petal protoplast membrane fluidity with age is enhanced by low pH.

The present results, together with those reported previously (3, 4), suggest some of the biophysical and biochemical processes which may occur in the rose petal membranes during aging. The apparent decrease in the membrane fluidity with age appears to be caused by the increase of the free sterol to phospholipid ratio (4), brought about by a decreased phospholipid content (Table I). This process was found both in cells aged in the tissue and in those aged after isolation (Ref. 4; Table I). The observed similar behavior of protoplasts which were aged *in situ* to those held after isolation at 37°C (pH 4.5), with regard to their lipid metabolism, makes protoplast *in vitro* aging a suitable model for plant membrane senescence studies. Senescence of rose petals appears to be characterized by a decrease in the ability of cells to synthesize phospholipids (Table III) and an increase in phospholipase A activity (Table IV). These two changes may be responsible for the reduction in phospholipid content which, in turn, results in an increase in the ratio of free sterol to phospholipid, leading to a decrease in the membrane fluidity.

These senescence-related changes in phospholipid metabolism and membrane fluidity are probably affecting metabolic processes of the cells and their hormonal responses, as has been shown for mammalian tissues (25).

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LITERATURE CITED

1. AMAR A, S ROTTEM, S RAZIN 1979 Is the vertical disposition of micoplasma membrane proteins affected by membrane fluidity? Biochim Biophys Acta 552: 457–467
2. BEUTELMAN P, H KENDE 1977 Membrane lipids in senescing flower tissue of *Ipomea tricolor*. Plant Physiol 59: 888–893
3. BOROCHOV A, AH HALEVY, M SHINITZKY 1976 Increase in microviscosity with aging in protoplast plasmalemma of rose petals. Nature (Lond) 263: 158–159
4. BOROCHOV A, AH HALEVY, H BOROCHOV, M SHINITZKY 1978 Microviscosity of plasmalemmas in rose petals as affected by age and environmental factors. Plant Physiol 61: 812–815
5. BOROCHOV A, H BOROCHOV 1979 Increase in membrane fluidity in liposomes and plant protoplasts upon osmotic swelling. Biochim Biophys Acta 550: 546–549
6. BOSS W, AW RUESINK 1979 Isolation and characterization of concanavalin A-labeled plasma membranes of carrot protoplasts. Plant Physiol 64: 1005–1011
7. BRAMHALL S, N NOACK, M WU, J LOEWENBERG 1969 A simple colorimetric method for determination of protein. Anal Biochem 31: 146–148
8. COOPER RA, EC ARNER, JS WILEY, SJ SHATTIL 1975 Modification of red cell membrane structure by cholesterol-rich lipid dispersion. J Clin Invest 55: 115–126
9. DEMEL RH, B DEKRUYFF 1976 The function of sterols in membranes. Biochim Biophys Acta 452: 109–132
10. DONALDSON RP, H BEEVERS 1977 Lipid composition of organelles from germinating castor bean endosperm. Plant Physiol 59: 259–263
11. FERGUSON CHR, EW SIMON 1973 Membrane lipids in senescing green tissues. J Exp Bot 24: 307–316
12. GALBRAITH DW, DH NORTHCOTE 1977 The isolation of plasma membranes from protoplasts of soybean suspension cultures. J Cell Sci 24: 295–310
13. HALEVY AH, S MAYAK 1979 Senescence and post harvest physiology of cut flowers. Hortic Rev 1: 204–236
14. HODGES TK, RT LEONARD 1974 Purification of a plasma membrane bound ATPase from plant roots. Methods Enzymol 32: 392–406
15. JENSEN A 1978 Chlorophylls and carotenoids. *In* JA Hellebust, JS Craigie, eds, Handbook of Phycological Methods, Ed 1, Vol 2. Cambridge University Press, Cambridge, pp 60–70
16. KAGAWA T, JM LORD, H BEEVERS 1973 The origin and turnover of organelle membranes in castor bean endosperm. Plant Physiol 51: 61–65
17. LEHNINGER AL 1975 Lipids, lipoproteins and membranes. *In* Biochemistry, Ed 2. Worth Publishers, New York, pp 279–308
18. LORD JM, T KAGAWA, TS MOORE, H BEEVERS 1973 Endoplasmic reticulum as the site of lecithin formation in castor bean endosperm. J Cell Biol 57: 659–667
19. MACHER BA, CP BROWN, TT MCMANUS, JB MUDD 1975 Studies on phospholipids-synthesizing enzyme activities during the growth of etiolated cucumber cotyledons. Plant Physiol 55: 130–136
20. MCKERSIE BD, JE THOMPSON 1978 Phase behavior of chloroplast and microsomal membranes during leaf senescence. Plant Physiol 61: 639–643
21. MAYAK S, Y VAADIA, DR DILLEY 1977 Regulation of senescence in carnation (*Dianthus caryophyllus*) by ethylene. Mode of action. Plant Physiol 59: 591–593
22. ROUSER G, S FLEISCHER, A YAMAMOTO 1970 Two dimensional TLC separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. Lipids 5: 494–496
23. SHIGA T, N MAEDA, T SUDA, K KON, M SEKIYA 1979 Decreased membrane fluidity of *in vivo* aged human erythrocytes. A spin label study. Biochim Biophys Acta 553: 84–95
24. SHINITZKY M, Y BARENHOLZ 1978 Fluidity parameters of lipid regions determined by fluorescence polarization. Biochim Biophys Acta 515: 367–394
25. SHINITZKY M, P HENKART 1979 Fluidity of cell membrane—current concepts and trends. Int Rev Cytol 60: 121–169
26. SHINITZKY M, M INBAR 1976 Microviscosity parameters and protein mobility in biological membranes. Biochim Biophys Acta 433: 133–149
27. STAHL E 1969 Spray Reagents in Thin Layer Chromatography. Springer Verlag, New York, pp 854–905
28. SUTTLE J, H KENDE 1980 Ethylene action and loss of membrane integrity during petal senescence in *Tradescantia*. Plant Physiol 65: 1067–1072