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SUPEROXIDE RADICAL PRODUCTION BY MICROSOMAL MEMBRANES FROM SENESCING CARNATION FLOWERS: AN EFFECT ON MEMBRANE FLUIDITY

SHIMON MAYAK*, RAYMOND L. LEGGE and JOHN E. THOMPSON

Department of Biology, University of Waterloo, Waterloo, Ontario, Canada, N2L 3G1

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Key Word Index—*Dianthus caryophyllus*; Caryophyllaceae; carnation; flower; superoxide radical; senescence; membrane fluidity.

Abstract—The lipid fluidity of carnation microsomal membranes decreases during *in vitro* aging in a manner that correlates with enzymatic production of superoxide anion ($O_2^{\cdot-}$) by the membranes. Levels of $O_2^{\cdot-}$ were determined from ESR spectra of the semiquinone formed when $O_2^{\cdot-}$ reacts with Tiron (1,2-dihydroxybenzene-3,5-disulfonic acid). Heat denaturation of the microsomes or addition of *n*-propyl gallate, a free radical scavenger, eliminated the Tiron radical signal and prevented the change in membrane fluidity. By contrast, the addition of xanthine/xanthine oxidase, a reputed $O_2^{\cdot-}$-generating system, accentuated the decrease in membrane fluidity and greatly increased the Tiron radical signal. Superoxide dismutase, an enzyme that catalyses the dismutation of $O_2^{\cdot-}$, reduced the amplitude of the Tiron radical signal. When Tiron was used as a scavenger of $O_2^{\cdot-}$, there was no change in membrane fluidity. The data collectively suggest that $O_2^{\cdot-}$ or highly reactive derivatives of $O_2^{\cdot-}$ induce membrane rigidification. In addition, microsomes from senescent flowers produce more $O_2^{\cdot-}$ than those from freshly cut flowers, suggesting that this reactive species of oxygen also contributes to membrane rigidification during natural senescence.

INTRODUCTION

Membrane deterioration is an inherent feature of plant senescence and is marked by the onset of pronounced physical changes in the lipid bilayer that lead to the formation of a mixture of lipid phases and decreased lipid fluidity [1–5]. There are also chemical changes in the lipids of senescing membranes characterized, in particular, by a loss of phospholipid that is manifest as an increased sterol–phospholipid ratio [6]. The physiological consequences of these physical and chemical changes in membrane lipid include increased permeability [7], loss of membrane-associated enzyme activity [8] and lateral and vertical rearrangement of membrane enzymes and receptor proteins within the plane of the membrane [9].

Senescence of cut carnation flowers is characterized by a respiratory climacteric and a climacteric-like rise in ethylene production [10]. In addition, there is a pronounced increase in microsomal membrane viscosity that coincides with the climacteric rise in ethylene production and can be induced prematurely by treatment with exogenous ethylene [4]. Borochov *et al.* [3] have demonstrated that the microviscosity of the plasmalemma of rose flowers also rises with advancing senescence and, more recently, Legge *et al.* [5] have identified a temporal sequence of physical changes in the lipid bilayer of microsomal membranes from senescing rose flowers. Specifically, there is an initial molecular tightening at the membrane surface, which is followed by increased microviscosity in the hydrophobic core of the membrane and the appearance of gel phase lipid as senescence intensifies.

Recent studies demonstrating that fruit ripening and the vase life of flowers can be modulated by free radical scavengers [11–13] suggest that free radicals are involved in senescence. The consequences of free radical attack on membranes are numerous and include the induction of lipid peroxidation [14], loss of membrane integrity and lysis of erythrocytes [15–17] and fatty acid de-esterification [18]. Moreover, free radicals are known to induce the same physical and chemical changes in membrane lipid that are observed during natural senescence [6, 19, 20]. In the present study, we provide evidence that microsomal membranes produce increased levels of $O_2^{\cdot-}$ with advancing age, and that this $O_2^{\cdot-}$ causes a decrease in membrane fluidity.

