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# THE INFLUENCE OF CAROTENOIDS ON THE OXIDATIVE STABILITY AND PHASE BEHAVIOUR OF PLANT POLAR LIPIDS

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**Key Word Index**—*Nerium oleander*; Apocynaceae; polar lipids; liposomes; oxidation; phase transitions.

**Abstract**—Carotenoids and total neutral lipids from thylakoids of *Nerium oleander* were evaluated as antioxidants in liposomes prepared from soybean polar lipids. The extent of lipid oxidation was assessed from the formation of malondialdehyde and conjugated dienes after exposure of the liposomes to free radicals generated by $^{60}Co$ gamma radiolysis. The carotenoids incorporated into the bilayers were isolated from clones of oleander grown at 20° or 45°, growth conditions which are known to result in a difference in the thermal properties of the membrane lipids. The effect of carotenoids on the temperature of the phase transition of thylakoid polar lipids was also examined. The results showed that, in comparison with the effectiveness of a reference antioxidant, α-tocopherol, the carotenoids and total neutral lipids from thylakoids of oleander did not protect the soybean polar lipids from oxidation, nor did they influence the temperature of the phase transition of thylakoid polar lipids.

## INTRODUCTION

Photosynthesis is inhibited in most plants when their leaves are exposed to light intensities exceeding those at which the plants were grown [1]. However, for chilling-sensitive plants, photoinhibition also becomes evident when plants are chill-stressed and are exposed to relatively low light intensities [2]. It has been proposed that this chill-induced photoinhibition involves the formation of free radicals and their interaction with chloroplast membranes, presumably resulting in peroxidation of unsaturated lipids [1]. This view stems from the observation that injury to chloroplasts of tomato leaves can be alleviated by exogenous superoxide dismutase [3]. In addition, a chilling-sensitive strain of *Chlorella ellipsoidea* has been reported to contain less superoxide dismutase than a corresponding chilling-resistant strain [4], a finding consistent with this view. Since photoinhibition appears to involve peroxidation of membrane lipids [1], and β-carotene has been shown to protect bilayer lipids from peroxidation induced by singlet oxygen ($^1O_2^*$) [5–7], the carotenoids of chloroplast membranes might play an important role in protecting the polar lipids of thylakoid membranes from oxidation at chilling temperatures.

For chilling-sensitive plants, photoinhibition induced by low light intensities is accentuated at temperatures below that of the phase transition in the membrane lipids [2]. Thus the physical disordering of the lipids at chilling temperatures may augment the effects of light. Carotenoids have also been shown to depress and broaden the thermal phase transition of liposomes formed from pure phospholipids [8]. It is therefore possible that if carotenoids depress the phase transition of chloroplast membrane lipids they might indirectly reduce the susceptibility of plants to photoinhibition at chilling temperatures.

To investigate these possibilities, the effect of carotenoids, and the total neutral lipids of thylakoids, on the phase transition and oxidative stability of the polar lipids from thylakoids of *Nerium oleander* L. were studied. The transition temperature of polar lipids isolated from oleander thylakoids can be induced to occur at either −2° to 7° by growing the plants at 20° or 45°, respectively [9]. Thus, measuring the effect of carotenoids isolated from plants grown at 20°, on the phase behaviour of polar lipids from plants grown at 45°, avoids the uncertainties associated with the use of different plant species.

## RESULTS AND DISCUSSION

### *Irradiation conditions and assessment of peroxidation*

Initial studies were directed at determining the conditions for controlled irradiation as well as establishing a method of assessing the degree of lipid oxidation in liposomes. Gamma radiolysis of water at pH 4–11 produces a number of primary species which, in the presence of oxygen, result in the production of hydroxyl ($HO^\cdot$), perhydroxyl ($HOO^\cdot$) and superoxide ($O_2^{-\cdot}$) free radicals [10] which can react with the phospholipids of the lipsomes.

