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INTERACTIONS OF PHOSPHOLIPID MONOLAYERS WITH CARBOHYDRATES

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Surface pressure studies of phospholipid monomolecular films of dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) formed at an air/water interface have been made and the effects on the films studied when various carbohydrates are present in the subphase. The results obtained show that at a given temperature, the area per molecule in a monolayer of DPPC increases with increasing concentration of the carbohydrate in the subphase. The carbohydrate which has the greatest expanding effect on the phospholipid monolayer is glycerol, followed in turn by trehalose, sucrose, glucose, raffinose, and inositol. The mechanism of monolayer expansion by glycerol is different from that observed in other carbohydrates, as the following experiments demonstrate. Below the phase transition temperature of DPPC, the area per molecule of DPPC at a pressure of 12.5 dyn/cm is the same with and without glycerol in the subphase. However, when the monolayer is heated to a temperature above the phase transition temperature for DPPC, the area/molecule on glycerol is considerably greater than the area/molecule on water at the same surface pressure. Cooling the monolayer back to the lower temperature produces an area/molecule of DPPC which is identical on both water and glycerol subphases. Glycerol therefore has no effect on the low-temperature (condensed) monolayers but causes expansion of the high-temperature (expanded) monolayers. By contrast with glycerol, both trehalose and sucrose interact with the DPPC monolayer producing an increased area/molecule over that observed on water, both with low-temperature (condensed) monolayers and with the high-temperature (expanded) monolayers. The efficiency of these carbohydrates at expanding the monolayer films (with the exception of glycerol) shows a strong correlation with their ability to stabilize membrane structure and function at low water contents.

Introduction

Monomolecular films of phospholipids spread on aqueous subphases have long been utilized as models for the physical properties of biological membranes (see for example, Refs. 1–3). Furthermore, studies on the effects of addition of various substances to the subphase have yielded valuable information concerning the modes of interaction of those substances with membranes [4–7]. Thus, we have used such films as models for studies on interactions of carbohydrates and membranes. Our aim in the present paper is to report results of experiments which suggest a mechanism by which certain carbohydrates interact with the polar groups of the phospholipids and thereby affect packing of phospholipids in the monolayer. These results may have bearing on our knowledge of related phenomena involving membranes. These include modulation of membrane fluidity by carbohydrates [4–8], effects of carbohydrates on fusion between membranes [9], and preservation of membranes by freezing [10] and dehydration [11]. The latter possibilities will be considered further in the following paragraphs.

Upon dehydration, membrane phospholipids

undergo changes in their physical state (reviewed in Ref. 11) that lead to phase transitions [12] and disruptions in membrane structure and function [13]. However, we have recently shown that intact biological membranes, sarcoplasmic reticulum vesicles, can be reduced to extremely low water contents (less then 0.3 g water/g dry weight) without damage if the membranes are dried in the presence of the carbohydrate, trehalose. When these membrane vesicles were lyophilized in the presence of as little as 0.3 g trehalose/g membrane (one trehalose molecule/two phospholipid molecules), the rehydrated membranes were seen to have a morphology and biological activity similar to the freshly prepared membranes [13–15]. Trehalose is far superior in this regard to any of the other carbohydrates we tested [16], including sucrose, which had previously been known to have some stabilizing effects on dry membranes [17]. The physical basis for the remarkable effects of trehalose on membranes is unknown, although we have suggested elsewhere that membrane fluidity might be increased in the presence of carbohydrates [11]. This increase in fluidity could inhibit dehydration damage to the membrane through the following sequence of events: Phosphatidylcholine is known to enter gel phase during dehydration at temperatures at which it normally exists in the liquid crystalline phase [18]. When PC undergoes its transition to gel phase, one might expect lateral phase separations of membrane proteins and other phospholipids such as phosphatidylethanolamine from the PC gel phase crystals, as other workers have observed following thermotropic phase transitions in membranes [18]. The phase separation of PE would, in turn, be expected to result in formation of hexagonal II phase crystals [19–21]. We have, in fact, previously provided evidence based on freeze-fracture and ^{31}P-nuclear magnetic resonance that such phase separations of membrane proteins from lipids and transition of PE to hexagonal II phase do indeed occur during dehydration of a membrane in the absence of trehalose. In the presence of trehalose the phase separations and transitions were not observed [11,12,15]. An increase in the fluidity of the membrane in the presence of the carbohydrate would be expected to inhibit the initial event in this sequence, the transition of PC to gel phase, and thus would be expected to alleviate damage to the membrane from dehydration. In the present paper we will provide evidence that this is the case. In the accompanying paper [16] we showed that trehalose is the most effective carbohydrate tested in preserving structure and function of sarcoplasmic reticulum vesicles. It follows that if our hypothesis concerning effects of trehalose on fluidity of membranes and the consequences for stabilization of the membrane in the dry state is correct, we would expect a relationship to exist between the fluidizing properties of the various carbohydrates and their ability to preserve sarcoplasmic reticulum in the dry state. We will show that such a relationship exists.

