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Temperature-dependent perturbation of phospholipid bilayers by dimethylsulfoxide

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Dimethylsulfoxide (DMSO) is known to protect isolated enzymes during freezing while destabilizing proteins at high temperatures. This apparent paradox is the subject of a review by Arakawa et al. ((1990) Cryobiology 27, 401–415), who present evidence for a temperature-dependent, hydrophobic interaction between DMSO and non-polar moieties of proteins. The present study investigates the interaction of DMSO with phospholipid bilayers. Phospholipid vesicles containing carboxyfluorescein were exposed to several concentrations of DMSO at various temperatures. Leakage rates increased with DMSO concentration and temperature. This effect was not reduced in the presence of solutes that have been shown to neutralize DMSO toxicity in tissues. The increased leakage rates correlate well with the increased partitioning of DMSO from water to octanol at higher temperatures. Additionally, reductions in the CH_2 vibrations of the bilayer are also shown to depend on DMSO concentration and temperature. A similar reduction in CH_2 vibrations was observed in solutions of octanol and DMSO, suggesting that this effect is not mediated through an interaction with water. Furthermore, investigation of sulfoxide vibrations indicate that DMSO is not hydrogen bonded to the alcohol moiety of octanol, and therefore the interaction between DMSO and octanol is most likely due to a hydrophobic association. These results are consistent with a destabilization of phospholipid membranes at higher temperatures due to a hydrophobic association between DMSO and the bilayer.

Introduction

Dimethylsulfoxide (DMSO) is an aprotic compound that possesses both a hydrophilic sulfoxide moiety and a hydrophobic component (two methyl groups). As a result, DMSO is readily soluble in both polar and non-polar solvents, and is able to solubilize a wide range of compounds [1]. This dual solubility is largely responsible for DMSO's ability to penetrate biological membranes [2]. In addition, this molecule has been shown to have many other effects on biological systems ranging from anesthesia [3] to the induction of cellular differentiation [4–6]. One of DMSO's more paradoxical properties is its ability to act as a cellular cryoprotectant at low temperatures while proving toxic at higher temperatures [7,8]. This effect is also seen in studies on isolated proteins in which DMSO preserves enzyme function during freezing yet lowers the thermal denaturation temperature of proteins [9–11]. These temperature dependent effects on protein stability have been noted with other solutes [8,9] and have been addressed in a recent review by Arakawa et al. [9]. These authors propose that DMSO's hydrophobic character is responsible for its destabilizing effect on protein stability at high temperatures [9].

Theoretically, DMSO should interact hydrophobically with any macromolecular structure having a non-polar region to which the solute has access. This hypothesis is consistent with results from a recent study that provides evidence for a destabilizing interaction at high temperatures between phospholipid membranes and other known protein perturbants [12]. The purpose of this study was to determine if DMSO interacts with phospholipid bilayers via a mechanism similar to that proposed for proteins [9].

The results presented here are consistent with a temperature-dependent, hydrophobic interaction between DMSO and the bilayer. Furthermore, we show

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that concentrations of DMSO similar to those used in tissue preservation studies result in bilayer destabilization only at high temperatures. We suggest that such an interaction may explain why DMSO is known to be toxic to cells at higher temperatures [7,8]. This toxicity has been shown to be reduced by the addition of solutes that could potentially bind to DMSO and decrease its interaction with enzymes [7]. We show here that these compounds, termed 'toxicity neutralizers' [7,8,13–15], do not reduce the effects of DMSO on phospholipid bilayers.

Materials and Methods

Leakage. Dimethylsulfoxide, urea and formamide were purchased from Sigma Chemical Co. (St. Louis, MO). Egg phosphatidylcholine was acquired from Avanti Polar Lipids (Pelham, AL) and used without further purification. Carboxyfluorescein (CF) was obtained from Molecular Probes Inc. (Eugene, OR) and purified according to the methods of Weinstein et al. [16]. Lipid was dried under nitrogen gas and placed under vaccuum (30 millitorr) for one hour to remove residual chloroform. The hydrating solution (10 mM Tes, 0.1 mM EDTA) was slightly alkaline (pH = 7.2) to avoid CF precipitation, and contained 100 mM CF in addition to the appropriate concentration of DMSO and toxicity neutralizer. Each lipid preparation was sonicated to clarity in a bath sonicator at a concentration of 40 mg lipid/ml, and external CF was removed by centrifugation through a two-inch column (3 ml) of Bio-Gel P-6 (Bio-Rad, Richmond, CA) equilibrated with the appropriate solution [17]. Liposome suspensions were kept on ice and used immediately after centrifugation. All other solutions used in this study were made in 10 mM Tes, 0.1 mM EDTA buffer (pH = 7.0 at 22°C).