Two methods which are commonly employed to assess the extent of oxidation of lipids are based on (a) the formation of conjugated dienes associated with the peroxidation of unsaturated acyl chains during the propagation stage of oxidation, and (b) the generation of malondialdehyde, an end-product of lipid oxidation reactions. The formation of conjugated dienes and malondialdehyde in liposomes prepared from soybean polar lipids, as a function of radiation dose, is shown in Fig. 1. Both malondialdehyde and conjugated dienes increased with increasing radiation dose over the dose range used. Whereas malondialdehyde generation showed a linear

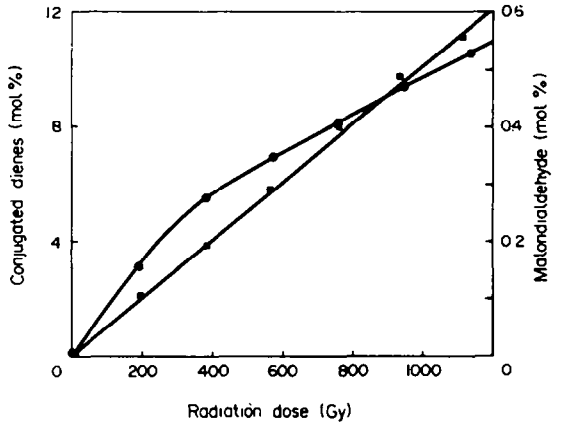


Fig. 1. Conjugated diene and malondialdehyde formation as a function of the radiation dose. Liposomes were irradiated with $^{60}$Co gamma rays at a dose rate of 47.31 Gy/min. Liposomes were prepared at a concentration of 1 mg phospholipid/ml in 20 mM Tris–acetate buffer, pH 7.2 (●) Conjugated diene development; (■) malondialdehyde generation.

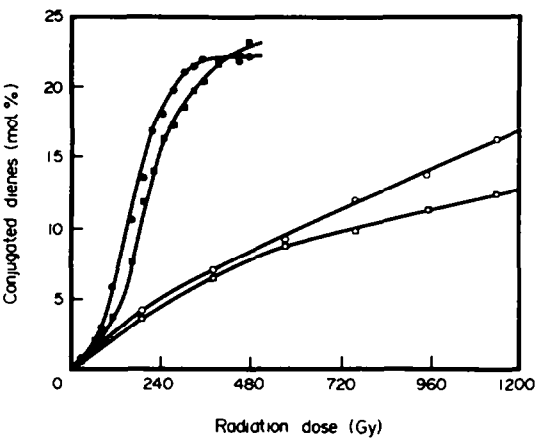


Fig. 2. Dependence of the rate of diene formation on the dose rate and type of buffer. Liposomes were prepared in 20 mM Tris–acetate (□) 5 mM Tris–acetate (○) 20 mM phosphate (■) and 5 mM phosphate (●). Each buffer was adjusted to pH 7.2 and irradiations were performed with $^{60}$Co gamma rays at a dose rate of 2.73 Gy/min.

increase with increasing radiation dose, the formation of conjugated diene was initially greater at low radiation dose than that of malondialdehyde generation, but above approximately 800 Gy it was less. This suggests that the oxidation products which contain the conjugated dienes are more labile to the continuing irradiation than the terminal product, malondialdehyde.

Initial irradiation studies also included a comparison of the effect of Tris and phosphate buffers. Although lipid oxidation was successfully induced in both of these buffers, in order to produce the same level of lipid oxidation, the liposomes suspended in Tris required an increased radiation dose. This could have been the result of two separate but interactive effects. The first is the possible interaction of HO· radicals with components of the medium in which the liposomes were suspended. For example, other studies with liposomes formed from unsaturated fatty acids [11] and rat liver microsomes [12] indicate that HO· radicals interact with Tris molecules. The second is the possibility that at high dose rates some of the species generated react with one another, or with relatively long-lived intermediates, causing termination of the chain reactions [13] and hence suppression of the propagation reactions associated with peroxidation. Both phenomena could therefore lead to a reduction in the dose of radiation absorbed by the target material. In preliminary experiments it was found that dose rates within the range 2.73–47.31 Gy/min did not produce significant differences in the course or extent of lipid oxidation. Therefore, in subsequent experiments, a dose rate of 2.73 Gy/min was used. Figure 2 compares the influence of two buffer types on the oxidation of the bilayer lipids. When the bilayers were prepared in phosphate buffer, a maximum diene formation of about 23 mol% resulted after irradiation with 400 Gy. However, when Tris buffer was used, no more than 15 mol% diene was generated after irradiation with about 1200 Gy. This indicated that some of the reactive species generated by gamma radiolysis had interacted with the Tris buffer. Because of this interaction, all subsequent experiments were performed using 5 mM phosphate buffer.