As we pointed out in the accompanying paper [16], the impetus for these investigations on the effects of trehalose on membranes is the observation that this molecule is widely distributed among organisms that are capable of surviving extreme dehydration (a phenomenon known as 'anhydrobiosis'; for references, see Refs. 11, 12 and 15). Survival of dehydration by at least some of these organisms has been linked with synthesis of this compound.

Materials and Methods

Materials. Phospholipids were purchased from Fluka AG (Switzerland), and their purity was checked with differential scanning calorimetry. Carbohydrates were purchased from Sigma Chemicals, and their purity was assayed with high performance liquid chromatography. Water used in the monolayer studies was purified with a Millipore Milli-Q filtering system with one carbon and two ion-exchange stages, which provides water with a resistivity of at least 10 Mohm · cm. The purified water is passed through a Millipore filter to remove any residual particles and used immediately after filtering.

Monolayer studies. The film balance used in these studies was essentially similar to that described by Albrecht et al. [3], except that a Wilhelmy plate was used as the pressure-sensing device. The important features of the balance are:
(a) All parts in contact with water are made of Teflon or are coated with Teflon sheets.

(b) The trough is built on a thermostated brass plate and the entire balance is surrounded by a

water jacket, providing the temperature control within 1 deg. C.

(c) The moving barrier is driven by a step motor which permits area resolution of $7.25 \cdot 10^{-2}$ cm^2.

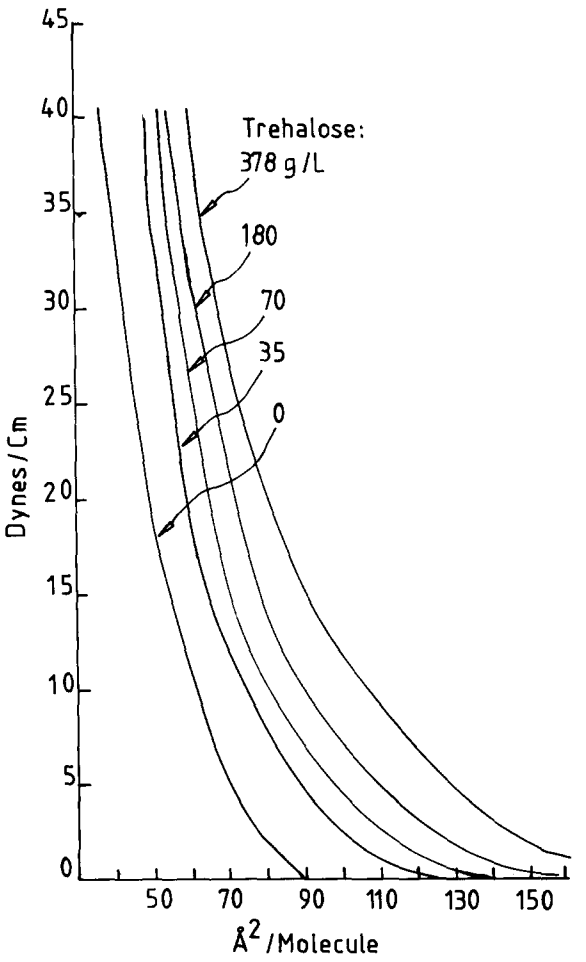
(d) The electronic control unit permits movement of the barrier at variable rates, isobaric measurements of changes in the area occupied by lipid molecules accompanying temperature changes, or pressure changes in lipids held in a constant area during alteration of temperature.