An aliquot (10–20 μl) of small unilamellar vesicles (SUVs) was added to a cuvette containing a solution of DMSO pre-equilibrated at a given temperature. Holding temperature constant, the samples were excited at 460 nm and the CF emission was monitored at 550 nm. Intravesicular CF concentrations of 100 mM are self quenching [16] and therefore the emission at 550 nm was due solely to external CF. Fluorescence was recorded every minute for 10 min after which 50 μl of 1% Triton X-100 was added to each sample to induce total CF leakage. The percent leakage was calculated at each time point and the data were fitted to a first-order regression line to calculate the leakage rate at each temperature.

Effects of DMSO on fusion during heating. In view of previous reports that DMSO induces cell fusion [18], we investigated the possibility that it might also affect liposome fusion at increased temperatures. Liposomes were prepared by sonicating egg PC (40 mg lipid/ml 10 mM Tes buffer + 1 mM EDTA) to clarity. 50 μl of this preparation were pipetted into a series of tubes containing 2.5 ml of 30% DMSO at 21°C or into buffer. The samples were then heated in a water bath to a maximum of 66°C. At intervals during the heating, tubes were removed and cooled to room temperature, thus providing a series of samples that had been heated to various temperatures. A small aliquot of each sample was diluted into buffer and the sizes of the vesicles were then measured, using a Brookhaven instruments quasielastic light scattering device, calibrated with polystyrene spheres. At least 1000 scans were made for each measurement, providing a precision of $\pm$10 nm for the vesicle diameters.

Octanol/water partitioning. A 40% solution (v/v) of DMSO in deionized water was mixed with a volume of octanol equal to that of the water in the DMSO/water solution. The resulting mixture (37.5% water, 37.5% octanol and 25% DMSO by volume) was equilibrated to the experimental temperature. After thermal equilibrium was achieved, the mixture was thoroughly mixed and incubated for 30 min at that temperature. Preliminary experiments showed that partitioning was complete in less than 15 min. After partitioning, the octanol phase was collected at the incubation temperature and stored overnight at 25°C. The following day, a 1 ml aliquot of the organic phase was repartitioned against an equal volume of water at 25°C. A 20-μl sample of this aqueous phase was diluted into 1 ml of water for concentration determination at 25°C. The amount of DMSO in each sample was quantified by generating a standard curve and measuring the absorbance at 218.6 nm in a Perkin-Elmer 550 spectrophotometer.

Fourier transform infrared spectroscopy. Lipids were dried as described above, and rehydrated in Tes-EDTA buffer. The lipids were sonicated to clarity at a lipid concentration of 100 mg/ml, and samples were prepared by incubating liposomes in an equal volume of DMSO solution for ten minutes before loading onto BaF_2 windows for infrared spectroscopy [19]. The temperature of the samples was controlled using a Peltier device as described previously [19], and samples were scanned at temperature intervals of approximately 2.5°C. The acyl chain region of the bilayer was investigated by monitoring the CH_2 symmetric stretch at 2852 cm^{-1} using a Perkin-Elmer 1750 Fourier transform infrared spectrometer. The location of the peak absorbance was quantified by calculating the center of gravity in the upper 10% of the peak height [19].

In the experiments without liposomes, solutions of water, octanol, and DMSO were scanned at room temperature. The CH_2 symmetric stretch of octanol (2586 cm^{-1}) and the S=O vibration of DMSO (1060–1010 cm^{-1}) were monitored and peak absorbance was located as described above.

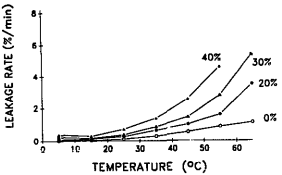

Fig. 1. Small unilamellar vesicles composed of egg phosphatidylcholine were incubated in DMSO solutions at different temperatures. The leakage of entrapped carboxyfluorescein was measured over time, and a leakage rate was calculated for each DMSO concentration and temperature.

