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Interactions of arbutin with dry and hydrated bilayers

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Abstract

The glycosylated hydroquinone arbutin (4-hydroxyphenyl-β-D-glucopyranoside) is abundant in certain resurrection plants, which can survive almost complete dehydration for prolonged periods. Little is known about the role of arbutin in vivo, but it is thought to contribute toward survival of the plants in the dry state. We have investigated the interactions of arbutin with model membranes under conditions of high and low hydration, as well as the possible participation of arbutin in carbohydrate glasses formed at low water contents. Retention of a trapped soluble marker inside large unilamellar vesicles and fusion of vesicles was monitored by fluorescence spectroscopy. Effects of arbutin on glass-transition temperatures and hydrated membrane phase-transition temperatures were measured by differential scanning calorimetry. The possible insertion of arbutin into membrane bilayers was estimated by following arbutin auto-fluorescence. Evidence is presented that arbutin does not change the glass-transition temperature of a sucrose/trehalose glass, but that arbutin does interact with hydrated membranes by insertion of the phenol moiety into the lipid bilayer. This interaction causes increased membrane leakage during air-drying by a mechanism other than vesicle–vesicle fusion. Implications of these effects on the dehydrated plant cells, as well as possible methods of obviating the damage, are discussed. © 1998 Elsevier Science B.V.

Keywords: Hydroquinone; Membrane bilayer; Liposome; Dehydration; Carbohydrate glass

1. Introduction

Arbutin (4-hydroxyphenyl-β-D-glucopyranoside) is a glycosylated hydroquinone, which consists of a phenol molecule with a glucose moiety in the para-position (see Fig. 1). Arbutin is well known as a substrate of the enzyme β-glucosidase [1] and also for its diuretic and urinary anti-infective properties. It has been found in the leaves of several plant species, such as *Vaccinium* sp. [2], but its physiological role in these plants is unknown. Arbutin constitutes as much as 25% of the dry weight in the leaves of *Myrothamnus flabellifolia* Welw. [2,3], a desiccation tolerant angiosperm that is found in regions of southern Africa subject to prolonged drought conditions. It is thought to play an important role in resistance to environmental stress, as it is present in several diverse plant taxa capable of withstanding such stresses as extreme low temperature and extended drought [[2], and references therein]. Although the possible contribution of arbutin to freezing-tolerance is also under current investigation, it is outside the scope of a single paper and will be reported separately.

In general, resurrection plants are able to survive nearly complete dehydration for extended periods of

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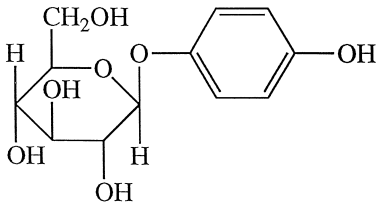

Fig. 1. Line drawing of the structure of arbutin.

time [see Ref. [4], for a review]. Although the precise mechanisms by which resurrection plants protect themselves from this severe environmental stress are not fully understood, certain adaptations are known to contribute to anhydrobiosis, or life without water. For instance, most anhydrobiotic organisms, including yeast and *Artemia*, accumulate trehalose in their cells prior to or during drying [5], and references therein]. Sucrose is considered to be the main functional analog of trehalose in the desiccation-tolerant seeds and pollen grains of angiosperms [6–8]. Dry *M. flabellifolia* leaves contain both, trehalose and sucrose [3,9] – an unexpected finding – as trehalose is not usually found in higher plants [6].

On account of the accumulation of disaccharides in desiccation-tolerant organisms, two independent protective effects may be observed. First, the sugars hydrogen bond with the phosphate groups on lipid molecules of the membrane in the dry state, thereby replacing water molecules. This reverses the tendency of the membrane lipids to go from the liquid crystalline into the gel phase as water is removed [5] (or, for a review, see Ref. [10]). The gel-to-liquid crystalline phase-transition temperature (T_m) of a membrane dried with sugar is similar to that of the hydrated membrane [11,12]. The bilayer is maintained in the liquid crystalline state during all phases of drying and rehydration, as has been shown in model, yeast, and bacterial systems [11–15]. Thus, catastrophic leakage of cell solutes associated with passage through the membrane phase transition is avoided [14,16–18].

A second protective effect of the disaccharides is their ability to vitrify at low water contents, forming a carbohydrate glass [19–23]. This glass is a high-viscosity amorphous solid, which prevents a host of time-dependent and diffusion-limited deterioration processes, primarily due to the extremely low molecular mobility within the glass [24]. At a characteristic glass-transition temperature (T_g), the glass undergoes a second-order transition to a more liquid, rubbery state, which is not protective [25–27]. Thus, it is important to maintain the dry sample at temperatures below T_g, in order to gain the protective effects of vitrification. This observation explains the accumulation of disaccharides rather than monosaccharides in the tissues of anhydrobiotic organisms, for although monosaccharides also form glasses, their T_gs are so low that, at ambient temperatures, the samples would almost always be devitrified [23,26] (and, for a review, [22]).