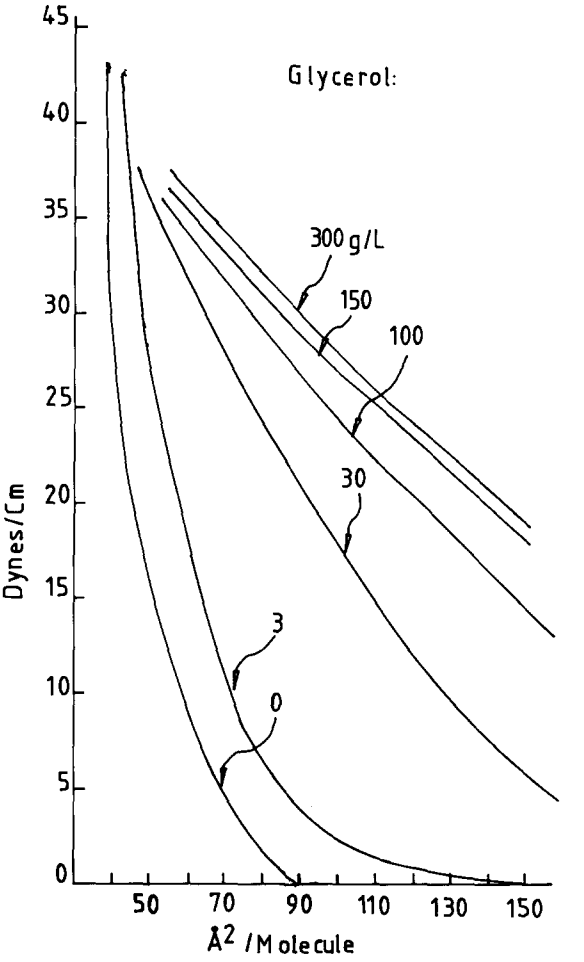
The monolayers were spread from a 9 : 1 mixture of hexane and ethanol at a concentration of 1.0 mg/ml. A point of special concern was the stability of the films, particularly in view of the fact that many of the measurements required considerable time. In our hands the films were found to be stable for at least two hours at 20 dyn/cm, with changes in surface pressure ≤ 1 dyn/cm. These observations concerning film stability agree with those of Cadenhead and Demchak [4] and Albrecht et al. [2].

Results

Isothermal measurements

Figs. 1 and 2 show the surface pressure-area curves obtained at 30°C for monolayers of dimyristoylphosphatidylcholine (DMPC) spread on subphases containing various concentrations of two





Figs. 1 and 2. Surface pressure/area curves for DMPC spread on aqueous solutions of trehalose (Fig. 1) and glycerol (Fig. 2) at the indicated concentrations. All measurements were made at 30°C.