Results

Effects on permeability

Leakage rates of carboxyfluorescein (CF) from phosphatidylcholine vesicles increased progressively with DMSO concentration (Fig. 1). The effect is minimal at room temperature but is greatly enhanced upon warming, especially at higher concentrations (Fig. 1). Bilayers incubated in 10% DMSO leaked at rates similar to the control (data not shown). The presence of 'toxicity neutralizers' in ratios reported to minimize the toxic effects of DMSO [7,8,13–15] did not reduce leakage rates of CF (Fig. 2). In fact, the presence of urea or formamide at 'neutralizing' ratios slightly increased leakage rates of CF from phospholipid vesicles (Fig. 2). The presence of formamide or urea alone at concentrations used in these experiments had little effect on CF leakage under the same conditions [12].

Effects on fusion

The possibility that the effect of DMSO on leakage could be accounted for by fusion was investigated. We found that when sonicated vesicles were heated in buffer, no size increase was evident at temperatures up to 66°C (Table I). When the vesicles were placed in 30% DMSO, they immediately increased in size, approximately doubling their diameter (Table I). However, when these vesicles were heated, no further size increase was observed. We note that both aggregation and fusion can lead to a measured size increase, but feel that aggregation is unlikely considering that the samples were diluted out of DMSO before determination of vesicle diameter. However, the observation that no size increase occurred upon heating excludes the possibility that fusion is enhanced upon warming. We conclude that the temperature dependent increase in permeability of the vesicles in the presence of DMSO cannot be accounted for by fusion between the vesicles as temperature increases.

TABLE I

Mean diameters (± standard deviation) of vesicles of egg PC previously heated to the indicated temperatures

Vesicles were sized at room temperature by quasielastic light scattering, following heating to the indicated temperatures.

Temperature (°C)	Mean diameter (nm)	
	in buffer	in 30% DMSO
21.4	110 ± 8	223 ± 14
40.6	113 ± 7	208 ± 9
66.2	105 ± 12	224 ± 19

Temperature dependent partitioning of DMSO into hydrocarbons

It is difficult to measure partitioning of small molecules like DMSO into phospholipid bilayers, so as a model we have measured the temperature dependent partitioning of DMSO between octanol and water. It has been previously reported that over 99% of the DMSO partitions with the aqueous phase at room temperature [20]. With such an overwhelming fraction of the DMSO in the aqueous phase, resolution of small changes in DMSO partitioning required measuring its concentration in the organic phase. Conducting such a

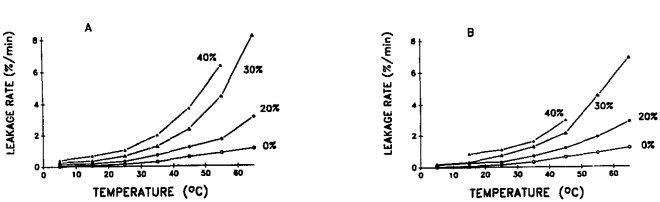

Fig. 2. Compounds that have been shown to neutralize DMSO toxicity in tissues were tested for their ability to reduce DMSO-induced leakage from liposomes. (A) DMSO in combination with urea at a weight ratio of 3:1 (DMSO/urea). (B) DMSO in combination with formamide at a mole ratio of 2:1 (DMSO/formamide).

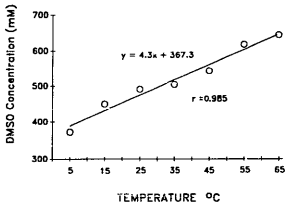

Fig. 3. The octanol/water partitioning of DMSO was measured at different temperatures. The concentration of DMSO that partitions into the organic phase (y-axis) increases with temperature.

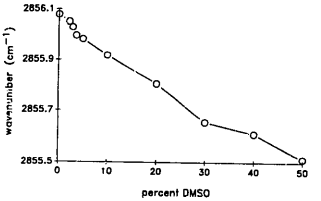

Fig. 5. The symmetric CH_2 vibration of octanol was monitored at room temparature. The presence of increasing amounts of DMSO dissolved in the octanol progressively decreased the wavenumber of the vibration.

measurement is complicated by water's solubility in octanol (approx. 2.3 M at room temperature, Ref. 20). As a result, the measured concentration of DMSO in the organic phase is the sum of the DMSO solvated by pure octanol and the DMSO associated with water molecules dissolved in the organic phase. It is possible that the solubility of water in the organic phase is enhanced due to the high DMSO concentrations used in this study. However, we assume that the organic phase mimics the hydrophobic region of the bilayer, and therefore any increased water in the organic phase under these conditions would simulate the environment within a membrane.