Recent work has shown that, in order to achieve full protection from dehydration, carbohydrate glass formation as well as maintenance of the membrane in the liquid crystalline state during drying and rehydration are required [28,10]. Since both these requirements would most likely be fulfilled by the trehalose and sucrose present in the dry *M. flabellifolia* leaves, the accumulation of large quantities of arbutin presents a puzzle. One possible function of arbutin could be the inhibition of membrane degradation. It has been shown previously that arbutin can act as an anti-oxidant [29] and can also inhibit phospholipase A_2 (PLA_2) in partially dehydrated systems [30]. However, as it is present in such large quantities in the dehydrated *M. flabellifolia* leaves (twice as much as trehalose [3]), arbutin warrants investigation into its interaction with the membrane, which may reveal other protective properties as well. The present study focuses on the interaction of arbutin with dry and hydrated bilayers, in a combined effort to elucidate arbutin's possible contribution to dehydration-resistance, and the physical characteristics of the membrane/arbutin system.

2. Materials and methods

2.1. Preparation of liposomes

Dipalmitoylphosphatidylcholine (DPPC), dielaidoylphosphatidylcholine (DEPC), dimyristoylphosphatidylcholine (DMPC), and 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Lipid was dried to a film

under nitrogen gas and placed under vacuum overnight to remove residual chloroform. Deionized water was added to the lipid, which was warmed and agitated gently to produce multilamellar vesicles. Large unilamellar vesicles (LUVs) were formed by extrusion with a hand-held extruder (Avestin; Ottawa, ON, Canada; [31]), using 0.1 μm pore polycarbonate filters (Poretics; Livermore, CA). Alternatively, sonicated vesicles were prepared by bath sonication (Laboratory Supplies, Hicksville, NY) of multilamellar vesicles until a clear suspension was obtained.

2.2. Differential scanning calorimetry

Glass transitions were measured using a Perkin–Elmer differential scanning calorimeter (DSC-7), cooled by a liquid-nitrogen reservoir. Carbohydrate samples (~ 2–5 mg) from stock solutions of 100 mg/ml were dried for several hours in small aluminum pans, in a dry box maintained at 0% relative humidity (RH). The pans were then either pressure-sealed inside the dry box or transferred to RH chambers, maintained at specific relative humidities over saturated salt solutions [32], overnight before sealing. The DSC-7 head was flushed with He, and the box with dry N_2. Carbohydrate samples were scanned between −100° and 200°C at 20°C/min on upscans and 100°C/min on downscans. Sample pans were weighed on a Cahn C-33 electrobalance before, and after DSC scanning to ensure that no water was lost during the procedure. Sample pans were then punctured and incubated at 120°C for at least 24 h and re-weighed. This incubation and re-weighing step was repeated until the sample weight remained constant for two consecutive days, thereby allowing accurate calculation of sample hydration during the DSC scan.

Gel-to-liquid crystalline phase transitions of hydrated membranes were measured with a second high sensitivity DSC from Calorimetry Sciences, temperature controlled by a Peltier device. Aqueous suspensions of DPPC, DMPC, or DEPC LUVs (0.5 ml of 40 mg/ml) in different arbutin concentrations were scanned in sealed ampules between 5° and 50°C at a rate of 20°C/h, and T_ms detected as the maxima of sharp endothermic peaks on thermal upscans.

2.3. Measurement of retention

Large unilamellar vesicles (LUVs) were prepared as described by extrusion in the presence of 100 mM carboxyfluorescein (CF, Molecular Probes, Eugene, OR; purified according to Weinstein [33]). External CF was removed by column chromatography (Sephadex G 50–80, 1 × 20 cm). Trehalose, sucrose (high purity disaccharides obtained from Pfanstiehl Laboratories, Waukegan, IL), arbutin (Fluka, Buchs, Switzerland: biochemistry grade), or a combination of excipients were added to the liposome suspension at various concentrations, as stated in the text. Liposome samples (10 μl) were placed into the lids of 1.5 ml microfuge tubes, and air-dried for at least 5 h in a dry box, maintained at 0% RH. The samples were then transferred to a Virtis Freezemobile 25SL freeze dryer (Gardiner, NY) and stored overnight under a 10 mtorr vacuum. Retention of carboxyfluorescein was either measured immediately, or after incubation at 22°C and 58% RH over a saturated solution of NaBr [32] for a specified length of time. After re-hydration with water, a small volume of LUVs (5 μl) was transferred to a cuvette containing 2.5 ml 10 mM TES, pH 7.5. Samples were excited at 460 nm, and emission was measured at 550 nm on a Perkin-Elmer LS-5 fluorescence spectrophotometer. Intravesicular CF concentrations are self-quenching, so the emission at 550 nm was due only to external CF. After an initial fluorescence measurement was taken, 50 μl of 1% Triton X-100 was added to each sample to cause total (100%) leakage. A final fluorescence measurement was taken at this point. Percent retention was calculated from the following two equations:

$$\%\ \text{Trapped} = \frac{\text{final fluorescence} - \text{initial fluorescence}}{\text{final fluorescence}} \times 100 \quad (1)$$

$$\%\ \text{Retention} = \frac{\%\ \text{trapped after drying}}{\text{trapped before drying}} \times 100 \quad (2)$$