RESULTS

Superoxide anion production by microsomes

Microsomal membranes from carnation flowers produce $O_2^{\cdot-}$ which will oxidize Tiron to form the Tiron semiquinone radical. This latter radical species is detectable by ESR spectroscopy as a narrow four line first derivative spectrum (Fig. 1A). The amplitude of the spectrum increases following initiation of the reaction to reach a plateau within 5 min which reflects the steady state level of $O_2^{\cdot-}$ production [21, 22]. No Tiron radical signal was observed in the absence of microsomes or when microsomes were heat-denatured (Fig. 1 and Table 1).

*On leave from the Department of Ornamental Horticulture, Hebrew University, Rehovot, Israel.

Abbreviations: CHES, cyclohexylaminoethane sulfonic acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; EPPS, *n*-2-hydroxyethylpiperazine propane sulfonic acid; Tiron, 1,2-dihydroxybenzene-3,5-disulfonic acid; $O_2^{\cdot-}$, superoxide anion.

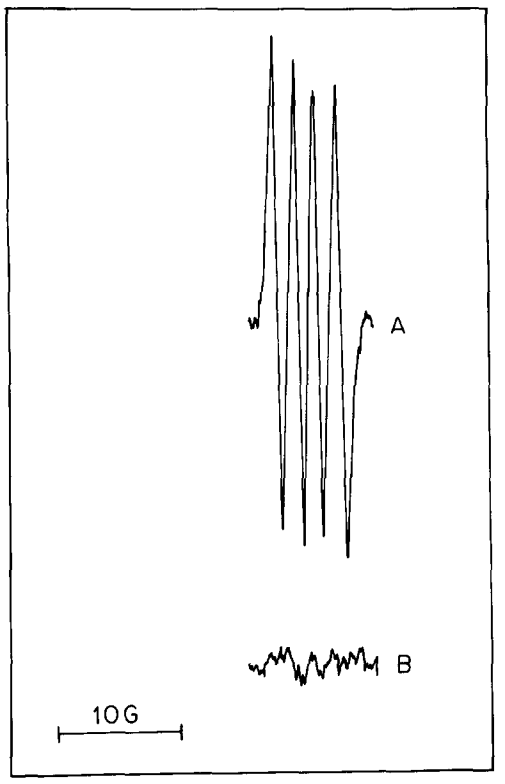

Fig. 1. ESR spectra of the Tiron radical recorded at 30° 5 min after the addition of Tiron. (A) Tiron (10 mM) and microsomal membranes from stage II carnation flowers (200 μg protein/ml) in 85 mM EPPS buffer, pH 8.5; (B) Tiron (10 mM) in 85 mM EPPS buffer, pH 8.5.

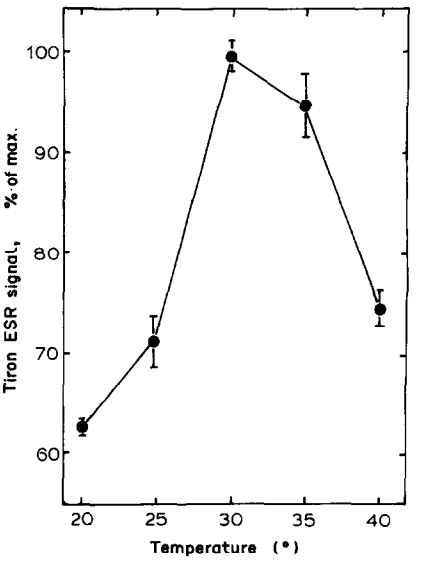

Fig. 2. Effect of temperature on the amplitude of the Tiron radical ESR signal obtained from microsomal membranes of stage II carnation flowers. Amplitudes are expressed as a percentage of the value at 30°. The reaction mixture contained Tiron (10 mM) and microsomal membranes (200 μg protein/ml) in 85 mM EPPS buffer, pH 8.5. s.e. values are indicated; $n = 3$.