To mimic the conditions used in the leakage experiments, a 40% DMSO solution (v/v) was partitioned against a volume of octanol equal to the volume of water used in the DMSO/water solution. The results of measuring the DMSO concentration in the organic phase at different temperatures are shown in Fig. 3. The positive slope of the fitted line indicates that progressively more DMSO partitioned into the organic phase at higher temperatures. This trend could be predicted from the thermodynamics of hydrophobic

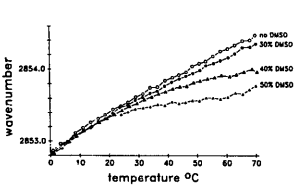

Fig. 4. The symmetric CH_2 vibration in egg phosphatidylcholine liposomes incubated in DMSO was monitored at different temperatures. The effect of DMSO is seen only at high concentrations and temperatures.

associations [21], and is consistent with a temperature dependent interaction of DMSO with the acyl chain region of phospholipid bilayers.

We wish to point out that our experimental design eliminates increased water content of the octanol due to high temperatures as a potential explanation for the effect seen in Fig. 3. As mentioned in Materials and Methods, the organic phase was stored at 25°C overnight, thus allowing any increased water dissolved at high temperatures to separate from the organic phase before the determination of DMSO content. No such separation was observed.

The results shown in Fig. 4 indicate that the CH_2 vibrations in the acyl chain region of the bilayer decrease progressively with increasing DMSO concentration. In addition, the reduction in CH_2 vibrations, as compared to the control, is more dramatic at higher temperatures. The CH_2 vibrations of bilayers in the

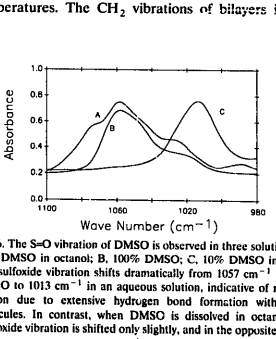

Fig. 6. The S=O vibration of DMSO is observed in three solutions: A, 10% DMSO in octanol; B, 100% DMSO; C, 10% DMSO in water. The sulfoxide vibration shifts dramatically from 1057 cm^{-1} in pure DMSO to 1013 cm^{-1} in an aqueous solution, indicative of reduced motion due to extensive hydrogen bond formation with water molecules. In contrast, when DMSO is dissolved in octanol, the sulfoxide vibration is shifted only slightly, and in the opposite direction.

presence of low DMSO concentrations (10% and 20%), were no different than that of the control over the temperature range of these experiments.

A similar effect of DMSO on CH_2 vibrations was seen in solutions of octanol (Fig. 5). The data demonstrate that increasing the number of DMSO molecules in contact with the CH_2 moieties of octanol progressively decreases their vibration.

It could be argued that the polar sulfoxide portion of DMSO is interacting with the alcohol moiety of octanol via a hydrogen bond, resulting in the data seen in Fig. 5. Fig. 6 shows the infrared absorption of the sulfoxide moiety on DMSO in different solutions. In pure DMSO, the sulfoxide moiety is relatively unrestricted, having no partner with which to hydrogen bond. In contrast, DMSO strongly interacts with water via hydrogen bonds [22,23]. As seen in Fig. 6, the sulfoxide vibration in water is dramatically decreased in wavenumber compared to that in pure DMSO, consistent with extensive hydrogen bonding with water molecules. The sulfoxide vibration of DMSO in octanol is almost identical to that in pure DMSO, indicating that it is not forming hydrogen bonds with the alcohol moiety of octanol.

Discussion

The goal of the present study was to determine if DMSO, which preserves the integrity of phospholipid bilayers during freezing [24,25], destabilizes membranes at higher temperatures. The results show that the presence of increasing concentrations of DMSO promotes CF leakage from phospholipid vesicles (Fig. 1). Furthermore, the increased leakage observed in solutions of DMSO is enhanced at higher temperatures (Fig. 1). Since DMSO apparently has no effect on fusion during a temperature increase over the same range (Table I), fusion cannot account for this effect. Instead, we suggest that at increased temperatures DMSO shows an increased hydrophobic interaction with the bilayer, resulting in packing defects.