2.4. Measurement of fusion

Fusion during air-drying was measured according to the method of Struck et al. [34]. Briefly, liposomes

were prepared, as already described, at 20 mg/ml in the absence, and presence of 0.5 mol% each of N-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE) and N-(lissamine Rhodamine B sulfonyl)dioleoylphosphatidylethanolamine (Rh-PE), which were purchased from Molecular Probes (Eugene, OR). Following extrusion, liposomes were combined at a ratio of 1:9 (labeled:unlabeled), air-dried, and stored as described. In order to measure fusion, dried samples were re-hydrated and mixed into 2.5 ml 10 mM TES, pH 7.5. Fluorescence measurements (excitation 450 nm, emission 530 nm) were made in the absence (F), and in the presence (F_0) of 50 μl 1% Triton X-100. The energy transfer efficiency (E) was calculated as follows:

$$E = 1 - F/F_0 \quad (3)$$

This energy-transfer efficiency was calculated for both unfused control vesicles (E_0), and for the dried experimental samples (E_s). Percent fusion was calculated as follows:

$$\%\ \text{Fusion} = 100 - \{(E_s/E_0) \times 100\} \quad (4)$$

Thus, fusion is detected as the decrease in energy transfer between the two fluorophores, as fused vesicles constitute a dilution of the probes by unlabeled lipid molecules [34].

2.5. Arbutin auto-fluorescence

Certain aromatic amino acids, such as tryptophan or tyrosine, will emit fluorescence when excited at 280 nm [35]. The fluorescence emission maximum will shift when these amino acids enter a hydrophobic environment. This phenomenon has been utilized in many studies to investigate the insertion of peptides or proteins containing these residues, into a lipid membrane [36,37]. Using aqueous quenchers such as $NaNO_3$, it is also possible to estimate the relative depth of insertion of a protein into a membrane, by measuring the protection from quenching afforded by the hydrophobic environment of the lipid phase [37,38]. Since arbutin also contains an aromatic ring structure, we conducted analogous experiments with this hydroquinone. To an aqueous solution of 4 μM arbutin in 10 mM TES (pH 7.5), either in the presence or absence of liposomes (0.4 mM), 5 μl aliquots of either buffer (10 mM TES) or $NaNO_3$ (5 M) were added stepwise, and the fluorescence (excitation 280 nm, emission 340 nm) was measured at each point. An emission wavelength of 340 nm was used in order to preferentially observe arbutin in the hydrophobic environment of the membrane. Fluorescence in the presence of buffer (F_0) and fluorescence in the presence of $NaNO_3$ (F) were used to measure the extent of quenching by the following calculation:

$$\text{Quenching} = F_0/F - 1 \quad (5)$$

Stern–Volmer plots were constructed by plotting the concentration of $NaNO_3$ against F_0/F-1 [36,38]. The slope of this plot yields the quenching constant (K_{sv}), which provides information about the extent of protection from quenching afforded arbutin by the liposomes and, thus, about insertion of the arbutin molecule into the membrane bilayer [37,38].

3. Results and discussion

3.1. Arbutin does not change T_g of the carbohydrate glass

As a first hypothesis, we considered that arbutin might participate in the carbohydrate glass formed at

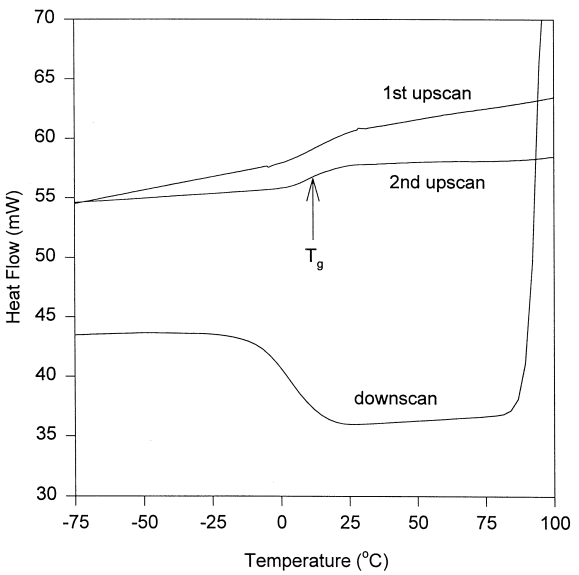

Fig. 2. Typical DSC thermograms showing the glass transition of a carbohydrate glass composed of sucrose, arbutin, and trehalose in a 3:2:1 mass ratio. The water content of this sample was 0.07 g water/g dry weight.

low water contents. If arbutin significantly raised the glass-transition temperature of this glass, the plant would be better protected during re-hydration, as more water could be adsorbed by the plant tissues before T_g dropped below the ambient temperature, resulting in devitrification of the glass [39–41].

Glass-transition temperatures were measured by differential scanning calorimetry in carbohydrate glasses composed of sucrose, arbutin and trehalose in the mass ratio of 3:2:1, which mimics the sugar composition in dry *M. flabellifolia* leaves [3]. Typical DSC data are shown in Fig. 2. The T_g of this combination–carbohydrate glass can be easily detected by a change in heat flow on both, up- and downscans, and T_g was taken as the midpoint of the shift. Crystallization of arbutin was prevented in this carbohydrate mixture as indicated by the disappearance of the characteristic arbutin crystalline melt at 199°C, which is clearly visible in thermal scans of pure arbutin samples (data not shown). This finding suggests that arbutin may participate in the carbohydrate glass.