representative carbohydrates. We chose to record these isotherms at 30°C since that temperature is well above the main transition temperature for DMPC in bilayer [18] and monolayer preparations [1,2]. The shapes of the isotherms both in the presence and absence of the carbohydrates conform most closely to the expanded isotherms of Type I recorded above the main transition temperature, as defined by Albrecht et al. [2]. The data for trehalose show that this molecule increases the surface pressure of the monolayer with increasing concentration of the carbohydrate. The limiting area (defined as the area/DMPC molecule below which the lateral pressure rises rapidly) increases with concentration of the carbohydrate in the subphase. We have recorded similar isotherms for a number of other carbohydrates and have found that they all affect packing of DMPC on monolayers in similar ways, although the magnitude of spreading of the monolayers varies with the carbohydrate. For example, the set of isotherms with glycerol in the subphase shows that this molecule spreads the monolayer to a remarkable extent; when DMPC was spread on water containing at little as 3 g glycerol/l, lateral pressure was detected even before compression of the monolayer was begun. Effects of glycerol on expansion of monolayer films have previously been reported [4,5,9], but the expansion effects reported by those workers are much less than those recorded here. This may be due to the fact that their measurements were made in most cases at much lower temperatures, often below the phase transition temperature. Cadenhead and his colleagues [4,5] note that if the monolayer film is too condensed before the glycerol is added, little further expansion of the monolayer occurs. We will explore that phenomenon further in the next section since we believe it has some significance in the mechanism by which the carbohydrates we have studied effect expansion of monolayer films.

When critical areas for DMPC monolayers with the several carbohydrates for which we have made such measurements were calculated, the results shown in Fig. 3 were obtained. With the exception of inositol, which has very little effect on the monolayer, all the curves are hyperbolic, with clear saturation of the expansion effect at high concentrations of the carbohydrate. Due to the low solubility of these carbohydrates in water, only relatively low concentrations of inositol and raffinose were used. Since most of the curves are hyperbolic, kinetic parameters for the expansion effects of the carbohydrates, formally similar to parameters for enzyme kinetics can be evaluated from linear transformations of the data. We have done such evaluations from Woolf plots of the data for the carbohydrates shown in Fig. 3 (with the exception of raffinose and inositol, for which the kinetic parameters were estimated). These parameters are indicated near each curve in Fig. 3.

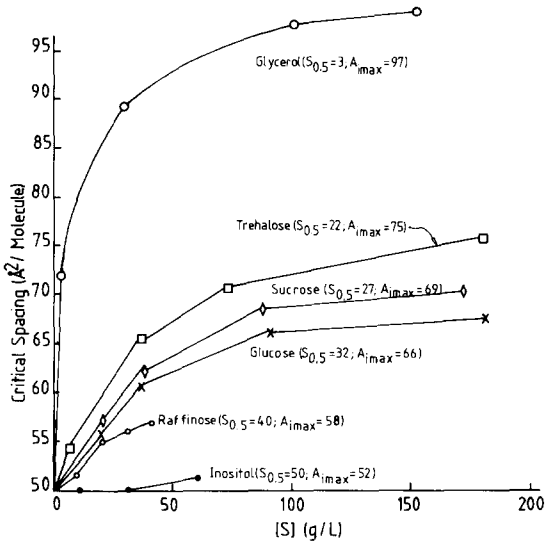


Fig. 3. Critical spacing for DMPC spread on aqueous solutions of various carbohydrates. Data derived from the isotherms of Figs. 1 and 2 and similar isotherms for the other carbohydrates indicated. The carbohydrate concentration which results in half maximal expansion on the monolayer ($S_{0.5}$) and maximal expansion (A_{imax}), evaluated from Woolf plots of the data, are shown near each curve. Parameters for inositol and raffinose were estimated since the curves for these two carbohydrates are not hyperbolic.

Carbohydrate	Symbol	$S_{0.5}$	A_{imax}
Glycerol	○	3	97
Trehalose	□	22	75
Sucrose	◇	27	69
Glucose	×	32	66
Raffinose	○	40	58
Inositol	●	50	52

Since glycerol appears to expand the monolayer so much more than the other carbohydrates shown in Fig. 3, we have investigated potential mechanisms for the expansion in the following experiments.

