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EFFECTS OF CARBOHYDRATES ON MEMBRANE STABILITY AT LOW WATER ACTIVITIES

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The relative effectiveness of a variety of carbohydrates in preserving the structural and functional integrity of membranes at low water activities was studied, using Ca-transporting microsomes from muscle as a model membrane. The order of effectiveness (greatest to lowest) was: trehalose, lactose, maltose, cellobiose, sucrose, glucose, fructose, sorbitol, raffinose, *myo*-inositol, glycerol. At the highest concentrations of the most effective sugars tested, microsomes were obtained upon rehydration that were similar structurally and functionally to fresh membranes. The least effective carbohydrates, alcohol sugars, all appear to be fusogenic. A structural explanation for relative effectiveness of the sugars was sought, but no clear relationship was found, except that effectiveness does not appear to be related to the number or position of hydroxyl groups available for hydrogen bonding.

Introduction

Phospholipids are maintained in the bilayer configuration by hydrophobic interactions [1]. In the absence of water, such interactions are minimized, and massive phase transitions result, the best known of which is the bilayer to hexagonal II transition [2]. We have recently shown that intact biological membranes, muscle microsomes, undergo lipid phase separations and phase transitions and fusion between vesicles when dried [3], the result of which is disruption of both structure and function in the rehydrated membranes [4]. The impetus for this work was our previous observation [5,6] that cell membranes appear to be maintained intact in some organisms that are capable of surviving total dehydration. Thus, we have sought an explanation for the mechanism by which membranes are stabilized in these dry organisms (said to be in a state of anhydrobiosis; see also Refs. 8 and 9), and we report further evidence in the present paper with this goal in mind. Using muscle microsomes derived from lobster (an animal which does not survive desiccation) we show that a number of carbohydrates will preserve structure and function in the microsomes during dessication. The most effective carbohydrate in this regard is trehalose.

Trehalose, a non-reducing disaccharide of glucose, is widely distributed among living systems, and has commonly been considered to be a reserve carbohydrate which is readily converted to glucose during activity or the breaking of dormancy. The widespread occurrence of trehalose at high concentrations (as much as 20% of the organism's dry weight) in anhydrobiotic organisms such as brine shrimps cysts (dry gastrulae of *Artemia salina* [10]), and the dry larvae or adults of several species of nematodes [11,12] as well as desiccation-tolerant

Abbreviation: Tes, 2-([2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino)ethanesulfonic acid.

dry active baker's yeast [13], ascospores of *Neurospora tetrasperma* [14], macrocysts of the slime mold *Dictyostelium mucoroides* [15], and the 'resurrection plant' *Selaginella lepidophylla* [16] suggests that this molecule may be involved in the desiccation tolerance of these organisms. We have recently provided evidence that this is the case; when muscle microsomes were dried in the presence of as little as 0.3 g trehalose/g membrane, the phase separations and transitions seen when the membranes were dried in the absence of trehalose were almost completely inhibited [4,17]. When membranes lyophilized in the presence of trehalose were rehydrated, they were seen to have a morphology and biological activity similar to fresh membranes [4,17]. Trehalose is in this regard far superior to sucrose, which is known to have some stabilizing properties for membranes at reduced water activities [18,19]. The physical basis for the remarkable stabilizing effect of trehalose is unknown, although we are beginning such physical studies in this laboratory. As a first step in this direction, we have undertaken a survey of the stabilizing effects of various commonly occurring reducing and non-reducing sugars and sugar alcohols. In the present paper, we will compare the stabilizing effects of these carbohydrates with those of trehalose, and we will present additional freeze-fracture evidence which suggests that the damage to the membranes which occurs at low carbohydrate concentrations is due to fusion of the membranes during the drying process. Furthermore, we will provide a discussion of molecular models of these carbohydrates that suggest further experiments on the modes of their interactions with membranes.

Materials and Methods

Vesicle preparation. Microsomal vesicles were isolated from the abdominal muscles of lobster (*Homarus americanus*) as described previously [3]. Protein content of the preparations was determined by the Coomassie blue method for use in calculating the results of all biological assays. For estimating the amount of membrane present when adding desired amounts of carbohydrates, the Lowry et al. [20] method for protein was used. The latter methods estimates approximately twice the amount of protein in a given sample of microsomes as does the Coomassie blue method. Bovine serum albumin was used as a standard.

