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Stabilization of Protein Structure by Sugars†

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ABSTRACT: The preferential interaction of proteins with solvent components was measured in aqueous lactose and glucose systems by using a high precision densimeter. In all cases, the protein was preferentially hydrated; i.e., addition of these sugars to an aqueous solution of the protein resulted in an unfavorable free-energy change. This effect was shown to increase with an increase in protein surface area, explaining the protein stabilizing action of these sugars and their enhancing effect of protein associations. Correlation of the preferential interaction parameter with the effect of the sugars on the surface tension of water, i.e., their positive surface tension increment, has led to the conclusion that the surface free energy perturbation by sugars plays a predominant role in their preferential interaction with proteins. Other contributing factors are the exclusion volume of the sugars and the chemical nature of the protein surface.

Polyhydric alcohols and sugars have been used for many years as stabilizing agents for the maintenance of the biological activity of macromolecules (Tanford et al., 1962; Utter et al., 1964; Gerlsma, 1968, 1970; Gerlsma & Sturr, 1972, 1974; Neucere & St. Angelo, 1972; Frigon & Lee, 1972). On the other hand, cautionary notes have been sounded about the use of sucrose at high concentration, as in sucrose density gradient centrifugation, on the basis of reports that sucrose decreased the activity of some enzymes (Hinton et al., 1969). Such an alteration of enzyme activity has been ascribed usually to conformational changes without, however, any experimental evidence. Gerlsma (1968, 1970) and Gerlsma & Sturr (1972, 1974) have shown that polyhydric alcohols and sugars increased the transition temperature of some proteins in aqueous solution, and they ascribed the stabilizing action of these substances to their induction in water of a decrease in hydrogen bond rupturing potency (Gerlsma, 1970).

Another source of structural stabilization can be the preferential interaction of protein with solvent components at high concentration of additives. For example, conformational changes induced by 2-chloroethanol and guanidine hydrochloride are linked to the binding of these substances to the protein (Timasheff & Inoue, 1968; Lee & Timasheff, 1974), while protein preferential hydration is observed in aqueous solutions of 2-methyl-2,4-pentanediol (Pittz & Timasheff, 1978), which has been used successfully to crystallize ribonuclease A in its native form (King et al., 1956). The same is true of other systems, such as glycerol (Timasheff et al., 1976; Gekko & Timasheff, 1981), some salts (Timasheff et al., 1976; Arakawa & Timasheff, 1982; Aune & Timasheff, 1970), and sucrose (Lee et al., 1975), which are known to be protein structure stabilizing agents. Lee & Timasheff (1981) have analyzed thermodynamically the aqueous sucrose system and shown that the stabilizing effect of sucrose stems from the preferential hydration of proteins in this medium and that this, in turn, may be related to the increase in the free energy of cavity formation induced by addition of sucrose to water. It seemed of interest, therefore, to examine whether preferential hydration is a common feature of sugar systems and, if so, to probe the causes of such preferential hydration. A study was carried out, therefore, of interactions of solvent components with proteins in aqueous lactose and glucose solutions, and the results are presented in this paper.

Materials and Methods

All the proteins used in this study were purchased from Sigma: ribonuclease A (RNase A)[1] (type II-A, lot 87C-0207), lysozyme (lot 57C-8025), chymotrypsinogen A (lot 66C-8125), β-lactoglobulin (lot 86c-8065, 106C-8070), bovine serum al-

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[1] Abbreviations: RNase A, ribonuclease A; CTG, chymotrypsinogen A; β-LG, β-lactoglobulin; BSA, bovine serum albumin; MPD, 2-methyl-2,4-pentanediol.

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bumin (lot 65c-7533, 58C-7172), and ovalbumin. These proteins were thoroughly deionized by dialyzing against distilled–deionized water (in some cases, against dilute aqueous HCl solution) or by passing through a mixed-bed ion-exchange column (Amberlite MB-J) and lyophilized. Lactose from Eastman and glucose from Sigma were used without further purification.