Isobaric measurements

Changes which occurs in the area/molecule of a phospholipid monolayer in the presence of various carbohydrates with changing temperature and constant pressure were also studied. DMPC has a relatively low transition temperature, as a result of which we encountered difficulty in readily obtaining condensed phase preparations of this phospholipid in monolayers. Therefore, dipalmitoylphosphatidylcholine (DPPC), which has a higher transition temperature, was used. In control studies, we established that DPPC behaves similarly to DMPC in isothermal measurements in the presence of the carbohydrates. Concentrations of trehalose and glycerol used for these measurements expanded the monolayers to a similar level at 30°C. The concentration of sucrose used expanded the monolayer less at 30°C than the equivalent concentration of trehalose or glycerol. Thus, we expected sucrose to have less effect than either trehalose or glycerol under isobaric conditions.

The results shown in Fig. 4 were obtained with DPPC monolayers. These data show that with increasing temperature up to about 22°C a broad plateau is seen in the response of area to changing temperature. There is a rapid increase in the area/DPPC, which begins at about 23°C, followed by a slower increase at temperatures above about 26°C. The rapid increase between 23 and 26°C has been shown by Albrecht et al. [2] to correspond to the main transition temperature in bilayer preparations. Their evaluation of the transition temperature (t_m, taken as the midpoint of the linear increase in area), about 25°C, agrees well with our own results (Fig. 4). During cooling, little or no hysteresis was seen with DPPC on water until the lowest temperatures were reached, again in reasonable with agreement with Albrecht et al. [2], who reported a somewhat larger hysteresis during cooling, however. When sucrose is added to the subphase, there is a small increase in area/DPPC compared with DPPC on water, even at the lowest temperatures. This expansion of the monolayer in the presence of sucrose is reflected by the decrease in t_m (Fig. 4). During cooling a large hysteresis is seen at all temperatures in the presence of sucrose. With trehalose in the subphase, expansion of the monolayer at low temperatures is even more pronounced, with a coincident decrease in t_m (Fig. 4).

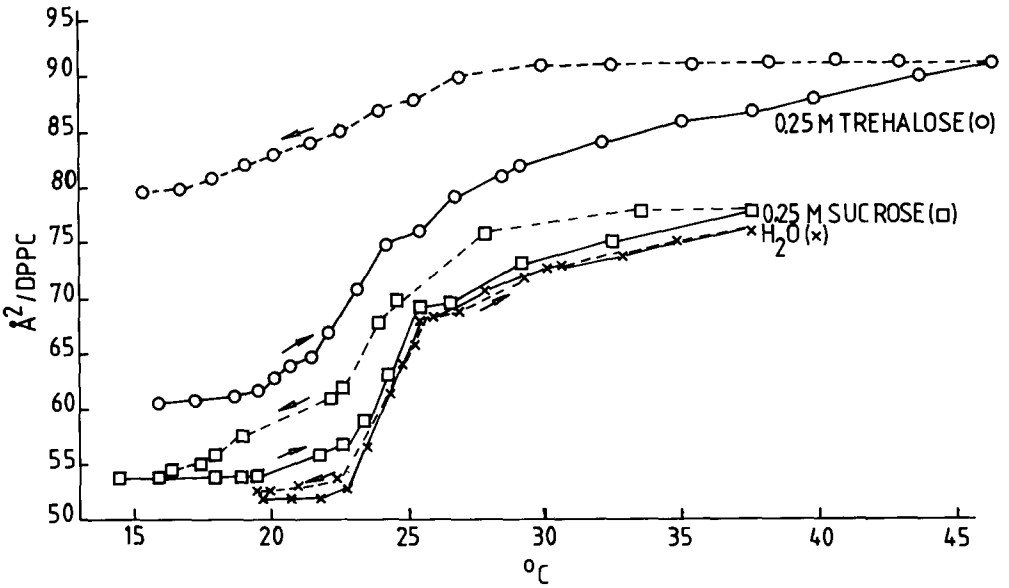


Fig. 4. Isobaric changes in area/molecule for DPPC spread on water and aqueous solutions of trehalose and sucrose. The arrows indicate the direction of temperature change. Pressure was held constant at 12.5 dyn/cm.