Arakawa et al. [9] have suggested that such an interaction may explain why DMSO protects enzymes against freezing damage, while destabilizing proteins at higher temperatures [9–11]. In their review, the authors suggest that DMSO is preferentially excluded from the hydration shell of the protein at low temperatures, due to its strong interaction with water [22,23]. This interaction of DMSO with water is favored at low temperatures [22], and results in stabilization of the native protein conformation [9]. Additionally, Arakawa et al. propose that high temperatures favor the interaction of DMSO's non-polar moiety with hydrophobic regions of the protein [9]. Such a hydrophobic association is favored at high temperatures [21,26] and could result in accumulation of DMSO at the protein/water interface, which favors protein unfolding. The authors provide evidence for a temperature dependent, hydrophobic interaction between DMSO and proteins that results in destabilization only at high temperatures [9]. Furthermore, they suggest that such a mechanism may be responsible for the cellular toxicity of DMSO observed at high temperatures.

Cryopreservation studies by Baxter and Lathe [7], and more recently by Fahy et al. [8,13–15], have documented that certain amides have the ability to neutralize DMSO toxicity. In particular, the presence of urea or formamide in certain ratios has been shown to significantly reduce DMSO toxicity in kidney tissues [7,13–15]. In the application of these findings to DMSO-induced liposome destabilization, neither urea nor formamide reduced CF leakage (Fig. 2). In fact, the presence of either of these compounds in combination with DMSO, promoted CF leakage (compare Figs. 1 and 2). In the studies mentioned above [7,8,13–15], DMSO toxicity, and therefore its reduction, was measured by its effects on enzyme function and may not involve a membrane component.

The mechanism by which DMSO fosters CF leakage from liposomes is unclear. Leakage of encapsulated solutes is known to occur when the lipids are undergoing a phase transition [27]. Alternatively, the interaction of DMSO with the acyl chains may alter lipid packing, resulting in defects that allow solutes to cross the bilayer. We favor the latter explanation because leakage due to a lipid phase transition is unlikely under the conditions used in our experiments since egg phosphatidylcholine has a T_m below 0°C [24].

A temperature dependent interaction with phosphatidylcholines has previously been observed with ethanol [28–30]. Rowe [28,29] has reported that low concentrations of ethanol decrease the main phase transition temperature (T_m) of phosphatidylcholines, while high concentrations increase T_m [28,29]. Her findings document the formation of an interdigitated gel phase in the presence of high concentrations of ethanol [28,29]. The induction of this interdigitated phase is favored at higher temperatures [28–30].

In addition to monitoring the induction of the interdigitated phase by ethanol, Rowe measured the transition temperatures and the corresponding enthalpies of a series of phosphatidylcholines [28]. Using these data, Rowe estimated the water/membrane partition coefficients of ethanol at different temperatures [28]. Her results indicate that more ethanol partitions into the membrane at higher temperatures.

To investigate the behavior of DMSO at high temperatures, we directly measured its partitioning between water and octanol at various temperatures (Fig. 3). Our findings are similar to those reported by Rowe

for ethanol [28,29], and demonstrate that more DMSO partitions into the organic phase at higher temperatures (Fig. 3). These data correlate well with the increased CF leakage observed in Fig. 1. We interpret this correlation as an indication that more DMSO partitions into the hydrophobic portion of the bilayer at higher temperatures, and increases the permeability of the membrane to CF.

The proposed hydrophobic interaction would be expected to affect vibrations of lipid chains in the nonpolar region of the membrane. As shown in Fig. 4, the wavenumber of the main CH_2 vibration is reduced by the presence of 30%–50% DMSO only at high temperatures. This non-linear shift in CH_2 vibration indicates reduced motion of the bonds in the acyl chain region relative to the control, and corresponds with the leakage data presented in Fig. 1. The reduced vibrations in the acyl chain region of the bilayer is indicative of decreased lipid fluidity, and is consistent with reports of increased T_m of phosphatidylcholine vesicles in the presence of DMSO [24].

A similar reduction in CH_2 vibrations due to DMSO is observed in solutions of octanol (Fig. 5). This provides evidence that the decreased CH_2 vibrations seen in Fig. 4 could be due to a direct interaction of DMSO with the acyl chain region and not mediated through DMSO's effect on water. Furthermore, the effect in Fig. 5 cannot be explained by the formation of hydrogen bonds between DMSO and the alcohol moiety of octanol. As depicted in Fig. 6, the sulfoxide vibration is greatly reduced in water as compared to that in pure DMSO due to the formation of hydrogen bonds with water. The sulfoxide absorption from a 10% solution of DMSO in octanol does not shift to a lower wavenumber, indicating that the sulfoxide group is not hydrogen bonded to the alcohol moiety of octanol. As a result, the decreased CH_2 vibrations observed in Figs. 4 and 5 are most likely due to a hydrophobic interaction between DMSO and methylene groups.