Preferential Interactions. The preferential interactions of the solvent components with the proteins were obtained from the partial specific volumes of the proteins, measured with a Precision DMA-02 density meter (Anton Paar, Gratz) (Stabinger et al., 1967; Kratky et al., 1973). These measurements were carried out at conditions (1) at which the molal concentrations of solvent components were identical in the solvent and in the protein solution and (2) at which the chemical potentials of diffusible components were kept identical in the solvent and in the protein solution. Operationally, the latter condition can be obtained to a close approximation by equilibrating the protein solution with the solvent by dialysis (Casassa & Eisenberg, 1964). According to the generally accepted notation of Scatchard (1946) and Stockmayer (1950), in a three-component system, water is designated as component 1, protein as component 2, and the additive as component 3. When the concentration is expressed in thermodynamic units, the preferential interaction parameter, $\xi_3 = (\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$, can be obtained from (Cohen & Eisenberg, 1968)

$$\xi_3 = \left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3} = \frac{(\partial\rho/\partial g_2)_{T,P,\mu_3} - (\partial\rho/\partial g_2)_{T,P,m_3}}{(\partial\rho/\partial g_3)_{T,P,m_2}} = \frac{(1 - \rho_0\phi'^0_2) - (1 - \rho_0\phi_2^0)}{1 - \rho_0\bar{v}_3} \quad (1)$$

where g_i is the concentration of component i, expressed as grams of component i per gram of water, μ_i is the chemical potential of component i, T is the thermodynamic (Kelvin) temperature, P is the pressure, ρ_0 and ρ are the densities of the reference solvent and solution, respectively, $\bar{v}_3$ is the partial specific volume of component 3, and ϕ'^0_2 and ϕ_2^0 are the apparent partial specific volumes of the protein measured at conditions of constant solvent chemical potential and at constant solvent molality, respectively. The values of ϕ_2^0 and ϕ'^0_2 are obtained by extrapolating to zero protein concentration the apparent specific volume, ϕ_{app}, measured at each set of conditions.

The interaction parameter, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$, is a measure of the excess of component 3 in the immediate domain of the protein over its concentration in the bulk solvent. A positive value of this parameter indicates an excess of component 3; a negative value means a deficiency of component 3, i.e., an excess of component 1, water, in the domain of the protein, namely, preferential hydration, since (Timasheff & Kronman, 1959)

$$\left(\frac{\partial g_1}{\partial g_2}\right)_{T,\mu_1,\mu_3} = \frac{1}{g_3}\left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3} \quad (2)$$

In molal units, this parameter becomes

$$\left(\frac{\partial m_3}{\partial m_2}\right)_{T,\mu_1,\mu_3} = \frac{M_2}{M_3}\left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3} \quad (3)$$

where m_i is the molality of component i and M_i is its molecular weight.

The preferential interaction parameter is related to the total amounts of the two solvent components present in the domain of the protein by (Inoue & Timasheff, 1972; Na & Timasheff, 1981)

$$\xi_3 = \left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3} = A_1\left(\frac{A_3}{A_1} - \frac{g_3}{g_1}\right) \quad (4)$$

where A_i is the total amount of component i bound to the protein, expressed as grams of component i per gram of protein. In the present context, "binding" or interaction does not imply stoichiometric complexation, but it accounts for the averaged amounts of the components present in the solvent volume perturbed by the protein, reflecting both strong and weak as well as attractive and repulsive interactions. Equation 4 shows clearly that ξ_3 is a measure of the difference between the solvent compositions in the immediate domain of the protein and in the bulk solvent.

Preferential interaction is also a direct expression of the change in the free energy of the system induced by the addition of component 3 into the aqueous protein solution, since, within close approximation (Casassa & Eisenberg, 1964)

$$\left(\frac{\partial m_3}{\partial m_2}\right)_{T,\mu_1,\mu_3} = -\frac{(\partial\mu_3/\partial m_2)_{T,P,m_3}}{(\partial\mu_3/\partial m_3)_{T,P,m_2}} \quad (5)$$