Preparation of dry microsomal membranes. Freshly prepared microsomal vesicles were diluted with 10 mM Tes buffer, pH 7, to a protein concentration of about 10 mg/ml. Concentrated solutions of the desired carbohydrate, made up in 10 mM Tes buffer, were added to subsamples of the vesicles to give a range of carbohydrate concentrations. Subsamples were frozen in liquid nitrogen in volumes no greater than 1.5 ml to facilitate drying, and were then lyophilized on a VirTis lyophilizer. In early experiments, the dry samples were stored over anhydrous $CaSO_4$. In later experiments, the dry samples were transferred to Pyrex tubing which had been sealed at one end, the tube was evacuated, and the tube sealed under vacuum. Since it could take several days to complete a series of assays, we wanted to avoid as much oxidative damage as possible in the samples. The carbohydrate concentrations of the subsamples were determined by the anthrone method of Dimler et al. [21], or by high performance liquid chromatography. Samples were rehydrated by the addition of approximately 1 ml of distilled water, and were suspended by use of a small hand held glass homogenizer.

Enzyme assays. ATPase activity of the vesicle preparations was determined by measuring the pH changes during hydrolysis of ATP [22]. Reactions were run in 4 ml of a medium consisting of 4 mM $MgCl_2$, 5 mM potassium oxalate, 100 mM KCl, 4 mM ATP, and approximately 0.1 to 0.2 mg total protein. The reaction was started by the addition of 1 μmol of $CaCl_2$. The coupling ratio, or Ca^{2+} transported per ATP hydrolyzed could then be calculated from the total ATP utilized in the reaction.

Measurement of Ca^{2+} transport. Ca^{2+} uptake by microsomal vesicles was measured by the Millipore filtration method of Martonosi and Feretos [23] with modifications as previously described [4].

Freeze-fracture. Freeze-fracture was carried out as described previously [4] on vesicles dried in various concentrations of sucrose. Vesicle diameters were estimated from half-shadowed vesicles, since replicas were shadowed at a 45° angle.

Results

Non-reducing sugars

The effect of concentration of sucrose in which the membranes were previously dried on Ca uptake, Ca transported/ATP, and vesicle diameter in the rehydrated membranes is shown in Fig. 1. Below about 0.9 g sucrose/g dry membrane (g carbohydrate/g, hereafter), both Ca uptake and Ca/ATP fall off rapidly, while vesicle diameter increases. These results confirm earlier results which showed a rapid rise in vesicle diameter at the same trehalose concentration where Ca uptake and Ca/ATP fall. The trehalose concentration at which these transitions occur, however, is about 0.3 g trehalose/g; thus, about 1/3 as much trehalose is required to achieve the same effect as sucrose. The increase in vesicle diameter is the result of fusion of membranes during the drying process, accompanied by phase separation of lipids and of proteins from the lipids [3]. If the concentration of sucrose is sufficiently high, lyophilized vesicles remain separated from each other, embedded in a matrix of carbohydrate (Fig. 2).

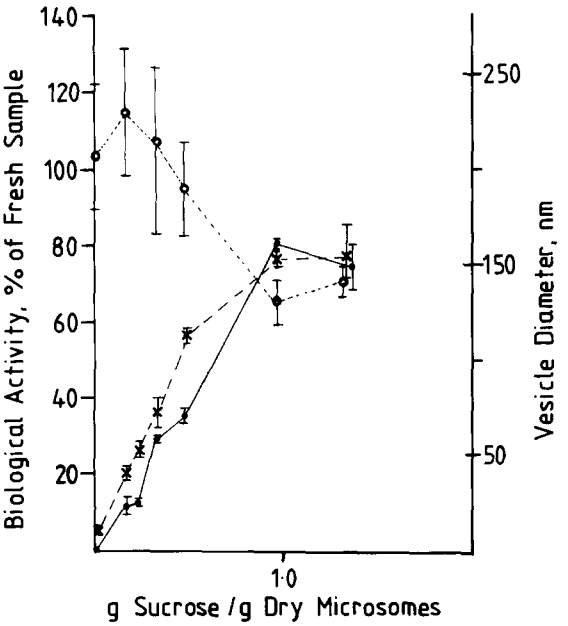

Fig. 1. Calcium uptake (×— — —×), Ca transported/ATP utilized (Ca/ATP, ●——●), and vesicle diameter (○····○) of muscle microsomes rehydrated after having been dried at the indicated concentration of sucrose.