Similarly, addition of *n*-propyl gallate, a non-specific radical scavenger, to the reaction mixture eliminated the Tiron radical signal (Table 1). Formation of the Tiron radical proved to be sensitive to superoxide dismutase (Table 1) and oxygen-dependent (data not shown). $O_2^{\cdot-}$ production by the microsomes was optimal at 30° (Fig. 2) and pH 8.5 (Fig. 3).

Effect of natural senescence on $O_2^{\cdot-}$ *production*

Microsomal membranes were isolated from cut carnation flowers at three stages of senescence, designated I–III, in order to determine the effects of natural senescence on $O_2^{\cdot-}$ production. The amplitude of the Tiron radical signal, reflecting $O_2^{\cdot-}$ production by microsomal membranes, increased by *ca* two-fold with advancing senescence of the flowers from stage I through stage III (Fig. 4) suggesting that the endogenous titre of $O_2^{\cdot-}$ increases as the flowers age.

Table 1. Factors affecting the amplitude of the Tiron semiquinone radical ESR signal

Components in the test reaction mixture	Amplitude of the Tiron radical signal (cm)	% reduction relative to complete reaction mixture
Complete reaction mixture	13.5 ± 1.2	—
85 mM EPPS buffer, pH 8.5	0	100
85 mM CHES buffer, pH 8.5	0	100
Complete reaction mixture, but with heat-denatured microsomes	0	100
Complete reaction mixture plus 10 mM *n*-propyl gallate	0	100
Complete reaction mixture plus superoxide dismutase (145 units/ml)	3.6 ± 0.5	73

Complete reaction mixture: 85 mM EPPS buffer, pH 8.5, containing 200 μg microsomal membrane protein/ml and 10 mM Tiron. Values expressed are means ± s.e.; $n = 3$.

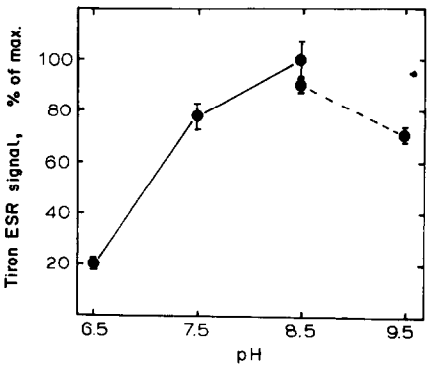

Fig. 3. Effect of pH on the amplitude of the Tiron radical ESR signal obtained from microsomal membranes of stage II carnation flowers. Spectra were recorded at 30° and amplitudes are expressed as a percentage of the value at pH 8.5 in EPPS buffer. The reaction mixture contained Tiron (10 mM) and microsomal membranes (200 μg protein/ml) in 85 mM EPPS buffer (pH 6.5–8.5) or 85 mM CHES buffer (pH 8.5–9.5). s.e. values are indicated; $n = 3$.

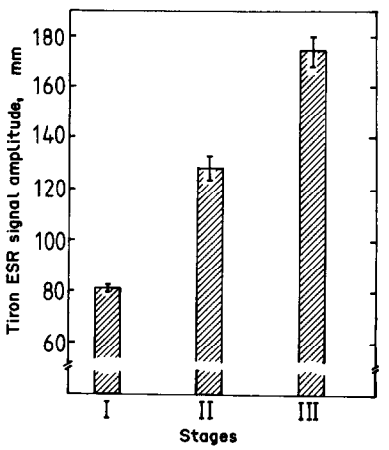

Fig. 4. Effect of natural senescence on the amplitude of the Tiron radical ESR signal obtained from microsomal membranes of cut carnation flowers aged in water. The reaction mixture contained Tiron (10 mM) and microsomal membranes (200 μg protein/ml) in 85 mM EPPS buffer, pH 8.5, and the spectra were recorded at 30°. The stages of flower senescence are described in the Experimental. s.e. values are indicated; $n = 3$.