The changes in the chemical potentials of components 2 and 3 induced by their mixing are then

$$\left(\frac{\partial\mu_2}{\partial m_3}\right)_{T,P,m_2} = \left(\frac{\partial\mu_3}{\partial m_2}\right)_{T,P,m_3} = -\left(\frac{\partial m_3}{\partial m_2}\right)_{T,\mu_1,\mu_3}\left(\frac{\partial\mu_3}{\partial m_3}\right)_{T,P,m_2} = -\left(\frac{\partial m_3}{\partial m_2}\right)_{T,\mu_1,\mu_3}\left[\frac{RT}{m_3} + RT\left(\frac{\partial \ln\gamma_3}{\partial m_3}\right)\right]_{T,P,m_2} \quad (6)$$

where γ_i is the activity coefficient of component i and R is the universal gas constant. $(\partial \ln\gamma_3/\partial m_3)_{T,P,m_2}$ can be calculated from osmotic coefficient data and is usually small relative to $1/m_3$ at low concentrations of component 3. We are not aware of data on the osmotic coefficient of lactose in aqueous solution and have, therefore, omitted the last term of eq 6 from calculations, although the full equation was used for glucose for which data are available (Stokes & Robinson, 1966).

Measurements of Partial Specific Volumes. The partial specific volume of a protein is obtained from density measurements with the equation

$$\phi_{app} = \frac{1}{\rho_0}\left(1 - \frac{\Delta\rho}{C_2}\right) \quad (7)$$

where ϕ_{app} is the apparent specific volume, $\Delta\rho$ is the difference between the densities of the protein solution and solvent, and C_2 is the protein concentration in grams per milliliter. Extrapolation of ϕ_{app} to zero protein concentration gives the partial specific volume.

Density measurements were carried out according to the previously described procedure (Lee & Timasheff, 1974; Lee et al., 1979; Gekko & Timasheff, 1981). The solvent conditions used for both the lactose and the glucose systems are listed in Table I. The buffer solutions containing the given sugar were filtered through a sintered-glass filter. In constant molality experiments, five samples (5–25 mg) of deionized and lyophilized protein were dried in small test tubes at 40 °C for 2 days in a vacuum oven. After being cooled to room temperature, about 1.1 mL of solvent was added to each tube. The tube was tightly sealed with parafilm and left to stand overnight at 4 °C prior to the densimetry measurement. In the constant chemical potential experiments, each of the five samples of protein (5–25 mg) was dissolved in about 1.1 mL

Table I: Absorbance Values of Proteins in Lactose Solution

protein	pH[a]	λ (nm)	absorptivity value [dL/(g cm)] 0	0.1	0.2	0.3	0.4	0.47
RNase A	8.8	278	7.38[b]		7.42	7.46	7.50	
CTG	2.0	282	19.7[c]	19.7	19.8	19.8	19.8	19.8
β-LG	2.0	278	9.60[d]				9.66	
	4.65	278	9.60[d]				9.66	
lysozyme	3.0	281	27.4[e]				27.4	
BSA	3.0	278	6.38	6.39		6.42	6.43	
	6.0	278	6.58[f]	6.59		6.61	6.63	
ovalbumin	7.0	280					7.34[g]	

[a] The solvents used at the indicated pHs were the following: pH 2.0, 0.01 M HCl; pH 3.0, 0.02 M glycine-HCl buffer; pH 4.65, 0.02 M acetate buffer; pH 6.0 and 7.0, 0.02 M phosphate buffer; pH 8.8, 0.02 M glycine-NaOH buffer. These solutions contained 0.01 M NaCl, except for chymotrypsinogen A at pH 2.0 and for bovine serum albumin at pH 3.0. [b] Scott & Sheraga (1963). [c] Jackson & Brandts (1970). [d] Townend et al. (1960a,b). [e] Roxby & Tanford (1971). [f] Noelken & Timasheff (1967). [g] Katz & Miller (1971).

of the same solvent and transferred with a Pasteur pipet into a dialysis bag which had been cleaned by boiling in 5% $NaHCO_3$ solution and then in water and finally rinsed with a small amount of the solvent. These samples were dialyzed at 4 °C for 48 h against two changes of solvent (400–500 mL of each) and then for 3–4 h at 20 °C, prior to densimetry. Density measurements were carried out at 20 °C in a constant temperature room following a sequence of water, solvent, sample, solvent, and water.