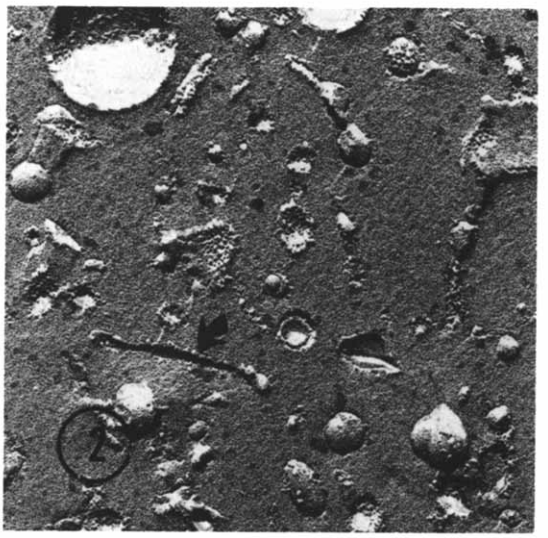

Fig. 2. Freeze-fracture of lyophilized muscle microsomes embedded in a matrix of dry sucrose. The arrow indicates a collapsed microsomal vesicle seen in cross fracture (see Ref. 4 for discussion). Magnification, 60000×.

When vesicles which had been dried in a low sucrose concentration were rehydrated, large areas of fused membrane are seen in freeze-fracture (Fig. 3). Since the vesicle diameter is measured only from vesicles, the values in Fig. 1 do not

Fig. 3. Freeze-fracture of rehydrated muscle microsomes previously lyophilized in a low (non-protective) concentration of sucrose. The starred area is part of a large expanse of fused membrane. A vesicle is indicated by V. Magnification, 60000×.

indicate the full extent of the fusion which occurs at lower sucrose concentrations.

Reducing sugars

Like trehalose, cellobiose is a disaccharide of glucose, but the monomers are joined through a 1,4-linkage, so that cellobiose is a reducing sugar. Results of lyophilizing microsomal vesicles at various cellobiose concentrations are shown in Fig. 4. Cellobiose appears to be about as effective as sucrose at preserving Ca uptake activity and Ca/ATP ratios; these values do not begin to fall off until the cellobiose concentration is less than 0.7 g/g. Thus, more than twice as much cellobiose as trehalose is required to stabilize the dry membranes. The ATPase activity begins to rise at low concentrations of the cellobiose where the membranes have become completely uncoupled. Although the membranes are uncoupled, it is possible to detect some slight Ca uptake activity due to the very high ATPase activity. The high ATPase activity, uncoupling, and low Ca uptake capacity occur at a point in the range of carbohydrate concentration where we have shown a concomitant increase in vesicle diameter with other sugars, e.g. sucrose, as shown in Fig. 1. This rise in ATPase activity in the range of carbohydrate concentration which also produces uncoupling and low Ca transport is a common feture to all of the preparations. In order to simplify the following discussion, data for ATPase activity and Ca uptake will be omitted from the remainder of the figures. Instead, Ca/ATP ratios will be compared with those for trehalose for the rest of the carbohydrates presented, since this coupling ratio provides a reliable assay for the structural and functional integrity of the membrane.