T_g values were measured at various water contents, allowing construction of a state diagram (Fig. 3). The state diagrams shown for pure trehalose and sucrose were taken from the literature [21,25], and are plotted as solid lines. The state diagram for a sucrose and trehalose combination (mass ratio 3:1 of sucrose:trehalose) is also shown, along with that of the sucrose/arbutin/trehalose mixture. As expected, the state diagram for the mixture of sucrose and trehalose falls in an intermediate range between the sucrose state diagram and that for trehalose. This effect has been previously documented with a wide

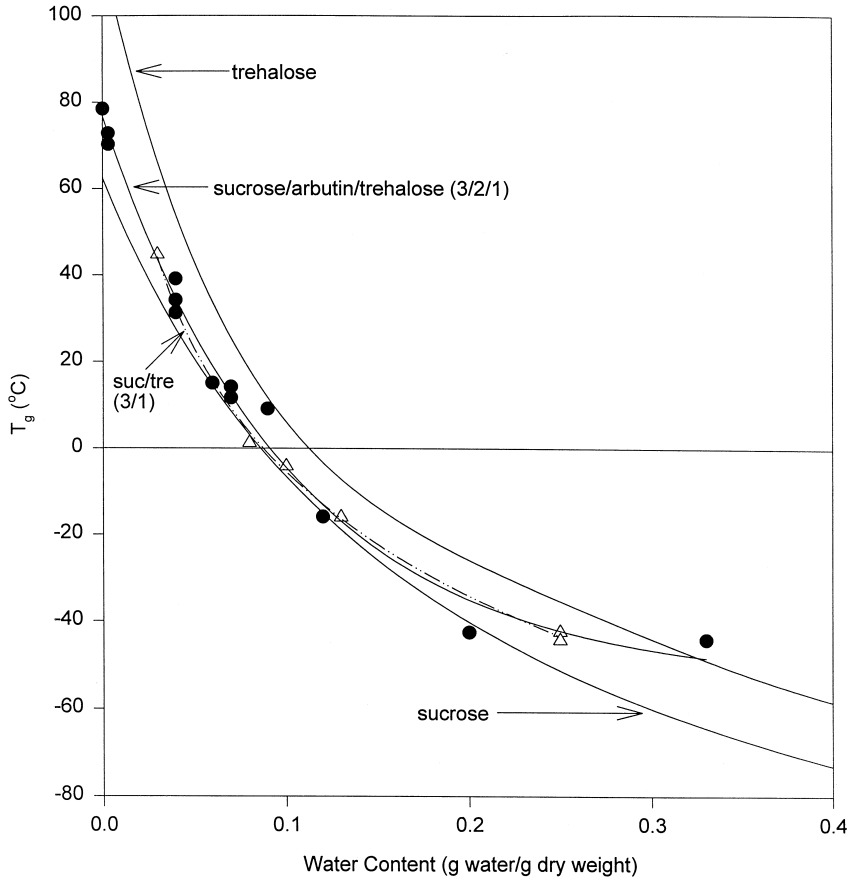

Fig. 3. State diagrams for sugar and sugar-mixture glasses showing decreasing glass-transition temperatures with increasing water contents. State diagrams for sucrose and trehalose were taken from Sun et al. [25] and Crowe et al. [21], respectively. The sucrose/trehalose (mass ratio 3/1) glass-transition temperature data points (△) were fitted with the broken line ($r^2 = 0.99$). The sucrose/trehalose/arbutin (mass ratio 3/2/1) glass-transition temperature data points (●) were fitted with the solid line ($r^2 = 0.99$).

variety of other sugar mixtures [42,43]. The interesting finding, however, is that the state diagram for the sucrose/arbutin/trehalose combination is nearly identical to that of the sucrose/trehalose mixture. This result indicates that, although arbutin may participate in the glass, it did not change the T_g of the sucrose/trehalose combination–carbohydrate glass.

3.2. Arbutin causes membrane leakage during air-drying

Since arbutin was found not to change T_g of the carbohydrate glass, we investigated the possibility that it might protect the dry membranes from leaking in a manner similar to that of the disaccharides [44]. This hypothesis was not unlikely, as arbutin had already been shown to depress the phase-transition temperature (T_m) of dry phospholipid bilayers as do sucrose and trehalose [30]. POPC LUVs were air-dried in the presence of various combinations of disaccharides and arbutin, and retention was measured immediately after re-hydration (Fig. 4A). Arbutin alone provided no protection to the liposomes, however, as samples dried with only arbutin showed the same negligible retention as those dried without any sugar present (Fig. 4A, samples E and F). Further, arbutin actually induced membrane leakage in liposomes that would have otherwise been protected by the disaccharides present (Fig. 4A, sample D compared to sample C; sample G, compared to samples A and B). The % retention of the liposome samples were followed for 8 h at 22°C and 58%RH, and they did not change significantly from measurements at time zero.

A dose–response effect of the different excipients on CF retention after air-drying is shown in Fig. 4B. It is clear that either sucrose or trehalose, or a combination of the two, provided maximum protection of the liposomes at an initial aqueous concentration of ≈30 mg/ml. When arbutin was present, however, either alone or in combination with the disaccharides, no significant CF retention was achieved at any initial concentration.