After the density measurement, each protein solution was taken out of the cell, transferred into a new test tube, and sealed tightly to avoid evaporation. The protein solutions were diluted gravimetrically with solvent to a final optical density of about 1.0, and the concentrations were determined spectrophotometrically with a Cary Model 118 spectrophotometer. The light scattering correction was made by careful extrapolation of the double logarithmic plot of the absorbance vs. wavelength from 320 to 380 nm. For determination of the absorbance values of the proteins in the sugar solutions, identical aliquots of an aqueous solution of the protein at high concentration were diluted volumetrically to the same extent with a solvent in which the absorbance value of the protein is known, and with the experimental solvent for which that value is not known. The absorbance value in the experimental solvent was obtained then from the ratio of the absorbances of the above two diluted protein solutions determined at the same wavelength and the known absorbance value in the first solvent. This method of determining absorbance may yield inexact absolute values of this parameter because of the experimental error in the volumetric dilution, leading to small variations in the absolute values of the partial specific volume of a protein. This, however, would affect, to an identical extent, the values at constant chemical potential, $\phi'_2{}^0$, and at constant molality, $\phi_2{}^0$, and no significant errors would be introduced into the preferential interaction parameter which is calculated from the difference between $\phi'_2{}^0$ and $\phi_2{}^0$.

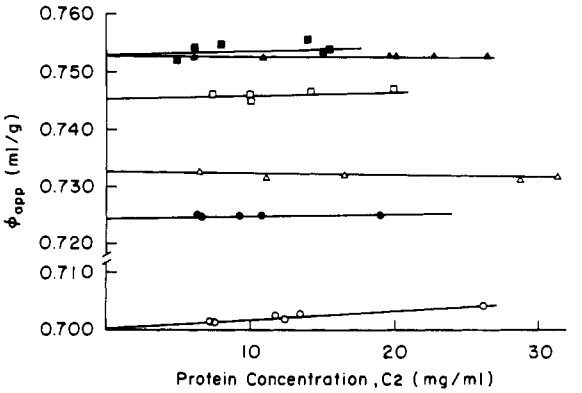


FIGURE 1: Apparent specific volume of proteins in 0.4 M lactose solution: ϕ_2 (open symbols); ϕ'_2 (closed symbols). Lysozyme (○, ●), ovalbumin (△, ▲), and β-lactoglobulin (□, ■).

Results

The absorbance values of the proteins are listed in Table I as a function of lactose concentration. In general, there is a slight increase in sugar solution, except for CTG which remains invariant, and RNase A for which the increase is considerable, just as in the sucrose (Lee & Timasheff, 1981) and the glycerol (Gekko & Timasheff, 1981) systems. Typical plots of the apparent specific volumes of proteins as a function of protein concentration, given in Figure 1, indicate in general little or no dependence of ϕ_{app} on protein concentration. Extrapolation to zero concentration gives $\phi'_2{}^0$ and $\phi_2{}^0$.

The partial specific volumes of all proteins in the native state were first determined in the given solvent without sugar. These values, listed in Table II, were similar to those reported in the

Table II: Partial Specific Volumes and Preferential Interaction Parameters of Proteins in Aqueous Lactose Solutions

[lactose] (M)	$\phi_2{}^0$ (mL/g)	$\phi'_2{}^0$ (mL/g)	$-\left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3}$ (g/g)	$-\left(\frac{\partial m_3}{\partial m_2}\right)_{T,\mu_1,\mu_3}$ (mol/mol)	g_3 (g/g)	$\left(\frac{\partial g_1}{\partial g_2}\right)_{T,\mu_1,\mu_3}$ (g/g)	$\left(\frac{\partial \mu_2}{\partial m_3}\right)_{T,P,m_2}$ [cal (mol of protein)$^{-1}$ (mol of sugar)$^{-1}$]
			Ribonuclease A, pH 8.8				
0	0.691	0.692					
0.2	0.692 ± 0.002	0.696 ± 0.001	0.0115 ± 0.0082	0.460 ± 0.329	0.072	0.160 ± 0.115	1300 ± 900
0.3	0.692	0.701 ± 0.001	0.0241 ± 0.0028	0.967 ± 0.112	0.110	0.220 ± 0.026	1800 ± 200
0.4	0.693 ± 0.002	0.704 ± 0.002	0.0343 ± 0.0118	1.37 ± 0.47	0.150	0.229 ± 0.079	1800 ± 650
			Chymotrypsinogen A, pH 2.0				
0	0.730	0.731					
0.1	0.729 ± 0.001	0.736 ± 0.001	0.0191 ± 0.0052	1.43 ± 0.39	0.035	0.545 ± 0.149	8200 ± 2000
0.2	0.727 ± 0.002	0.740 ± 0.002	0.0354 ± 0.0109	2.66 ± 0.82	0.072	0.495 ± 0.152	7400 ± 2300
0.3	0.727 ± 0.001	0.744 ± 0.002	0.0483 ± 0.0085	3.63 ± 0.64	0.110	0.440 ± 0.007	6600 ± 1200
0.4	0.728	0.753 ± 0.002	0.0743 ± 0.0059	5.58 ± 0.44	0.149	0.499 ± 0.040	7500 ± 600
0.47	0.729 ± 0.001	0.756 ± 0.002	0.0818 ± 0.0091	6.14 ± 0.68	0.179	0.457 ± 0.051	5900 ± 800
			Bovine Serum Albumin, pH 6.0				
0	0.729	0.728					
0.1	0.729 ± 0.001	0.733 ± 0.001	0.0109 ± 0.0053	2.17 ± 1.03	0.035	0.311 ± 0.151	12300 ± 6000
0.3	0.725 ± 0.001	0.736 ± 0.001	0.0300 ± 0.0057	5.97 ± 1.14	0.110	0.273 ± 0.052	10800 ± 2100
0.4	0.725 ± 0.002	0.742 ± 0.003	0.0482 ± 0.0149	9.63 ± 2.96	0.150	0.321 ± 0.099	12700 ± 4000