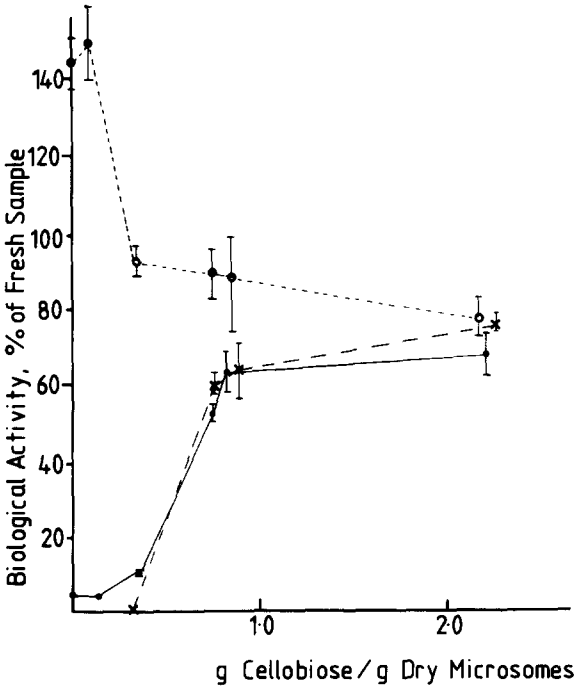

Fig. 4. Ca uptake (●——●), ATPase activity (○·····○), and Ca/ATP (×— — —×) in rehydrated muscle microsomes previously dried at the indicated concentration of cellobiose.

Lactose monohydrate and maltose monohydrate were assayed at only two concentrations, but were as effective as trehalose at concentrations of 0.4 g/g and 0.5 g/g, respectively. Although no lower concentrations were assayed, it appears that lactose and maltose may be as effective as trehalose and more effective than sucrose in preserving biological activity in lyophilized membranes. Like cellobiose, however, lactose and maltose are reducing sugars, in contrast to trehalose, and may have other deleterious effects on the lyophilized membranes.

Stabilizing effects of glucose, a subunit of both trehalose and sucrose, fructose, a subunit of sucrose, and the trisaccharide, raffinose which has a sucrose subunit, are compared to trehalose in Fig. 5. Although all of these carbohydrates do as well as stabilizing the dry microsomes at high concentrations, none are as effective as trehalose at lower concentrations. Glucose is the only one of these sugars besides trehalose which has protective ability at concentrations below 0.5 g carbohydrate/g. Cellobiose (Fig. 4), seems somewhat more effective than glucose, and sucrose (Fig. 1) appears to be slightly more effective than cellobiose. In section 4 on statistical analysis of the data we will provide a more rigorous analysis of these apparent trends.

Sugar alcohols

myo-Inositol, a cyclitol, and sorbitol, an open chain polyalcohol, are compared with trehalose in Fig. 6. Sorbitol is as effective as trehalose at high concentrations, but membranes dried at 0.3 g

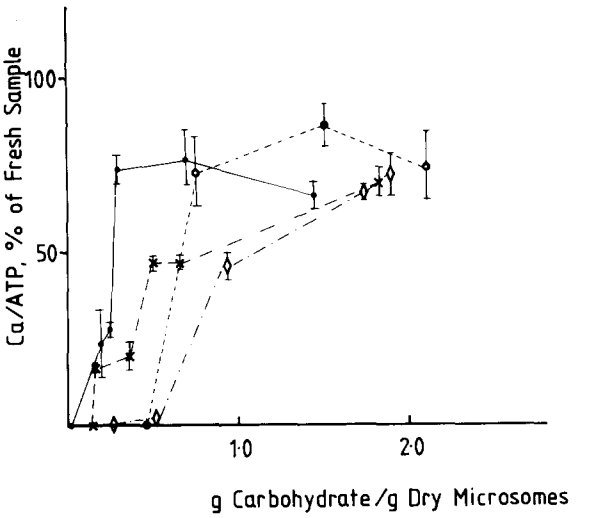

Fig. 5. Ca/ATP of rehydrated muscle microsomes previously dried at the indicated concentrations of trehalose (●———●), fructose (○------○), glucose (×— — —×), or raffinose (◇-·-·-◇).

sorbitol/g are completely uncoupled, while microsomes dried in trehalose at that concentration are maximally protected. Sorbitol is maximally effective, and as effective as trehalose, at 1 g sorbitol/g. *myo*-Inositol is ineffective for stabilizing dry membranes. At the highest concentration tested (about 1.2 g inositol/g), *myo*-inositol preserves only about 20% of the Ca/ATP ratio found in fresh membranes, and at about 1 g inositol/g, the membranes are uncoupled.