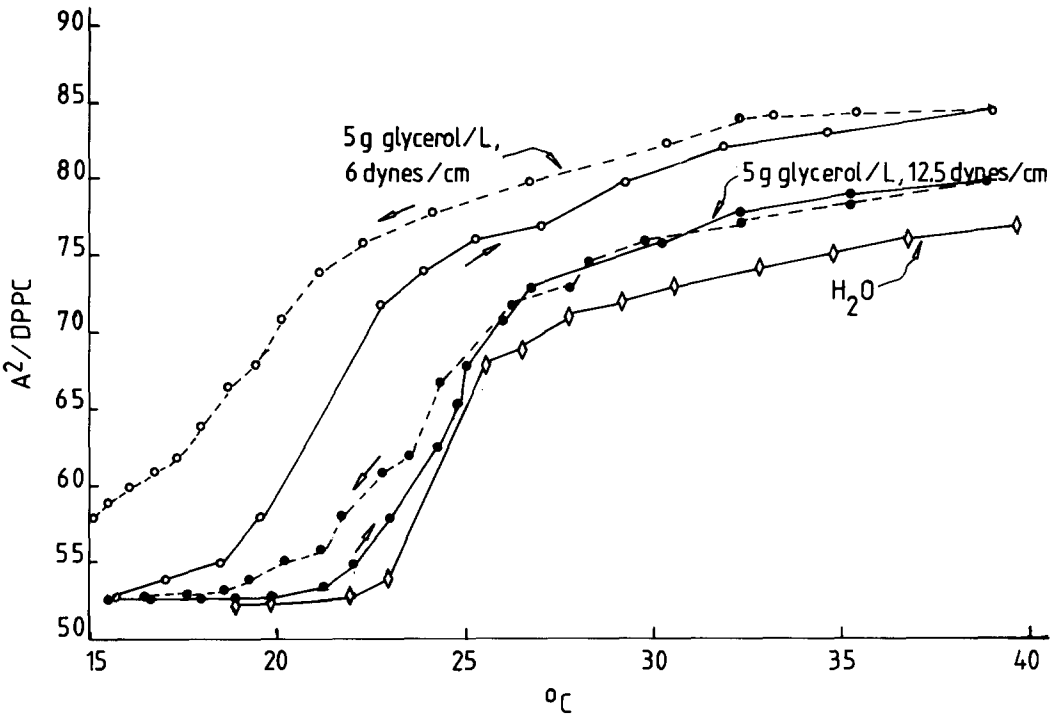


Fig. 5. Isobaric changes in area/molecule for DPPC spread on water and aqueous solutions of glycerol. The arrows indicate the direction of temperature change. Pressure was held constant at 12.5 dyn/cm or 6 dyn/cm.

Cooling of this preparation shows a large hysteresis and expansion of the monolayer by about 20 Å^2/DPPC, even at the lowest temperatures.

The pattern observed with glycerol is considerably different from that seen for sucrose and trehalose. The fully condensed monolayer at low temperatures shows no detectable expansion in the presence of glycerol (Fig. 5). As the temperature is increased, a slight expansion is seen as t_m is approached, but we could measure no significant change in t_m. At temperatures above t_m, significant expansion of the monolayer was seen, but during cooling the hysteresis seen with sucrose and trehalose was almost entirely missing, except at temperatures near t_m (Fig. 5). At the lowest temperatures, even this small hysteresis disappears, and the area/DPPC in these cooled preparations is not different from the area/DPPC on water. When similar experiments were made with DPPC on glycerol, but with decreased pressure on the monolayer, glycerol was seen to have an expanding effect on the monolayer except at the lowest temperatures. At 15°C, the monolayer was not expanded at all compared with that on water, but as the temperature was increased expansion increased until at the highest temperatures expansion similar to that seen under influence of trehalose was seen, as expected from the pressure area curves made above t_m. When these expanded monolayers were cooled, however, the enormous hysteresis seen in the presence of trehalose was missing (Fig. 5). There is a hysteresis, with expansion from about 53 to about 57 Å^2/DPPC during warming and recooling to 19°C, but in the presence of trehalose, by contrast, expansion following warming and recooling to 19°C gave an expansion from about 62 to about 82 Å^2/DPPC.