Table III: Preferential Interaction Parameters of Proteins with Solvent Components in Aqueous Glucose Solutions

[glucose] (M)	ϕ_2^0 (mL/g)	ϕ'^0_2 (mL/g)	$-\left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3}$ (g/g)	$\left(\frac{\partial g_1}{\partial g_2}\right)_{T,\mu_1,\mu_3}$ (g/g)	$-\left(\frac{\partial m_3}{\partial m_2}\right)_{T,\mu_1,\mu_3}$ (mol/mol)	$\left(\frac{\partial \mu_2}{\partial m_3}\right)^{\text{exptl}}_{T,P,m_2}$ [cal (mol of protein)$^{-1}$ (mol of sugar)$^{-1}$]	$\left(\frac{\partial \mu_2}{\partial m_3}\right)^{\text{calcd}}_{T,P,m_2}$ [cal (mol of protein)$^{-1}$ (mol of sugar)$^{-1}$]	$\frac{\left(\frac{\partial \mu_2}{\partial m_3}\right)^{\text{exptl}}_{T,P,m_2}}{\left(\frac{\partial \mu_2}{\partial m_3}\right)^{\text{calcd}}_{T,P,m_2}}$
				Chymotrypsinogen A, pH 2.0				
0.5	0.725	0.737	0.0353	0.370	5.03	5600	10500	0.536
1.0	0.727	0.752	0.0802	0.394	11.4	6200	9300	0.668
1.5	0.729	0.758	0.103	0.317	14.5	5200	7900	0.657
2.0	0.726	0.760	0.138	0.297	19.7	5100	7000	0.725
3.0	0.727	0.760	0.168	0.205	24.0	3700	5700	0.656
				Lysozyme, pH 3.0				
0.5	0.698	0.714	0.0456	0.458	3.69	4100	6900	0.599
1.0	0.699	0.720	0.0704	0.346	5.59	3000	5900	0.515
1.5	0.700	0.726	0.0958	0.294	7.60	2700	5200	0.515
2.0	0.703	0.728	0.101	0.215	8.01	2100	4600	0.450
				Ribonuclease A, pH 8.8				
0.5	0.695	0.700	0.0157	0.164	1.19	1300	6600	0.202
1.0	0.695	0.707	0.0406	0.199	3.09	1700	5700	0.295
1.5	0.694	0.711	0.0580	0.178	4.41	1600	5000	0.312
2.0	0.700	0.716	0.0610	0.131	4.64	1200	4400	0.273
				Bovine Serum Albumin, pH 6.0				
0.5	0.726	0.736	0.0295	0.308	11.1	12400	20100	0.618
1.0	0.726	0.741	0.0509	0.250	19.2	10400	17200	0.606
1.5	0.726	0.747	0.0737	0.226	27.8	9800	15100	0.649
2.0	0.727	0.750	0.0990	0.212	37.4	9600	13400	0.718
				Bovine Serum Albumin, pH 3.0				
0.5	0.721	0.734	0.0377	0.394	14.2	15900		
1.0	0.722	0.745	0.0755	0.371	28.5	15500		
1.5	0.723	0.754	0.113	0.346	42.6	15000		
2.0	0.728	0.759	0.137	0.294	51.7	13300		