*Effectiveness of carotenoids as antioxidants*

α-Tocopherol is an effective scavenger of free radicals in biological systems [14], and was used in these studies as a reference for comparing the antioxidant effectiveness of the carotenoids and neutral lipids from thylakoid membranes when incorporated into liposomes of soybean polar lipids. As shown in Fig. 3, when α-tocopherol was incorporated into the bilayer of the liposomes, at a concentration of 0.4 mol%, it almost completely inhibited the formation of conjugated dienes over the range of radiation absorbed. In contrast, pure β-carotene, when incorporated at the same concentration, provided comparatively little protection against lipid oxidation. The effectiveness of various compounds as protective agents against radiation-induced lipid oxidation can be conveniently compared using the dose-reduction ratio [15]. This is the ratio of the dose required to produce a specific level of oxidation in a test sample, containing the protective agent, to that required to produce the same effect in an unprotected control. For the experiments described here, the level of oxidation used for comparison was 10 mol%. At this level, diene formation was directly proportional to the absorbed radiation dose. From the data in Fig. 3 the dose-reduction factor for β-carotene is 1.6 while for α-tocopherol it approaches infinity. Thus, although β-carotene was capable of providing some protection against radiation-induced lipid oxidation, it was not as effective as α-tocopherol. It was of interest to note that although the liposome suspensions were air-saturated before irradiation, β-carotene did not produce any autocatalytic or pro-oxidant effect, as has been reported by Burton and Ingold [16] when hyperbaric conditions were employed.

Although the results shown in Fig. 3 indicate that β-carotene did not effectively protect polar lipid bilayers from oxidation induced by HO·, HOO· and O·⁻ radicals, other studies [5–7] have shown it to be an efficient scavenger of singlet oxygen ($^1O_2^*$) generated from photodynamic reactions. The location of the carotenoids within the chloroplast membrane may therefore be an important

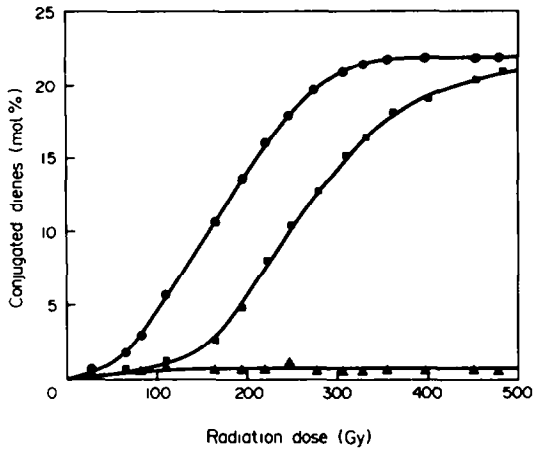


Fig. 3. Effect of β-carotene or α-tocopherol on diene formation Liposomes were irradiated with $^{60}Co$ gamma rays at a dose rate of 2.73 Gy/min. β-Carotene and α-tocopherol were incorporated into liposome membranes at a concentration of 0.4 mol % during their preparation in 5 mM phosphate–saline buffer, pH 7.2. (●) Control soybean polar lipid liposomes; (■) liposomes with incorporated β-carotene; (▲) liposomes with incorporated α-tocopherol.

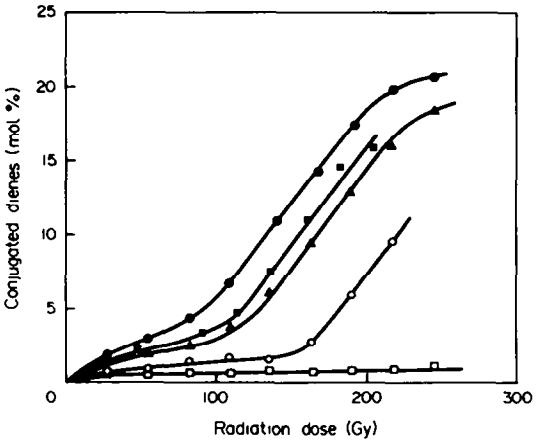


Fig. 4. Effect of carotenoids from oleander thylakoids on diene formation. Liposomes were irradiated with $^{60}Co$ gamma rays at a dose rate of 2.73 Gy/min. The mixed carotenoids were incorporated into liposome membranes at a concentration of 0.4 mol % and α-tocopherol at concentrations of 0.1 and 0.05 mol % during preparation of the liposomes in 5 mM phosphate–saline buffer, pH 7.2. (●) Control; (■) 0.4 mol % of 20°-grown oleander carotenoids; (▲) 0.4 mol % of 45° -grown oleander carotenoids; (○) 0.05 mol % α-tocopherol; (□) 0.10 mol % α-tocopherol.