Discussion

The results presented above suggest that glycerol and the other carbohydrates studied interact with monomolecular films of phospholipids by different mechanisms. Thus, we would like to discuss further the potential mechanisms by which these interactions occur.

Our results are generally in agreement with and extend those of previous workers concerning effects of glycerol on monolayer films [4–6,9]. Cadenhead and Bean [5] suggested that the mechanism by which glycerol expands the monolayer involves mixing of the lipid and glycerol. The evidence for this suggestion came primarily from the finding that when glycerol was added at temperatures below the phase transition it had little or no expanding effect. They interpreted this result to mean that glycerol is incapable of penetrating the condensed monolayer. Our results are consistent with this notion; the isobaric measurements (Fig. 5) show that the condensed monolayer under pressure is expanded little, if any, in the presence of glycerol. As the transition temperature is reached, the monolayer on glycerol expands relative to that on water, but upon cooling this expansion is lost. We interpret this effect to be due to mixing and de-mixing of the glycerol and monolayer during warming and cooling, respectively. Similarly, when the monolayer is spread on glycerol at reduced pressure the expansion of the monolayer is enhanced.

Calorimetric data from other workers also seem consistent with the idea that glycerol and other alcohols expand monolayer films by mixing with the lipids. The lateral expansion of the monolayer would be expected to increase the fluidity of the hydrocarbon chains, i.e. decrease the main transition temperature, and there is good evidence in the literature that this is the case. Eliasz et al. [22] showed that *n*-alcohols containing fewer than ten carbons all lowered the transition temperature of phospholipid bilayers, while longer chained *n*-alcohols had the opposite effect. Similar measurements with a series of phenylalcohols indicated that increasing the number of methylene groups between the phenyl group and the terminal -OH decreased the transition temperature, a finding which seems consistent with the possibility that the alcohol may insert between the polar head groups. Similar data have been published by Hui and Barton [23] and Ebihara et al. [24], with similar conclusions.

By contrast with glycerol, sucrose and trehalose are capable of expanding the monolayer film even below the transition temperature and in monolayer films under lateral pressure (Fig. 4). These findings suggest that sucrose and trehalose interact with monolayers by means of a mechanism different from that of glycerol. A likely explanation is that trehalose might interact with monolayers by means of hydrogen bonding to the polar head group or to the hydration shell centered around it [25]. The hydrogen bonding between -OH groups on the carbohydrate and the polar head groups could result in altered mobility and packing density of the head groups, depending on the spacing of the -OH groups on the carbohydrate. From what we know about the conformational state of the head group both in bulk solution [26] and at low water activities [25] hydrogen bonding to the head group or to water in the main hydration shell is feasible. Experimental data that can test this idea have not yet been obtained, nor has rigorous modeling been attempted. However, with this explanation for the interactions in mind, other effects of trehalose on the monolayer films may be explained. In contrast with glycerol, when the monolayer is heated, the expansion is increased, and upon cooling the monolayer remains expanded. As the monolayer is warmed and becomes less condensed, phosphate groups might be expected to become increasingly available for interaction with the trehalose, and these interactions might be maintained upon re-cooling. In support of this hypothesis is our finding that when DPPC is spread on an aqueous subphase containing trehalose at high temperature and then cooled under isobaric conditions only a small decrease in the area/molecule of DPPC is seen (data not shown).