Table IV: Thermodynamic Parameters of Glucose in Aqueous Solution

[glucose] (M)	g_3 (g/g)	m_3 (mol/1000 g of H_2O)	a_3	$RT\left(\frac{\partial \ln \gamma_3}{\partial m_3}\right)_{T,P,m_2}$ (cal/mol^2)	$\left(\frac{\partial \mu_3}{\partial m_3}\right)_{T,P,m_2}$ [cal (mol of sugar)$^{-2}$]	$\left(\frac{\partial \sigma}{\partial m_3}\right)_{T,P,m_2}$ (dyn cm^{-1} mol^{-1})	$\bar{v}_3$ (mL/g)
0.5	0.0956	0.531	0.540	22	1119	1.528	0.623
1.0	0.203	1.13	1.18	28	543	1.309	0.628
1.5	0.326	1.81	1.96	31	353	1.148	0.629
2.0	0.466	2.59	2.92	33	258	1.020	0.633
3.0	0.820	4.55	5.69	28	156	0.825	0.638

literature. In all solvents, ϕ_2^0 was identical with ϕ'^0_2 in the absence of the third component. This is an essential requirement for measurements of preferential interactions, since the interaction parameter is obtained from the difference between ϕ_2^0 and ϕ'^0_2 when the third component is added to an aqueous solution of the protein. In Table II, the values of ϕ_2^0 and ϕ'^0_2 as a function of lactose concentration are shown for RNase A, CTG, and BSA. From these values, the preferential interaction parameter $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ was calculated with eq 1, by using values of the partial specific volume of lactose, $\bar{v}_3$, in aqueous medium, determined by densimetry. These values for $\bar{v}_3$ (in units of milliliters per gram) were the following: 0.606 for 0.1 M lactose solution, 0.608 for 0.2 M, 0.609 for 0.3 M, 0.610 for 0.4 M, and 0.613 for 0.47 M. The results for the glucose system are shown in Table III. In this system, the absorbance values, used for the calculation of ϕ_2^0 and ϕ'^0_2, were estimated from those determined at the highest glucose concentration in each protein, assuming their linear dependence on glucose concentration, as is nearly true of lactose, sucrose, and glycerol. The determined values were 20.3 dL/(g cm) for CTG in 3 M glucose, 29.4 dL/(g cm) for lysozyme, 7.85 dL/(g cm) for RNase A, and 6.88 dL/(g cm) at pH 6.0 and 6.66 dL/(g cm) at pH 3.0 for BSA, all measured in 2 M glucose. The values of ϕ_2^0 were nearly constant within experimental error and close to those observed in the presence of lactose.

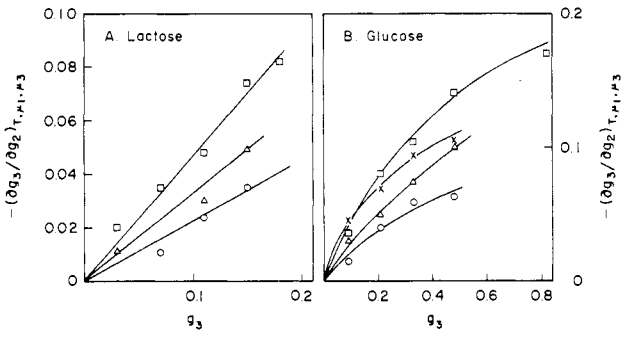


FIGURE 2: Dependence of preferential interaction on sugar concentration in lactose (A) and glucose (B) solutions: RNase A (○); BSA at pH 6.0 (△); chymotrypsinogen (□); lysozyme (×).