factor in the maintenance of membrane integrity. The early removal of $^1O_2^*$ may also be important if there is a danger of other radicals, such as $HO^*$, being generated [17] and against which the carotenoids might have less antioxidant activity. Under the environmental conditions that inhibit photosynthesis and that cause the electron transport system of the chloroplast to become reduced, prolonged illumination can potentiate the formation of activated oxygen species and excited-state chlorophyll [1]. Under such conditions, a close association of carotenoid with the chlorophylls within the thylakoid, as documented by Anderson [18], might enable β-carotene to function more effectively as a radical scavenger at the site of radical production. That is, the relative inability of carotenoids to prevent oxidation in bilayers of soybean polar lipids might not be representative of their protective capacity in chloroplast membranes.

*Antioxidant effectiveness of oleander neutral lipids*

Following the evaluation of pure β-carotene, the antioxidant properties of the carotenoids and other neutral lipids from thylakoids of oleander plants grown at 20° or 45° were investigated. Oleander provides a convenient model because when grown at 20° or 45° the temperature of the phase transition of the thylakoid polar lipids occurs at −2° or 7°, respectively [9]. Accompanying this change, there is a corresponding increase in the susceptibility of the leaf tissue to chill-temperature induced photoinhibition [19]. If the insensitivity to photoinhibition shown by plants grown at 20° is due to the antioxidant properties of the carotenoids, or other neutral lipids, then the incorporation of carotenoids and neutral lipids from the 20°-grown plants into bilayers of soybean polar lipids should protect the lipids from oxidation.

Figure 4 shows the relation between diene formation and radiation dose in irradiated liposomes after a portion of the mixed carotenoids, from 20°- or 45° -grown oleander, was incorporated into the bilayers. For production of up to 3 mol % diene, the radiation dose required to produce 1 mol % was about 25 Gy. However, after the formation of 5 mol % diene, irradiation became more effective such that only about 7 Gy was required to effect the same change. This suggests that the antioxidant capacity of the carotenoids became depleted during the early phase of irradiation. Compared with the effectiveness of α-tocopherol, the incorporated carotenoids from either oleander clone produced little protection against lipid oxidation. The dose-reduction factors resulting from the incorporation of 0.4 mol % carotenoid, from 20°- and 45°-grown clones, were 1.2 and 1.3, respectively. For β-carotene and the oleander carotenoids the dose-reduction factors did not exceed 1.6 after 0.4 mol % incorporation (Figs. 3 and 4) whereas, for α-tocopherol, a factor of 1.7 was obtained after incorporation of only 0.05 mol % (Fig. 4). Therefore, both β-carotene and the oleander carotenoids were approximately eight times less effective than α-tocopherol. Similar results were obtained when a portion of the total neutral lipid fraction was incorporated into the soybean liposomes. There was no evidence that the carotenoids, subfractions, or the total mixed neutral lipids from the 20°-grown plants were more effective antioxidants than those isolated from the 45°-grown plants.

The observation that the neutral lipid fractions from the 20°-grown oleander plants did not provide greater antioxidant protection than similar fractions from the 45°-grown plants suggests that the carotenoids do not have a significant role in scavenging the free radicals generated by oxidation reactions within the lipid bilayer of the chloroplast membrane.

*Differential scanning calorimetry*

If carotenoids play a role in protecting membrane lipids from oxidation at chilling temperatures through a mechanism of lowering the temperature of the phase transition, it should be possible to demonstrate such an effect in the isolated polar lipids from 45°-grown plants by addition of carotenoids, or total neutral lipids, from the 20°-grown plants.

Figure 5 shows thermograms of polar lipids extracted from thylakoids of oleander grown at 45°. Trace (a) shows that the exothermic transition is initiated at 7° and, as shown in Table 1, the enthalpy of the transition ($\Delta H$), between 7° and −10°, was 6.18 mJ for the 7.32 mg of lipid contained in the calorimeter pan. Assuming an average enthalpy of 25 kJ/mol for the mixed polar lipids [9], it was calculated that if all the lipid was transformed (via a first-order phase transition) from a liquid crystalline to a gel phase, the heat evolved would be 244 mJ. Thus, it can be calculated that at −10°, only 2.5% of the polar lipids were in the gel phase. Trace (b) of Fig. 5 shows that when carotenoids from the 20°-grown plants were mixed in the proportion of 1 mol% with polar lipids from the 45°-grown plants, the temperature of the transition did not change significantly. It is unlikely that the amount of carotenoid incorporated was excessive and caused disruption to the bilayers since other related studies [8] have successfully incorporated 4 mol%. The fact that the carotenoids did not alter the phase transition of the polar lipids indicates their role in altering the phase transition of membranes would be minor. This would be consistent with the location of carotenoids being within the thylakoid where they are bound to protein and in close association with the chlorophylls [18]. Table 1 further demonstrates that by mixing 1 mol% β-carotene, α-tocopherol or oleander carotenoids (isolated from leaf chloroplasts of plants grown at 20° or 45°) with the polar lipids from leaf chloroplasts of plants acclimated at 45°, neither the temperature of the transition (7°) nor the proportion of lipid involved in the transition, to −10°, is altered.