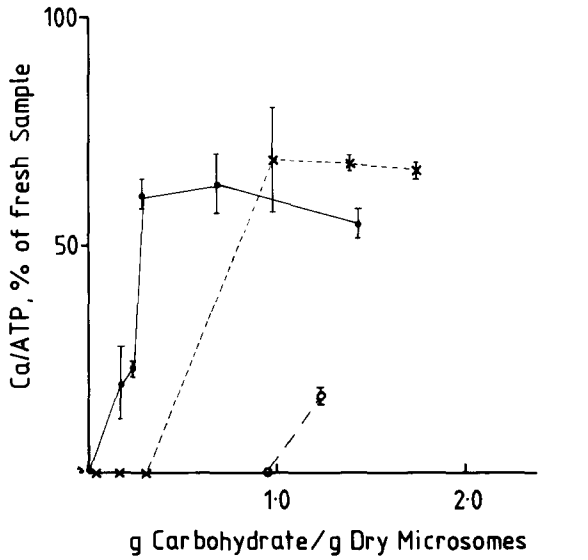

Fig. 6. Ca/ATP of rehydrated muscle microsomes previously dried at the indicated concentrations of trehalose (●———●), *myo*-inositol (○— — —○), or sorbitol (×------×).

Glycerol is found in some anhydrobiotic nematodes (11) and in brine shrimp cysts [24,25] in concentrations up to 5% of the dry weight of the organisms. Glycerol is also a widely used and effective cryoprotectant [26]; its effectiveness has been ascribed to the large number of hydrogen-bonding groups/volume and to the fact that it may be able to fit into the water structure [27]. We have tested glycerol both separately and in combination with trehalose as a stabilizer in the dry state. Even at low concentrations, it causes fusion of dry microsomal membranes, and in higher concentrations (0.27 g glycerol/g) causes such extensive fusion that the microsomes will not resuspend upon rehydration. The presence of 0.01 g glycerol/g in a preparation which also contained 0.67 g trehalose/g reduced the coupling ratio to 23% of the freshly made sample. Microsomes dried at that concentration of trehalose by itself could be expected to be maximally protected (cf. Fig. 6).

Statistical analysis

As shown in Figs. 1, 4, and 5, all the curves illustrating relationships between biological activity (Ca/ATP) in the rehydrated membranes and concentration of the carbohydrate used are sigmoidal, or at least roughly so. For example, the curve for glucose fits a sigmoidal shape according to a third order polynomial curve fitting with a regression coefficient of 0.92. Similar or even more significant regression coefficients were obtained for curves for all the non-reducing and reducing carbohydrates. Since most sugar alcohols had no significant protective effects, they were omitted from the analysis (with the exception of sorbitol, Fig. 6).

Sigmoidal curves can be adequately described by the Hill equation, which is commonly used in analyzing kinetic data for enzymes and for transport functions [28]. While the present data are not formally analogous to kinetic data for enzymes, the sort of mathematical analysis used in Hill plots can be applied here with a view towards providing a convenient method for comparing curves. One

useful form of the Hill equation is:

$$\log \frac{A_i}{(A_{max} - A_i)} = n \log S - \log K$$

where A_{max} is the maximal rate, A_i is the rate at a given concentration of substrate, S (the carbohydrate in the present analysis), n is the number of binding sites (meaningless for this analysis), and K is a constant. A plot of $\log A_i/(A_{max} - A_i)$ against S should yield a straight line. As shown in Fig. 7, this is the case. For all the carbohydrates for which sufficient data are available, the data are linearized in a Hill plot, with regression coefficients of 0.95 or greater. When $A_i/(A_{max} - A_i)$ is set equal to zero, the S which results in $A_{max}/2$ can be evaluated. This parameter, which we will call $S_{0.5}$, provides a convenient way of comparing the efficiency of these carbohydrates in stabilizing the dry membranes.