In conclusion, our results show that concentrations of DMSO known to be toxic to cells can destabilize phospholipid bilayers at high temperatures. This destabilization was not reduced by compounds known to neutralize DMSO toxicity in cellular systems [7,13–15]. Our partitioning measurements indicate that DMSO has increasingly hydrophobic character at higher temperatures, and we propose that the association of DMSO with bilayers is enhanced upon warming. This is consistent with our infrared spectroscopy data which indicates that the acyl chain region of the bilayer is progressively more perturbed by DMSO at higher temperatures. We suggest that future studies on DMSO toxicity consider the profound effects that this solute has on bilayer stability.

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References

1 Kligman, A.M. (1965) J. Am. Med. Assoc. 193, 796–804.
2 Jacob, S.W., Bischel, M. and Herschler, R.J. (1964) Curr. Ther. Res. 6, 193–198.
3 Jacob, S.W. and Herschler, R. (1986) Cryobiology 23, 14–27.
4 Priestler, H.D. and Lyman, G. (1975) Cell Differentiation 4, 179–185.
5 Lyman, G.H., Priestler, H.D. and Papahadjopoulos, D. (1976) Nature 262, 360–363.
6 Friend, C. and Freedman, H.A. (1978) Biochem. Pharmacol. 27, 1309–1313.
7 Baxter, S.J. and Lathe, G.H. (1971) Biochem. Pharmacol. 20, 1079–1091.
8 Fahy, G.M. (1986) Cryobiology 23, 1–13.
9 Arakawa, T., Carpenter, J.F., Kita, Y.A. and Crowe, J.H. (1990) Cryobiology 27, 401–415.
10 Fujita, Y., Izumiguchi, S. and Noda, Y. (1982) Int. J. Peptide Protein Res. 19, 25–32.
11 Carpenter, J.F. and Crowe, J.H. (1988) Cryobiology 25, 244–255.
12 Anchordoguy, T.J., Carpenter, J.F., Cecchini, C.A., Crowe, J.H. and Crowe, L.M. (1990) Arch. Biochem. Biophys. 283, 356–361.
13 Fahy, G.M. (1983) Cryo-Lett. 4, 309–314.
14 Fahy, G.M. (1984) Cryo-Lett. 5, 287–294.
15 Fahy, G.M., Levy, D.I. and Ali, S.E. (1987) Cryobiology 24, 196–213.
16 Weinstein, J.N., Ralston, E., Leserman, L.D., Klausner, R.D., Dragsten, P., Henkart, P. and Blumenthal, R. (1984) in Liposome Technology (Gregoriadis, G., ed.), Vol. 3, pp. 183–204, CRC Press, Boca Raton, FL.
17 Fry, D.W., White, J.C. and Goldman, I.D. (1978) Anal. Biochem. 90, 809–815.
18 Ahkong, Q.F., Fisher, D., Tampion, W. and Lucy, J.A. (1975) Nature 253, 194–195.
19 Crowe, J.H., Hoekstra, F.A., Crowe, L.M., Anchordoguy, T.J. and Drobis, E. (1989) Cryobiology 26, 76–84.
20 Leo, A., Hansch, C. and Elkins, D. (1971) Chem. Rev. 71, 525–616.
21 Tanford, C. (1980) The Hydrophobic Effect, Wiley-Interscience, New York.
22 Schichman, S.A. and Amey, R.L. (1971) J. Phys. Chem. 75, 98–102.
23 Szmant, H.H. (1975) Ann. N.Y. Acad. Sci. 243, 20–23.
24 Strauss, G. and Ingenito, E.P. (1980) Cryobiology 17, 508–515.
25 Anchordoguy, T.J., Cecchini, C.A., Crowe, J.H. and Crowe, L.M. (1991) Cryobiology 28, 467–473.
26 Scheraga, H.A., Nemethy, G. and Steinberg, I.Z. (1962) J. Biol. Chem. 237, 2506–2508.
27 Crowe, J.H., Crowe, L.M. and Hoekstra, F.A. (1989) J. Bioenerg. Biomem. 21, 77–91.
28 Rowe, E. (1983) Biochemistry 22, 3299–3305.
29 Rowe, E. (1985) Biochim. Biophys. Acta 813, 321–330.
30 Nambi, P., Rowe, E. and McIntosh, T.J. (1988) Biochemistry 27, 9175–9182.