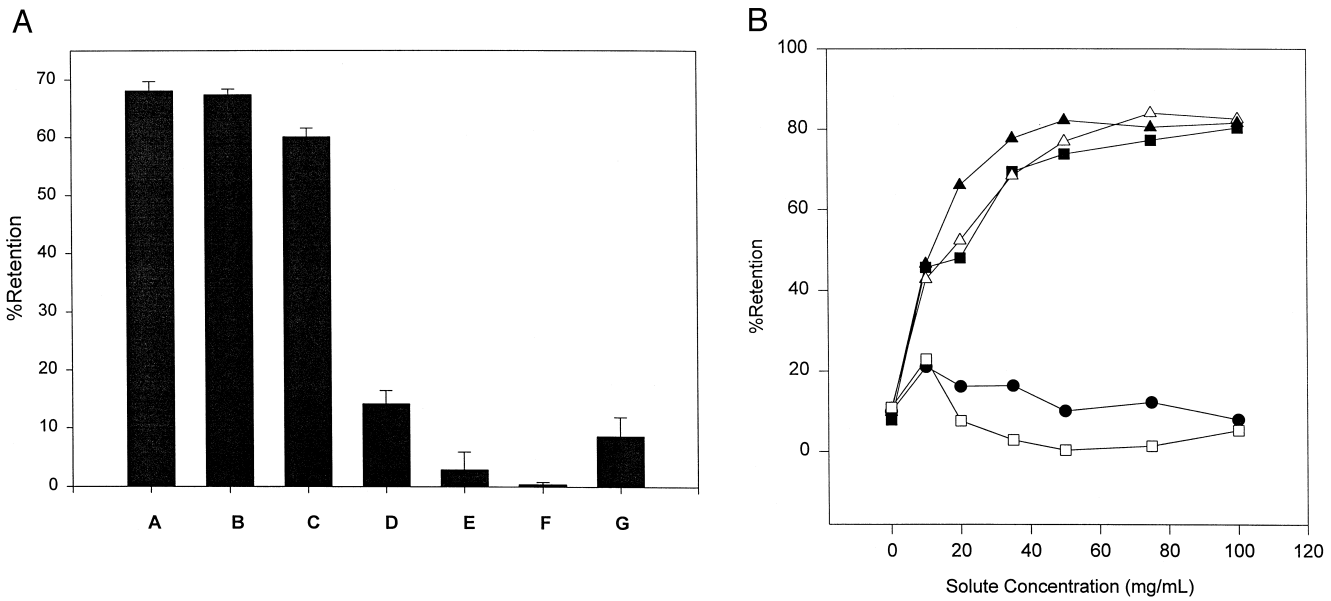

Fig. 4. A – Carboxyfluorescein retention (± S.D., $n = 3$) of air-dried POPC LUVs in sugar or combination of sugars. In all samples, the lipid concentration before drying was 10 mg/ml. Excipients were present in initial concentrations as follows: (A) – 50 mg/ml trehalose; (B) – 50 mg/ml sucrose; (C) – 33 mg/ml sucrose/trehalose (3/1 mass ratio); (D) – 50 mg/ml sucrose/arbutin/trehalose (3/2/1 mass ratio); (E) – no sugar; (F) – 50 mg/ml arbutin; and (G) — 50 mg/ml sucrose/trehalose (3/1 mass ratio) + 25 mg/ml arbutin. B – Dose–response curve showing carboxyfluorescein retention of POPC LUVs, air-dried in the presence of various combinations and concentrations of solute. In all samples, the lipid concentration before drying was 10 mg/ml. The solute concentrations shown represent initial concentrations before air-drying: (▲) – trehalose; (△) – sucrose; (■) – sucrose/trehalose (3/1 mass ratio); (●) – sucrose/arbutin/trehalose (3/2/1 mass ratio); and (□) – arbutin.

3.3. Arbutin is non-fusogenic

The negligible retention seen in liposomes air-dried in the presence of arbutin raised the possibility that arbutin might cause fusion, inducing nearly complete leakage in the liposome samples. Fusion was measured, using a resonance energy-transfer assay, according to the method of Struck et al. [34] as described in Section 2. POPC LUVs were air-dried in the presence of sucrose, trehalose, arbutin, or a combination of all three, and fusion was measured immediately after re-hydration (Fig. 5). Although arbutin, by itself, did not prevent fusion, its presence in the combination with sucrose and trehalose also did not cause fusion in the liposome samples.

The percentages of fusion of the liposome samples were also followed for 8 h at 22°C and 58% RH, and they did not change significantly from the measurements at time zero. The finding that neither CF leakage nor vesicle–vesicle fusion changed significantly over time at 58% RH, provides additional evidence that the effects of arbutin are unrelated to its

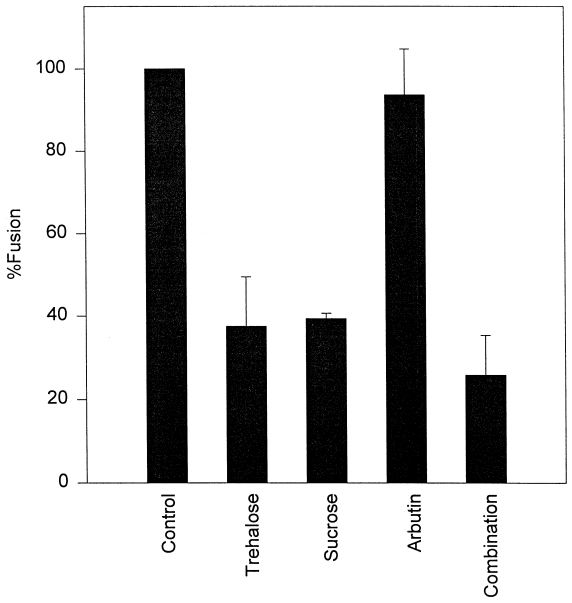

Fig. 5. Fusion in POPC LUVs, air-dried in the presence of different excipients. In all samples, the concentration of lipid before drying was 10 mg/ml. The initial solute concentration before air-drying was 50 mg/ml for each. In the control sample, no sugar was present. Experimental samples contained trehalose, sucrose, arbutin, or the combination of sucrose/arbutin/trehalose (3/2/1 mass ratio).