As shown in Fig. 7, sucrose and glucose which have $S_{0.5}$ values of 0.58 and 0.60, respectively, appear to have similar properties; not only are their $S_{0.5}$ values similar, the linear transformations have nearly identical slopes. When the transformed data were compared by means of paired t-tests, the lines for these two carbohydrates were shown not to be significantly different ($p > 0.95$). We do not have sufficient data for cellobiose and sorbitol to carry out the linear transformation, but the data we do have fall near the lines for sucrose

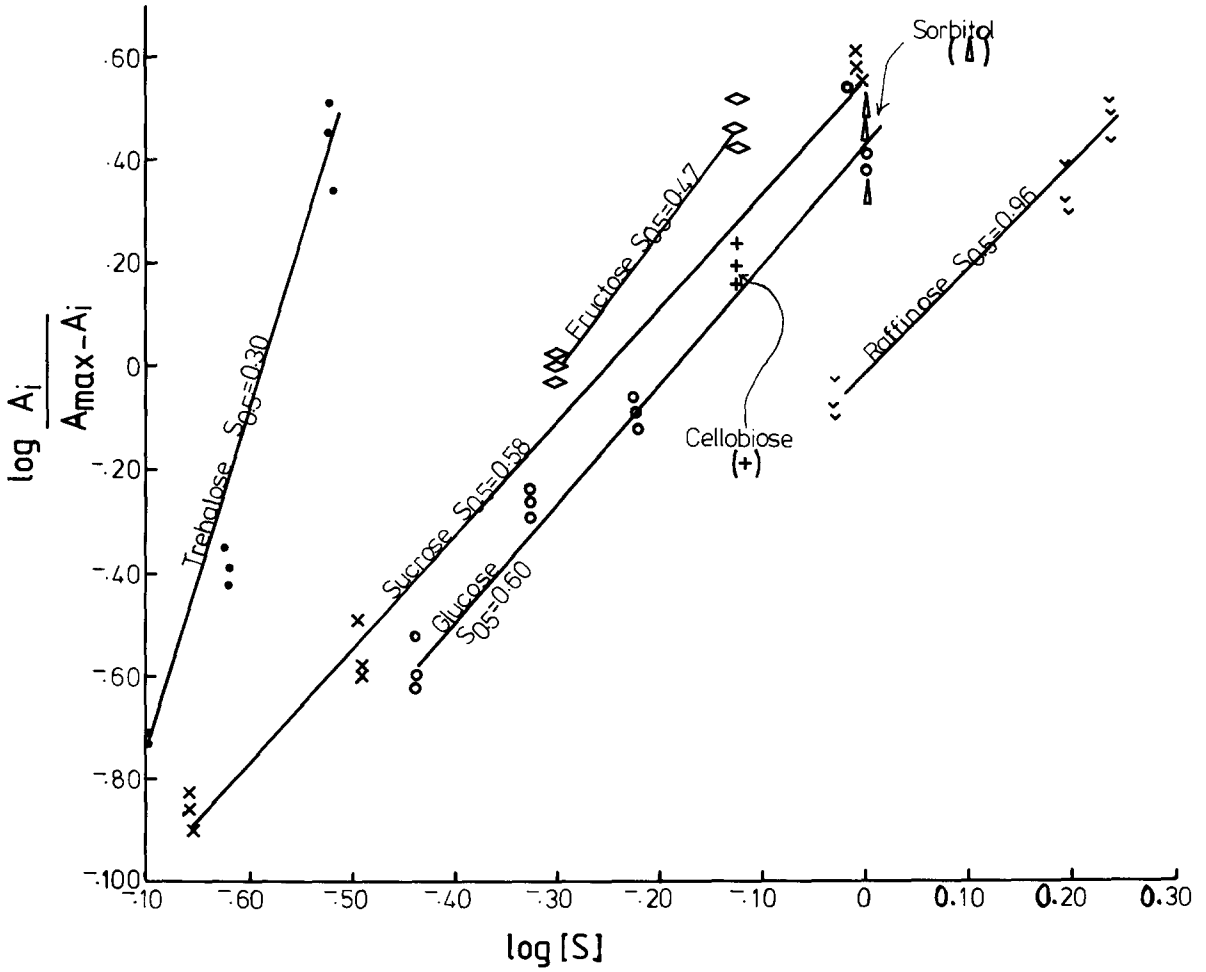

Fig. 7. Linear transformation of the data shown in Figs. 1 and 4–6, according to the Hill equation. $S_{0.5}$ represents the concentration of carbohydrate necessary to recover vesicles that show 50% Ca/ATP of fresh vesicles. $S_{0.5}$ value for: trehalose (●), 0.30; fructose (◇), 0.47; sucrose (×), 0.58; glucose (○), 0.60; raffinose (v), 0.96.