Effects of in vitro *aging on* $O_2^{\cdot-}$ *production and membrane lipid fluidity*

It has been previously demonstrated, by fluorescence depolarization, that the lipid fluidity of microsomal membranes from carnation flowers decreases with advancing senescence [4]. Given the reactivity of free radicals and the sensitivity of membranes to free radical attack, we sought to determine if the production of $O_2^{\cdot-}$ by microsomal membranes and changes in membrane fluidity were related. This was achieved by artificially modulating $O_2^{\cdot-}$ production by microsomes during *in vitro* aging in buffer and, subsequently, evaluating the effects of this modulation on membrane lipid fluidity as measured by fluorescence depolarization.

Changes in lipid fluidity during *in vitro* aging of the isolated membranes are illustrated in Fig. 5 in terms of anisotropy parameter. Fluidity is inversely related to the anisotropy parameter [23]; thus, an increase in the anisotropy parameter reflects rigidification or reduced fluidity. Within 15 hr the anisotropy parameter had increased by *ca* two-fold (Fig. 5). Addition of *n*-propyl gallate to the incubation mixture prevented this decrease in fluidity and, as well, heat-denatured microsomes showed virtually no change in fluidity over the 15 hr period (Fig. 5). These observations are consistent with the fact that *n*-propyl gallate and heat denaturation both totally prevent the formation of $O_2^{\cdot-}$ by these membranes (Table 1), and suggest that the decrease in membrane fluidity during *in vitro* aging is attributable to either the superoxide anion itself or to the hydroxyl radical, which can be chemically derived from $O_2^{\cdot-}$ in the presence of trace concentrations of metal ions [24].

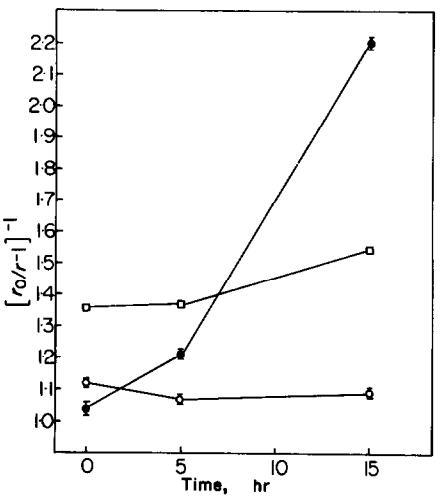

Fig. 5. Effect of *in vitro* aging at 30° on the lipid fluidity of microsomal membranes from stage II carnation flowers. Fluidity is expressed in terms of anisotropy parameter $[(r_0/r)-1]^{-1}$. (●) Control; (○) *n*-propyl gallate; (□) heat denatured microsomes. s.e. values are indicated; $n = 3$.

This contention is further supported by experiments in which xanthine/xanthine oxidase, which are known to generate $O_2^{\cdot-}$ [25], were used to increase $O_2^{\cdot-}$ levels during *in vitro* aging of the membranes. In the presence of xanthine/xanthine oxidase there was an increase in the amplitude of the Tiron radical signal reflecting increased $O_2^{\cdot-}$ levels over a corresponding control to which xanthine/xanthine oxidase had not been added (Fig. 6B). Of particular interest is the finding that, in the presence of xanthine/xanthine oxidase, the anisotropy parameter also increased relative to the control (Fig. 6A). As well, the addition of Tiron (0.1 or 10 mM) to the *in vitro* aging incubation mixture effectively eliminated the rise in anisotropy parameter reflecting decreased membrane fluidity (Fig. 7), an observation that is consistent with the ability of Tiron to scavenge $O_2^{\cdot-}$ [21].