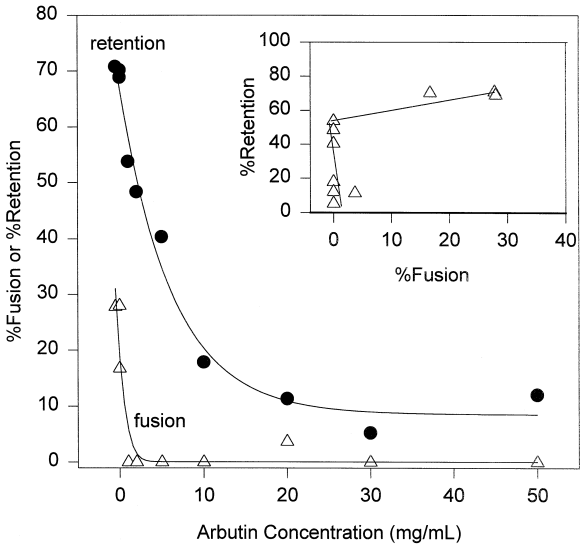

Fig. 6. Dose–response curve showing the effects of increasing arbutin concentration on (△) fusion and (●) CF retention of air-dried POPC LUVs. In all samples, initial lipid concentration before air-drying was 10 mg/ml, and the initial trehalose concentration was 50 mg/ml. The concentration of arbutin before air-drying was varied as shown. Inset: lack of correlation between fusion and retention in this study. This plot of CF retention as a function of fusion shows the presence of arbutin in the carbohydrate glasses caused a decrease in CF retention that was not due to vesicle–vesicle fusion.

effect on the carbohydrate glass-transition temperature. If arbutin were compromising the glass in some way, then, as water was adsorbed by the liposome samples during incubation at elevated relative humidity, the glass would devitrify, and the CF retention and/or fusion values would be expected to change.

A summary of the effects of arbutin on liposome CF retention and fusion during air-drying is presented in an arbutin dose–response curve (Fig. 6). Trehalose was present in all samples at an initial aqueous concentration of 50 mg/ml, and the arbutin concentration was subsequently varied. The inset shows the total lack of correlation between fusion and decreasing retention in these experiments. In fact, the samples showing the highest level of fusion also had the highest retention. Although we do not have a satisfactory explanation for this effect, it is clear evidence that arbutin caused leakage by some mechanism other than vesicle–vesicle fusion.

It is interesting to note that arbutin, in combination with the disaccharides, caused a moderate decrease in

fusion compared to samples with only trehalose or sucrose (Figs. 5 and 6). Although it could be argued that this is a protective effect, the principal benefit of reducing fusion is the improvement of membrane integrity, leading to an increase in % retention. Since the CF retention was decreased so drastically, even by the lowest concentrations of arbutin, this small reduction in fusion seems unlikely to provide any significant protection to dry membranes. In addition, the possibility that arbutin might interfere with the fusion assay was eliminated by control experiments showing that arbutin had no fluorescent properties in the range of either fluorescent probe used (data not shown).

3.4. Arbutin decreases T_m of hydrated lipid membranes

Since arbutin caused leakage but not fusion in the air-dried liposomes, we investigated the possibility that arbutin might interact directly with the lipid bilayer, which would help to explain the decrease in CF retention seen in liposomes dried in the presence of arbutin. Fig. 7 shows that arbutin decreased the phase-transition temperature, as measured by DSC, in hydrated LUVs composed of DPPC, DMPC, or DEPC, in a dose-dependent manner. The T_m values in the absence of arbutin were 42.7°C for DPPC, 25.6°C for DMPC, and 12.9°C for DEPC, which are similar to those found in the literature [45]. The effect caused by arbutin, of decreasing the hydrated membrane T_m, is opposite to the osmotic dehydration effect caused by trehalose, which raises the hydrated membrane T_m in a concentration-dependent manner [46].

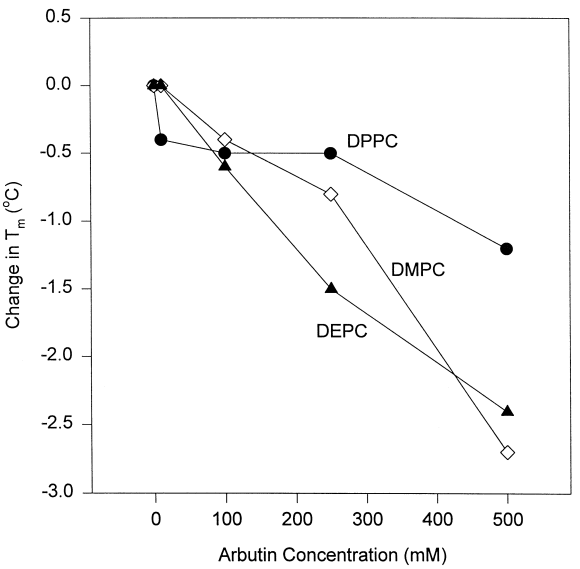

Fig. 7. Dose–response effect of arbutin on the thermotropic phase transition in hydrated PC LUVs of different chain length and saturation as measured by differential scanning calorimetry.