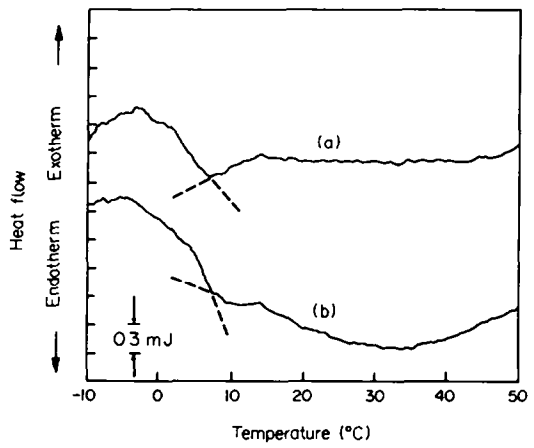


Fig. 5. Thermograms of oleander thylakoid polar lipids. Polar lipids from (a) 45°-grown oleander and (b) 45°-grown oleander mixed with carotenoids from 20°-grown oleander. The calorimeter trace was obtained at a cooling rate of 10°/min and a sensitivity of 0.2 mcal/s for the amounts of lipid presented in Table 1.

## GENERAL CONCLUSION

When liposomes of soybean polar lipids were exposed to gamma radiation, both conjugated diene and malondialdehyde concentrations increased with increasing radiation dose showing that, under these conditions, the plant polar lipids were oxidized. The extent of lipid oxidation induced by radiation exposure was dependent on the type of buffer used in the preparation and suspension of the liposomes. Incorporation of α-tocopherol effectively inhibited lipid oxidation, whereas pure β-carotene provided comparatively little protection. Neither the carotenoids nor the neutral lipids extracted from oleander grown at either 20° or 45° provided liposomes with any significant protection against lipid oxidation induced by gamma radiation. Nor did these extracts, when combined with the polar lipids isolated from 45°-grown plants, alter the temperature of the phase transition.

If photoinhibition at chilling temperatures is associated with uncontrolled free radical reactions, the supposition that carotenoids provide membranes with effective protection against lipid oxidation cannot be substantiated. In addition, the resistance to photoinhibition by 20°-grown oleander cannot be attributed to a lowering of the temperature of the phase transition by carotenoids, or other neutral lipids, of the chloroplast membrane.

Table 1. Effect of neutral lipids on the enthalpy and temperature of the phase transition of 45°-grown oleander polar lipids

| Addition | Transition point* (°) | Lipid (mg) | Enthalpy of transition (mJ) | Total expected enthalpy† (mJ) | Estimate of lipid in gel phase (%) |
|---|---|---|---|---|---|
| None (control) | 7.0 | 7.32 | 6 | 244 | 2.5 |
| 1 mol% carotenoids from plants grown at 20° | 7.5 | 6.86 | 6 | 228 | 2.5 |
| 1 mol% carotenoids from plants grown at 45° | 7.0 | 6.08 | 6 | 203 | 2.8 |
| 1 mol% β-carotene | 7.0 | 6.83 | 9 | 228 | 3.9 |
| 1 mol% α-tocopherol | 7.0 | 6.37 | 5 | 212 | 2.3 |

*Transition detected by differential scanning calorimetry, examples of which are shown in Fig. 5.
†An average $M_r$ of 750 was assumed for the mixed polar lipids from oleander.

## EXPERIMENTAL

*Plants.* Oleander (*Nerium oleader*) plants were cloned from a single genotype. The plants were raised in growth cabinets at 20°/15° (day/night) or 45°/32° temps. at a light intensity of 400 $\mu$E/s/m$^2$.