and glucose and are not significantly different from data for sucrose and glucose, according to a paired *t*-test. Fructose, with a slightly lower $S_{0.5}$, and a slightly different slope, is significantly different from sucrose, glucose, cellobiose, and sorbitol but only at the $p > 0.90$ level; we suspect that fructose actually is quite similar in its protective efficiencies to the other sugars discussed so far, even though statistical differences can be demonstrated. Raffinose appears to be in a category by itself, with $S_{0.5}$ of 0.96, and while the slope of the transformed data is nearly identical to that for the previously discussed sugars, the line is so displaced on the concentration axis that raffinose is highly significantly different ($P < 0.99$).

By contrast, with the other carbohydrates studied, trehalose has a much lower $S_{0.5}$ and a slope that is clearly different (Fig. 7). The data for trehalose are highly significantly different from the aggregated data for sucrose, cellobiose, glucose, fructose and sorbitol ($p > 0.99$) and from those for raffinose. We have not obtained sufficient data for maltose and lactose to do a rigorous analysis, but the data we do have fall on the extrapolated line for trehalose (Fig. 7).

Thus, this analysis strongly suggests that the carbohydrates we have studied here fall into four distinct groups in terms of their effectiveness, in decreasing order: (1) trehalose, with maltose and lactose potentially having similar properties, at least for the short term; (2) sucrose, glucose, cellobiose, fructose, and sorbitol; (3) raffinose; and (4) the sugar alcohols, with the curious exception of sorbitol.

Discussion

All of the sugars and sugar alcohols used in the present study, with the single exception of trehalose (which has not been studied in this regard), have been shown to interact strongly with water. It has been postulated that these carbohydrates participate in the water lattice in bulk water through hydrogen bonding. In support of this hypothesis, thermodynamic analyses of solutions of glucose and sucrose indicate stronger hydrogen bonding between these sugars and water than there is between water molecules themselves [29]. Such increase in hydrogen bonding strength in sugar solutions has been used to account for the effects of glucose and sucrose on the solubility of aromatic amino acids. Lakshmi and Nandi [30] suggested that the increased water structuring leads to an increase in hydrophobic interactions in non-polar portions of molecules. Along the same lines, a number of sugars and sugar alcohols, including some used in the present study, have been shown to increase the thermal stability of proteins. The ability to increase the temperature of the thermal phase transition is apparently related to partial molar volume of the solute, which is a rough measure of the 'structure-making' or 'structure-breaking' ability of the solute [31]. There is some suggestion in the literature that the interactions between carbohydrates and water are altered dramatically with changes in the position of the -OH group: thermodynamic studies have shown that at concentrations up to about 0.3 M, mannitol has a structuring effect on water, while sorbitol has a structure breaking effect; these two stereoisomers differ in the position of only one of their -OH groups [32]. There is also considerable evidence that the most favorable carbohydrate-water interactions involve equatorial -OH groups [33–35]. Franks et al. [36] have shown that *myo*-inositol, which has five equatorial -OH groups, would be expected to have a strong hydration, leading to a large negative molar compressibility, which in fact was found to be so. Some of the best evidence for this hypothesis is based on stereochemical modeling; Warner [27] suggested that since the second nearest neighbor oxygen distance in water is about 4.86 Å, which is the distance between equatorial -OH groups on pyranose and cyclitol rings, these -OH groups could fit precisely into the water structure.

The notion that sugars and sugar alcohols may increase the structuring of water by entering into the water structure is attractive in the present context, since it also suggests that these carbohydrates may be able to substitute for water, particularly for that small fraction (approx. 0.3 g H_2O/g dry weight) which is strongly bound [37–40]. Thermodynamic studies have shown that this primary hydration water is important for the stability of isolated proteins and phospholipid bilayers, with larger changes occurring in the temperature of denaturation or phase transition, and

the enthalpy and entropy of denaturation in the range of 0–0.3 g H_2O/g dry material, with little or no change occurring at higher hydration levels [37,38,41]. Direct evidence concerning the water replacement hypothesis is still lacking, but Clegg et al. [39] have provided some evidence that is suggestive; using H-nuclear magnetic resonance, they have recorded relaxation times for H_2O protons in protein/water mixtures of variable water contents. As water contents declined, relaxation times declined linearly, as expected. When similar measurements were done with protein/glycerol/water mixtures, relaxation time declined with H_2O content, but reached a minimal value at about 0.3 g H_2O/g protein. At lower water contents the relaxation time increased. A possible interpretation of this finding is that at low water contents glycerol hydrogen bonds to polar residues in the protein, displacing water. In support of this suggestion is the work of Gekko and Timasheff [42,43], who have interpreted their thermodynamic data on protein-glycerol interactions to be consistent with the existence of water-glycerol structures around polar residues in the protein.