Since arbutin has been shown previously to decrease the phase-transition temperature of dry bilayers [30], the finding that it did so in hydrated membranes as well was not surprising, but it does provide more information regarding the nature of the interaction between membranes and arbutin. This result indicates that it is not necessary to remove the water molecules present, in order to force an interaction between arbutin and the lipid bilayer, as has been assumed for mono- and disaccharides [46], but that arbutin can interact with the membrane in the hydrated state as well.

3.5. Arbutin inserts into hydrated lipid bilayers

The direct interaction between arbutin molecules and the membrane might be explained by the insertion of the phenol moiety into the lipid bilayer, which would generate a larger surface area per lipid molecule, allowing for greater mobility of the hydrocarbon chains, and thus a decrease in the gel-to-liquid crystalline phase-transition temperature. We tested the possibility that the phenol group of arbutin might have an auto-fluorescence, similar to that of tryptophan or other compounds with one or more aromatic rings [37]. Indeed, when an aqueous solution of arbutin was excited at 280 nm, it emitted fluorescence with a maximum at 319 nm. In addition, the emission maximum of arbutin shifted to longer wavelengths, depending upon the hydrophobicity of the solution (Fig. 8A). Although the direction of this shift differs from that seen with tryptophan, which undergoes a blue-shift upon insertion into the membrane [35], the effect is, nonetheless, consistent. Therefore, the emission maximum of arbutin (when excited at 280 nm) can be used to monitor the hydrophobicity of the environment in which arbutin exists.

The fluorescence maximum of an aqueous solution of arbutin (20 μM in 10 mM TES, pH 7.5) excited at

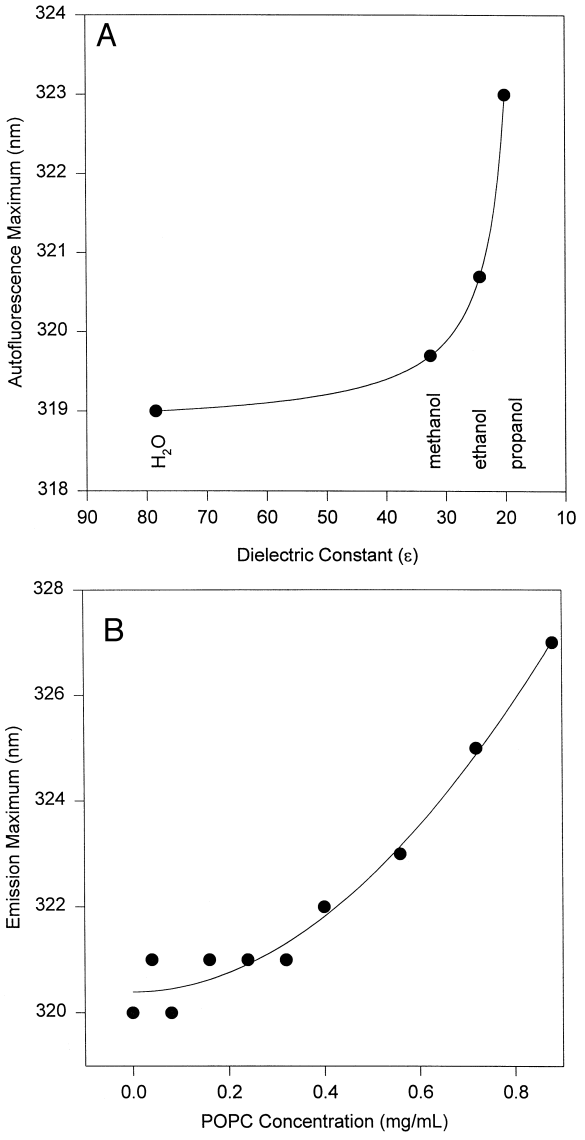

Fig. 8. A – Effect of the dielectric constant of the medium on the fluorescence maximum of arbutin when excited at 280 nm. Measurements were taken on 2 μM solutions of arbutin in deionized water, methanol, ethanol, or propanol. Values of the dielectric constant of each were taken from Ref. [32]. B – Effect of increasing lipid concentration on the fluorescence maximum of arbutin. Aliquots of POPC sonicated vesicles in 10 mM TES (pH 7.5) were added stepwise to a 20 μM solution of arbutin in 10 mM TES (pH 7.5), and an emission spectrum was taken, while exciting at 280 nm.

280 nm was monitored in the presence of different concentrations of POPC sonicated vesicles. As shown in Fig. 8B, the fluorescence maximum shifted steadily to higher wavelengths with increasing POPC concentration.

As a control, a fluorescence spectrum was run on pure POPC LUVs in 10 mM TES. When excited at 280 nm, a fluorescence maximum was detected at 356 nm in the POPC sample. This maximum did not interfere with the arbutin fluorescence maximum, however, until a relatively high concentration of POPC (1.2 mg/ml) was reached. The arbutin and POPC maxima were clearly distinguishable at lower POPC concentrations (data not shown). The TES buffer had a slight (1 nm) effect on the arbutin emission maximum as compared to arbutin in water (compare the first data points of Fig. 8(A and B). Because the TES concentration was constant at 10 mM throughout the experiment, any effect of TES on arbutin fluorescence would also be constant throughout the experiment and, thus, internally controlled.