*Reagents.* The buffers used in radiolysis expts were prepared from recrystallized AR-grade chemicals and using glass distilled water which had been further purified by passage through a Milli-Q (Millipore, Sydney) ion exchange/charcoal filtration unit. All prepared solns were filtered through 0.2 m Millipore filters. Soybean polar lipids were prepared from soybean lecithin (Sigma) using silicic acid chromatography [9] to remove the neutral lipids. $\beta$-Carotene was obtained from Roche Products and $\alpha$-tocopherol from Calbiochem.

*Isolation of oleander neutral and polar lipids.* Thylakoids were isolated and the lipids extracted as described by Raison *et al.* [20]. Fractions containing total neutral or polar lipids were separated by CC using silica gel as described by Raison and Wright [9]. A portion of the total neutral lipids was further separated to obtain subfractions of carotenoids. The presence of carotenoids in these fractions was confirmed by spectral analysis [21]. In some expts the subfractions were pooled to produce a total, mixed carotenoid fraction. All separation procedures were conducted under subdued lighting and using filters which eliminated wavelengths below 340 nm. All fractions were stored at −20° under $N_2$ until required for use.

*Preparation of liposomes.* Liposomes were prepared from soybean polar lipids in either 20 mM Tris–acetate buffer, pH 7.2, or 5 mM phosphate buffer, pH 7.2. The phosphate buffer also contained 25 mM NaCl, 1 mM KCl and 0.15 mM $MgCl_2$. The lipids were initially solubilized in $CHCl_3$ then combined with carotenoids or $\alpha$-tocopherol (also in $CHCl_3$) in the required proportions. An average $M_r$ of 750 was assumed for soybean polar lipids and the amount of protective material incorporated into the liposomes was expressed as mol%; that is, the number of molecules of introduced material per 100 molecules of soybean polar lipids. The amount of carotenoid incorporated into the bilayers was chosen after examination of a range of liposome suspensions containing various concns of $\beta$-carotene. Stable suspensions were obtained using up to 4.0 mol%, an amount also used by Yamamoto and Bangham [8]; however, beyond this amount aggregation was found to occur. Since an ideal antioxidant should be effective at low concn, and in order to avoid disruption of the bilayer structure, a level of 0.4 mol% was used, unless otherwise stated. Solvent was removed from the lipid mixtures by vacuum and the lipid dispersed in buffer by sonication under $N_2$ for 3 min using a Bransen B12 sonicator. The liposome suspension (10 mg lipid/ml) was diluted with a buffer to a final concn of 1 mg/ml and sonicated for a further 1 min. During this procedure, $N_2$ was bubbled through the suspension and the temp. was maintained at 30°.

*Gamma irradiation.* Liposomes were irradiated using a $^{60}$Co gamma source. The suspensions were saturated with air then exposed to radiation at a dose rate of 2.73 Gy/min, unless otherwise stated. Dose rate was measured by $FeSO_4$ dosimetry using Fricke soln [22] having $G(Fe^{3+})$ equal to 15.5 ions per 100 eV.

*Determination of lipid oxidation.* Samples of irradiated liposome suspension were diluted 1:7 (v/v) with MeOH and the concn of conjugated dienes calculated from absorbance measurements at 234 nm and using $\varepsilon = 2.8 \times 10^4$ [23]. The presence of malondialdehyde was determined using a modification of the method by Ohkawa *et al.* [24]. Portions (0.5 ml) of irradiated liposome suspension were mixed with 0.2 ml aq. 8% (w/v) sodium dodecyl sulphate, 1.0 ml 20% (v/v) HOAc and 1.0 ml 0.8% (w/v) thio-barbituric acid, then heated for 60 min at 90° in sealed tubes. After cooling, 2 ml of 6.25% (v/v) $C_5H_5N$ in BuOH was added, the tube contents mixed, then centrifuged for 10 min at 800 *g*. Malondialdehyde concn in the organic layer was calculated from absorbance measurements at 532 nm and using $\varepsilon = 1.48 \times 10^5$ [25].

*Differential scanning calorimetry* was performed using a Model DSC-2 differential scanning calorimeter (Perkin–Elmer Corp., Norwalk, CT) as described by Raison and Wright [9]. Polar lipids from oleander leaf thylakoids, together with any added component as indicated, were dissolved in $CHCl_3$ and placed in stainless-steel pans (170 $\mu$l). Solvent was removed under vacuum and 150% (v/v) of 5 mM Pi buffer, pH 7.2, added. The pans were sealed and the samples equilibrated for at least 2 hr at 36° before examination by calorimetry.

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