It seems likely that useful information could be obtained from comparisons of the structure of the carbohydrates we have used in this study. The conformation of the sugars and sugar alcohols used in conjunction with dry muscle microsomes is of course, unknown, but is more likely to be close to that of the crystalline state than the conformation in aqueous solution, so we have undertaken a survey of the crystal structure of all of the carbohydrates used in this study, as well as trehalose. We paid particular attention to the number of -OH groups available for forming hydrogen bonds, and their density in the crystal structure, as well as the number of axial and equatorial -OH groups. A summary of the data and a list of the sources are shown in Table I, with the sugars and alcohols listed in order of relative effectiveness in preserving activity in dried microsomes. There appears to be no clear relationship between any of the parameters of the crystal structure and effectiveness of the carbohydrate, but the data do suggest that the number of equatorial -OH groups available for hydrogen bonding has little to do with the relative effectiveness of these compounds. For example,

TABLE I

SOME PHYSICAL PROPERTIES OF CARBOHYDRATES THAT MAY BE RELATED TO THEIR ABILITY TO HYDROGEN BOND TO SURFACES

Parameters for glycerol were calculated from known density and molecular weight. Carbohydrates are listed in their apparent order of effectiveness at stabilizing membranes at low water activities. Superscripts indicate carbohydrates that are not significantly different from each other in their stabilizing capacity, but are significantly different from all carbohydrates with different superscripts. n.a., not applicable.

Carbohydrate	Number of OH available for intermolecular bonding per nm^3	Number of equatorial OH groups available for intermolecular bonding per nm^3	Equatorial OH/ Total OH	Refs.
Trehalose dihydrate [a]	19	19	1	44, 45
Lactose monohydrate [a]	20	10	0.5	46, 47
Maltose monohydrate [a]	18	15	0.83	48, 49
Cellobiose [b]	19	19	1	50–52
Sucrose [b]	14	11	0.8	53, 54
Glucose [b]	26	20	0.77	55, 56
Fructose [b]	27	5	0.2	57, 58
Sorbitol [b]	30	n.a.		59
Raffinose [c]	17	17	1	60
myo-Inositol [d]	30	25	0.83	61
Glycerol [d]	25	n.a.		

trehalose and cellobiose have the same number of -OH groups available for intermolecular bonding, the same number of -OH groups/unit volume, and all of the -OH groups are equatorial, yet trehalose is more than twice as effective as cellobiose in preserving coupling between ATPase activity and Ca transport. Sucrose, glucose and fructose have 11, 5, and 20 equatorial -OH groups/unit volume, respectively, but all have nearly the same ability to protect dry microsomes. *myo*-Inositol, which has five of its six -OH groups in an equatorial position (more equatorial -OH groups/unit volume than trehalose) and which forms strong bonds with water [35] is poorest of the carbohydrates tested in preserving function of dried microsomes. Sorbitol, which does not exist in a ring structure, cannot be said to have equatorial and axial hydroxyl groups at all, yet it is more effective than *myo*-inositol in preserving function.

Speculations as to the ability of sugars and sugar alcohols to substitute for water in dry systems, thus preserving structure and function, suffer from the lack of thermodynamic or physical studies on the effects of carbohydrates on phospholipids, proteins, or membranes at low water contents. As far as we know, there is no information available on such effects. Further, trehalose, despite its widespread occurrence in many organisms, has not been the subject of the sorts of studies in hydrated systems or aqueous solutions that have been carried out for other carbohydrates. We are now undertaking physical studies on trehalose/phospholipid preparations at various trehalose and water contents with this end in mind.

Acknowledgments

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