Another method for measuring the insertion of a fluorescent compound into the hydrophobic phase of a membrane is the use of aqueous quenchers, such as $NaNO_3$ [36,38]. We, therefore, titrated the fluorescence emitted by arbutin in the absence, or presence of liposomes with increasing concentrations of sodium nitrate. The data were analyzed in the form of a Stern–Volmer plot, in which the relative change in fluorescence emission is plotted as a function of $NaNO_3$ concentration in solution (Fig. 9). The slopes of the resulting lines yield the quenching coefficients (K_{sv}). The decrease in K_{sv} seen in the presence of DPPC or POPC sonicated vesicles (0.32 mg/ml) shows that both types of liposomes protected arbutin from quenching. This result, along with the increase in the fluorescence maximum seen in the presence of lipid (Fig. 8B), provides evidence that arbutin does indeed insert into the membrane with the phenol moiety. Such a finding is consistent with the high lipid solubility of phenol [47] as well as the bilayer adhesion behavior of certain polyphenolic compounds, due to their simultaneously partitioning into closely apposing membranes [48]. This result, therefore, suggests that the leakage caused in liposomes during air-drying in the presence of arbutin is likely to be due to its insertion into the bilayer.

It is interesting to note that POPC vesicles provided slightly better quenching protection to arbutin than did DPPC vesicles (Fig. 9). This interpretation is

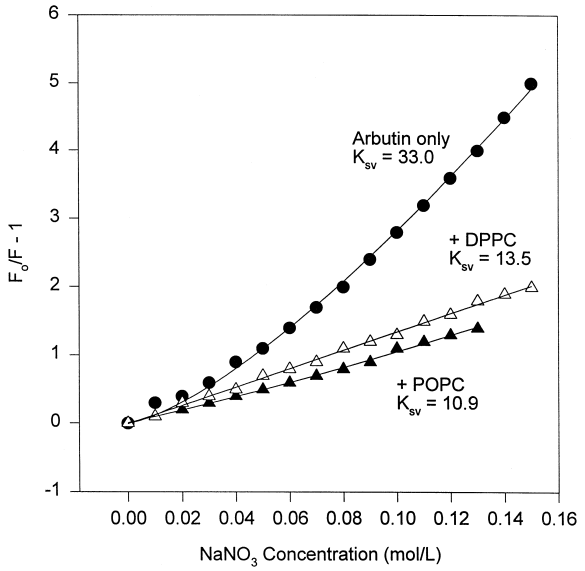

Fig. 9. Stern–Volmer plots for arbutin (4 μM in 10 mM TES, pH 7.5) in (●) the absence, or presence of (△) 0.32 mg/mL DPPC or (▲) POPC sonicated vesicles. This method (see text for details) monitors the quenching of arbutin auto-fluorescence caused by increasing concentrations of $NaNO_3$. The decrease in K_{sv} seen in the presence of DPPC or POPC indicates that both types of liposomes protected arbutin from the aqueous quencher.

warranted, as the plot constructed in the presence of POPC ($K_{sv} = 10.9$) was significantly different from that of DPPC ($K_{sv} = 13.5$) at a 99% level of confidence, as determined by the SigmaPlot regression analysis (SigmaPlot, Jandel Scientific, San Rafael, CA). This finding correlates well with the result shown in Fig. 7 that arbutin decreased the T_m of hydrated DMPC and DEPC more than that of DPPC. These phenomena can both be explained by the differences amongst the lipids in surface area per molecule. Molecular area is inversely proportional to chain length and level of saturation of the fatty acyl chains (for review, see Ref. [49]). Since the molecular area for DPPC is smaller than that of DMPC, DEPC, or POPC, and also because DPPC is in the gel phase at room temperature, it is likely to be more difficult for arbutin to insert into a DPPC membrane than into a membrane composed of DMPC, DEPC, or POPC. Thus, because arbutin can insert more easily into the bilayers with looser head-group packing, it can have a greater effect on their phase transition temperatures, and can be better protected by them from $NaNO_3$-induced quenching.

4. Conclusions

Evidence has been presented that arbutin may participate in a carbohydrate glass formed from sucrose and trehalose without changing the T_g, that arbutin can cause leakage from phospholipid vesicles during air-drying by a mechanism other than fusion, and that arbutin interacts directly with membranes by insertion of the phenol moiety into the lipid bilayer. This information, although obtained in an in-vitro system, will likely provide important clues in the effort to explain the role of arbutin in the resurrection plant *M. flabellifolia*. Several difficult questions remain, however. For instance, why is arbutin present in the plant if it causes so much damage in the form of increased membrane permeability? Although it is impossible to answer this question at the current time, the solution may be related to the location of arbutin within the plant cells, which is unknown. Recently, we have gathered evidence that not all lipids are equally susceptible to damage from drying in the presence of arbutin. Monogalactosyl-diacylglyceride (MGDG), which is the predominant lipid in the thylakoid membranes of plant cells [50], greatly ameliorates the destructive effects of arbutin (unpublished results). Thus, the location of arbutin within the cell, and the composition of specific membranes are likely to have a large impact on the effect of arbutin at that site. Experiments using MGDG and other plant-cell lipids are in progress, but the results of the current study give information about the interaction of arbutin with phosphatidylcholine membranes as a first approximation, and will be valuable as a comparison with membranes of varying compositions.

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