Values of the partial specific volume of glucose are listed in Table IV. The results, given in Tables II and III, show that, in all the cases examined, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ was negative, indicating a deficiency of lactose or glucose molecules in the immediate domain of the protein, just as was the case in the

Table V: Preferential Interaction Parameters in 0.4 M Lactose Solution

protein	ϕ_2^0 (mL/g)	ϕ'^0_2 (mL/g)	$-\left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3}$ (g/g)	$-\left(\frac{\partial m_3}{\partial m_2}\right)_{T,\mu_1,\mu_3}$ (mol/mol)	$\left(\frac{\partial g_1}{\partial g_2}\right)_{T,\mu_1,\mu_3}$ (g/g)	$\left(\frac{\partial \mu_2}{\partial m_3}\right)_{T,P,m_2}$ [cal (mol of protein)$^{-1}$ (mol of sugar)$^{-1}$]
ribonuclease A	0.693 ± 0.002	0.704 ± 0.002	0.0343 ± 0.0118	1.37 ± 0.47	0.229 ± 0.0079	1800 ± 700
lysozyme	0.700	0.724	0.0705	2.94	0.470	3900
β-lactoglobulin	0.749 ± 0.002	0.778 ± 0.002	0.0838 ± 0.0088	4.51 ± 0.47	0.559 ± 0.059	6000 ± 700
chymotrypsinogen A	0.728	0.753 ± 0.002	0.0743 ± 0.0059	5.58 ± 0.44	0.499 ± 0.040	7500 ± 600
ovalbumin	0.733 ± 0.001	0.753	0.0589 ± 0.030	7.91 ± 0.40	0.392 ± 0.002	10500 ± 600
bovine serum albumin, pH 6.0	0.725 ± 0.002	0.742 ± 0.003	0.0482 ± 0.0149	9.63 ± 2.96	0.321 ± 0.099	12800 ± 4000

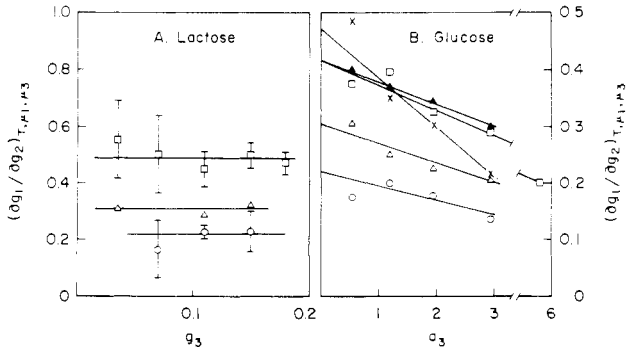


FIGURE 3: Dependence of preferential hydration on lactose (A) and glucose (B) concentrations: RNase A (○); BSA at pH 6.0 (△) and pH 3.0 (▲); CTG (□); lysozyme (×).

sucrose system (Lee & Timasheff, 1981). Figure 2A,B shows $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ as a function of g_3 for the two sugar systems. For the lactose system, this interaction parameter increased linearly with lactose concentration within the relatively narrow accessible sugar concentration range (0.1–0.47 M) while for the glucose system, which could be studied over a wider concentration range (0.5–2.0 M), all of the plots showed curvature. The extent of preferential hydration, $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$, was calculated from these results by using eq 2, and the values are given in Tables II and III. Figure 3A,B shows the dependence of $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ on sugar concentration for the two systems. It is evident that this parameter has little dependence on lactose concentration. It is, however, a linear function of glucose activity. This last quantity was calculated from the osmotic coefficient data of Stokes & Robinson (1966) and is summarized in Table IV together with other thermodynamic parameters of glucose solutions.

The above results indicate that the preferential interaction parameter is a strong function of the nature of the protein in both the lactose and the glucose systems. Measurements were extended to other proteins in 0.4 M lactose solutions to define better this dependence. The results obtained are summarized in Table V and show that all proteins were preferentially hydrated. From this table, as well as Table III for the glucose system, it is clear that the preferential interaction parameter, expressed in molal units, $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$, increases with the molecular weights of the proteins, which are listed in Table VI along with other molecular parameters. The preferential interaction parameter, however, is not proportional to the mass of the protein, since the results expressed on the gram basis, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$, i.e., normalized for molecular weight, also differ significantly from one protein to another. With the assumption as a limit of total exclusion of lactose or glucose molecules from the vicinity of the protein, namely, setting $A_3 = 0$ in eq 4, $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ becomes equal to the total extent of protein hydration, A_1. With the exception of β-LG, the A_1 value of all proteins fell within the range of 0.2–0.4 g of water per g