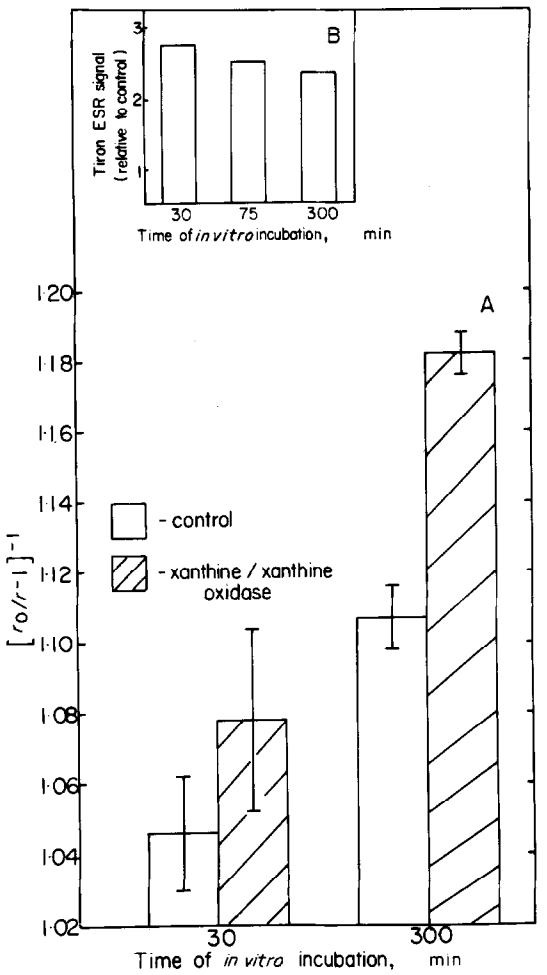

Fig. 6. Effect of xanthine/xanthine oxidase on lipid fluidity (A) and the amplitude of the Tiron radical ESR signal (B) during *in vitro* aging at 30° of microsomal membranes from stage II carnation flowers. Membrane fluidity is expressed in terms of anisotropy parameter $[(r_0/r)-1]^{-1}$. s.e. values are indicated; $n = 3$.

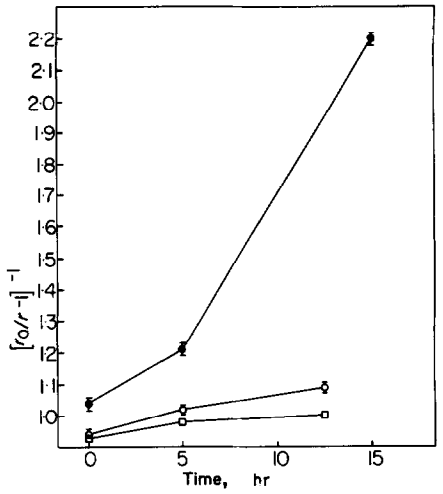

Fig. 7. Effect of Tiron on lipid fluidity during *in vitro* aging at 30° of microsomal membranes from stage II carnation flowers. Fluidity is expressed in terms of anisotropy parameter $[(r_0/r)-1]^{-1}$. (●) Control; (○) Tiron (0.1 mM); (□) Tiron (10 mM).

DISCUSSION

Tiron has been used previously to detect and quantify levels of $O_2^{\cdot-}$ produced in biological systems [21, 22, 26] and in the present study has been used to monitor $O_2^{\cdot-}$ formation by microsomal membranes isolated from senescing carnation flowers. Formation of the Tiron semiquinone results from oxidation of Tiron by $O_2^{\cdot-}$ [21], and the sensitivity of the Tiron radical signal originating from carnation microsomes to superoxide dismutase confirms that the radical species being produced is the superoxide anion.

The Tiron radical signal was eliminated by *n*-propyl gallate, a non-specific radical scavenger, and proved to be sensitive to oxygen (data not shown) suggesting that the $O_2^{\cdot-}$ is being generated enzymatically, presumably by a membrane-associated oxidase. This contention is further supported by the observation that the amplitude of the Tiron radical signal can be modulated by changes in pH and temperature and by heat denaturation. Reduction of oxygen to the superoxide radical by membrane-bound enzymes has been demonstrated previously [27, 28], and the increased propensity of microsomal membranes from senescing carnations to produce superoxide with advancing age may reflect deterioration in the molecular organization of membranes known to accompany senescence [2, 4, 29]. Moreover, since an external electron donor is not required for $O_2^{\cdot-}$ production by microsomes, the reaction may well be driven by peroxidation of unsaturated fatty acids.