The data shown in Fig. 4 indicate that both sucrose and trehalose significantly alter the main transition temperature for DPPC monolayers. There is also some calorimetric evidence in the literature that carbohydrates may alter transition temperatures of phospholipid vesicles. Chen et al. [27] have published scanning calorimetry data on DPPC in the presence of various carbohydrates. They showed, for example, that when the galactose concentration around the lamellar phase lipids was increased, the enthalpy of the gel to liquid crystalline phase transition at t_m was apparently decreased, with a spreading of the endotherm towards lower temperatures. More recently, McDaniel et al. [28] published remarkable results

showing that glycerol decreases the main transition temperature of dry DPPC and increases the enthalpy of the main transition to values similar to those for the fully hydrated lipid. We have produced similar results for glycerol, and we have also shown that trehalose decreases the phase transition temperature of dry DPPC. The dry DPPC has an elevated main transition temperature (t_m), as others have previously reported [18], but when dried in the presence of trehalose t_m is depressed. In fact, at the highest concentrations of trehalose t_m for the dry DPPC is actually below that in bulk water. These data will constitute the subject of a forthcoming publication.

In the introduction we indicated that these studies were being conducted with a view towards elucidating the mechanism by which trehalose stabilizes the structure and function of biological membranes at low water contents. Therefore, we wish next to comment on the relationship between the relative abilities of carbohydrates to expand monolayer films of phospholipids and to stabilize dry biological membranes. When the values for maximal monolayer expansion in the presence of carbohydrates and similar values for preservation of dry sarcoplasmic reticulum (taken from the accompanying paper [16]) are compared, Fig. 6 is obtained. There is a clear correlation between the efficiency of the carbohydrates at expanding the monolayer films and stabilizing membrane structure and function at low water contents; inositol has little or no expanding effects on the monolayers and does not stabilize membranes at low water contents, while trehalose expands the monolayers more than any of the carbohydrates tested and is the most effective at preserving membrane structure and function in the absence of water. The only exception to this pattern is glycerol, which probably interacts with membranes by means of a different mechanism from the other carbohydrates.

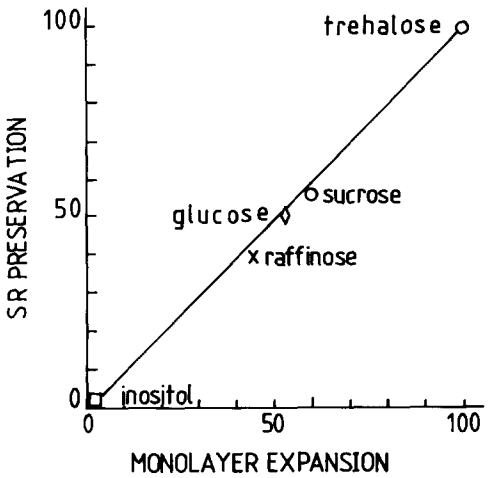


Fig. 6. Correlation between preservation of sarcoplasmic reticulum (SR) in the dry state by various carbohydrates and expansion of DMPC monolayers by the same carbohydrates. All values are presented relative to those for trehalose (taken as 100) and were derived from data for half saturation constants for sarcoplasmic reticulum preservation ($S_{0.5}$, from Ref. 16) and data for maximal expansion of monolayers (A_{imax}, from Fig. 4).

In conclusion, the data presented here not only support our hypothesis that increased membrane fluidity under the influence of carbohydrates could stabilize membranes at low water contents, they have other ramifications: They may provide an explanation for the well known cryoprotective effects of carbohydrates. Furthermore, in view of the findings reported here that carbohydrates normally found in significant concentrations in cells profoundly alter membrane fluidity, we suggest that the role of carbohydrates in modulating membrane fluidity in intact cells warrants further investigation.

Acknowledgments

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