Thompson *et al.* [4] have recently reported that the lipid fluidity of microsomal membranes from carnations decreases during natural senescence of the cut flower. This change in fluidity is abrupt and coincides with the climacteric-like rise in ethylene production. In the present study, we demonstrate that this decrease in fluidity can be simulated by *in vitro* aging of membranes from young flowers in buffer for up to 15 hr. Of particular interest is the finding that this decrease in fluidity appears to be caused by $O_2^{\cdot-}$, or highly reactive derivatives of $O_2^{\cdot-}$, such as the hydroxyl radical, formed by the membranes when they are incubated aerobically in buffer. The evidence for this correlation lies mainly in the finding that the decrease in membrane lipid fluidity is prevented by *n*-propyl gallate, heat denaturation of the microsomes and Tiron, each of which either scavenges or prevents the formation of $O_2^{\cdot-}$. In addition, the decrease in fluidity is hastened by xanthine/xanthine oxidase, which is known to generate the superoxide anion [25]. Superoxide dismutase (145 units/ml) consistently reduced the degree of fluidity change during *in vitro* aging (by an average of 26% over 24 hr), but not to the extent afforded by *n*-propyl gallate or Tiron. This may reflect the larger size of superoxide dismutase in comparison with *n*-propyl gallate or Tiron, which renders it less able to reach the site of $O_2^{\cdot-}$ production. In addition, it has been previously reported for other systems that enzymatically generated $O_2^{\cdot-}$ is not readily accessible to superoxide dismutase-catalysed dismutation [30].

A key feature of membrane deterioration in senescing plant tissues is loss of membrane phospholipid, which

becomes manifest as an increased membrane sterol–phospholipid ratio. This has been demonstrated for *Ipomoea* [31], rose flowers [3], bean cotyledons [29], bean leaves [6] and carnation flowers [4]. These observations imply a role for phospholipase in membrane deterioration. Measurements of phospholipase activity during the course of senescence in *Tradescantia* suggest that there is no increase in the activity of this group of enzymes [32], but increased phospholipase A activity has been reported for senescing rose petals [33]. It has also been demonstrated that $O_2^{\cdot}$ can bring about de-esterification of phospholipids by acting as a nucleophile [18]. Accordingly, the increased production of $O_2^{\cdot}$ by one or more membrane-associated oxidases with advancing senescence, documented in the present study, may well contribute to the breakdown of membrane phospholipid that is such a consistent feature of membrane deterioration. The free fatty acids so liberated may then be more prone to lipid peroxidation, a process that is known to rigidify membrane lipid [6, 19]. Thus, the effect of $O_2^{\cdot}$ on membrane fluidity may either be indirect through induced de-esterification or directly attributable to free radical attack (by $O_2^{\cdot}$ or the hydroxyl radical formed chemically from $O_2^{\cdot}$) on the membrane phospholipid.

EXPERIMENTAL

Plant material. Carnation flowers (*Dianthus caryophyllus* L. cv White-Sim; Yoder Atkin, Leamington, Ontario, Canada) were grown in a greenhouse and cut at a young stage after the petals had expanded *ca* 2 cm beyond the sepals. They were cut to 20 cm in length, placed in glass tubes containing deionized H_2O and maintained under continuous illumination at 22°. The flowers were used at three stages subsequent to cutting as follows: stage I, 1 day after being placed in H_2O when the flowers were only partially open; stage II, 3 days after being placed in H_2O when the flowers were mature and fully expanded but not showing any visual symptoms of senescence, such as petal in-rolling; stage III, 5–6 days after being placed in H_2O when the flowers were visibly senescent and showing extensive petal in-rolling.

Membrane isolation. 40 g of flower petals from 8–10 flowers were homogenized in a Sorvall omnimixer at 4° in 100 ml 0.01 M EPPS buffer, pH 8.5, for six periods of 10 sec with 30 sec intervening cooling periods. The resulting slurry was filtered through four layers of cheesecloth and rehomogenized with 15 strokes of a tight-fitting Potter–Elvehjem homogenizer. The final homogenate was centrifuged at 10 000 *g* for 20 min. The resulting supernatant was centrifuged at 131 000 *g* for 1 hr to yield a pellet of microsomal membranes. The pellet was resuspended to form a membrane suspension (2 mg protein/ml) in 0.002 M EPPS (pH 8.5). Protein was measured as described by Bradford [34].

ESR spectroscopy. ESR spectra were recorded at 30° with a Varian E-12 spectrometer equipped with a Varian variable temp. control accessory. Aliquots of the reaction mixture were placed in 100 μl capillary tubes sealed at one end, which were inserted into a quartz sample holder in the microwave cavity. A copper-constantan thermocouple was placed beside the capillary tube in the quartz sample holder. Spectra were recorded at a microwave power setting of 10 mW, microwave frequency of 9.164 GHz, time constant of 1 sec and modulation amplitude of 1.0 gauss.

Measurements of superoxide anion. For measurements of $O_2^{\cdot}$ production, Tiron, which was freshly prepared prior to each expt and protected from light, was brought to a final concn of 10 mM in a suspension containing 200 μg microsomal membrane protein/ml in 85 mM EPPS buffer (pH 8.5). The reaction was timed from the point at which the microsomal suspension and Tiron were mixed. Aliquots of the reaction mixture were placed immediately into the microwave cavity of the ESR spectrometer and the sample was scanned repeatedly at 1 min intervals for *ca* 15 min. Relative amplitudes of the Tiron radical spectra were determined by measuring the peak to peak height in cm of the low field hyperfine resonance.

In some expts, the membrane suspension was heat-denatured in boiling H_2O for 15 min, cooled on ice and used in the same manner as the regular membrane suspension. When required, *n*-propyl gallate was added directly to the reaction mixture at a final concn of 10 mM. For expts with superoxide dismutase, the microsomal suspension was preincubated with the enzyme (145 units/ml) for 15 min at 20° before the addition of Tiron. Modulation of pH was achieved by using two buffers: EPPS for the range pH 6.5–8.5 and CHES for the range 8.5–9.5. For temp. determinations, the membrane suspension and Tiron were pre-equilibrated to the specified temp. before being mixed together, and the temp. of the microwave cavity in the ESR spectrometer was adjusted accordingly.

Modulation of superoxide anion levels during in vitro *aging.* For *in vitro* aging studies, 0.1 ml microsomal membrane suspension was mixed with 0.9 ml EPPS buffer (final concn 68 mM), pH 8.5, and incubated in foam plugged tubes at 30° for up to 15 hr. Levels of $O_2^{\cdot}$ were modified during *in vitro* aging by the addition of 10 mM *n*-propyl gallate to the incubation medium or by the addition of 2 mM xanthine and 0.045 units xanthine oxidase [(EC 1.2.3.2) sp. act. 0.76 units/mg protein/ml] as described by Fridovich [25].

Measurements of membrane lipid fluidity. Membrane lipid fluidity was measured by steady-state fluorescence depolarization of DPH-labelled membranes in an SLM 8000 fluorescence spectrofluorometer as previously described [4]. Fluidity is expressed in terms of anisotropy parameter $[(r_0/r) - 1]^{-1}$ where r_0 and r are the limiting (0.362) and measured fluorescence anisotropy, respectively [23].

Chemicals. Xanthine oxidase, xanthine, DPH, EPPS, *n*-propyl gallate and superoxide dismutase were purchased from Sigma, Tiron from Fisher and CHES from Calbiochem Behring.

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