Table VI: Molecular Parameters of Proteins

protein	M_2[a]	R_0[b] (A)	s_2[c] (A^2)	p[d]
RNase A	13 700	15.54	3050	1.73
lysozyme	14 300	15.83	3150	1.18
β-LG,[e] pH 2.0	18 400	17.61	3900	0.96
β-LG, pH 4.65	36 800	22.20	6200	0.96
CTG	25 700	19.52	4800	0.83
ovalbumin	46 000	23.73	7050	0.92
BSA	68 000	26.98	9150	1.22

[a] Molecular weight. [b] Radius of the protein molecule. [c] Surface area calculated by taking a spherical approximation. [d] Polarity. [e] β-LG was assumed to be monomeric at pH 2.0 and dimeric at pH 4.65.

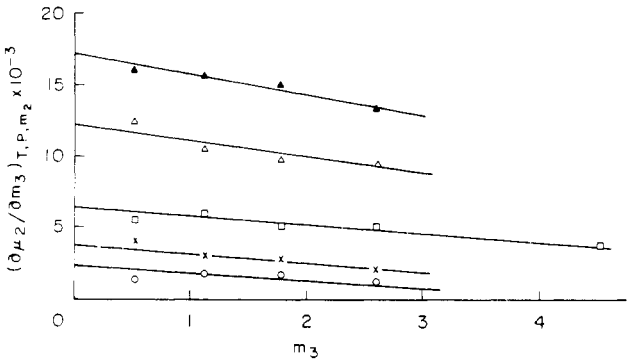


FIGURE 4: Dependence of chemical potential gradient on glucose concentration at 20 °C: RNase A (○); BSA at pH 6.0 (△) and pH 3.0 (▲); CTG (□); lysozyme (×).

of protein, which is the normal hydration value of proteins (Bull & Breese, 1968; Kuntz, 1971; Kuntz & Kauzmann, 1974), suggesting that sugar molecules may be close to totally excluded from the vicinity of the protein.

The chemical potential change, $(\partial \mu_2/\partial m_3)_{T,P,m_2}$, induced by the addition of these sugars to the aqueous protein solution, was calculated with eq 6, by using for the glucose system the values of $(\partial \ln \gamma_3/\partial m_3)_{T,P,m_2}$ listed in Table IV, and the results are given in Tables II, III, and V. The positive values of this parameter are a measure of the increase in the activity of the protein. This unfavorable thermodynamic effect must have as a consequence a decrease in the solubility of the proteins in the sugar solutions. For the three proteins listed in Table II, $(\partial \mu_2/\partial m_3)_{T,P,m_2}$ is independent of lactose concentration within experimental error, making it possible to evaluate the free energy of transfer of the protein from water to an aqueous lactose solution of a given composition, since this change increases linearly with the molal concentration of lactose. For BSA, the appropriate relation is

$$\mu_{2,1} - \mu_{2,w} = 12000 m_3 \quad (8)$$

where $\mu_{2,1}$ and $\mu_{2,w}$ are the chemical potentials of the protein in the lactose solution and in water, respectively. On the other

Table VII: Effects of Unfolding and Association on the Partial Specific Volumes and Preferential Interaction Parameters of Proteins

[lactose] (M)	ϕ_2^0 (mL/g)	ϕ'^0_2 (mL/g)	$-\left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3}$ (g/g)	$-\left(\frac{\partial m_3}{\partial m_2}\right)_{T,\mu_1,\mu_3}$ (mol/mol)	$\left(\frac{\partial g_1}{\partial g_2}\right)_{T,\mu_1,\mu_3}$ (g/g)	$\left(\frac{\partial \mu_2}{\partial m_3}\right)_{T,P,m_2}$ [cal (mol of protein)$^{-1}$ (mol of sugar)$^{-1}$]
			Bovine Serum Albumin, pH 3.0			
0	0.721	0.722				
0.1	0.720 ± 0.001	0.731 ± 0.002	0.0304 ± 0.0079	6.04 ± 1.57	0.866 ± 0.224	34300 ± 8900
0.3	0.719 ± 0.002	0.740 ± 0.001	0.0601 ± 0.0085	12.0 ± 1.7	0.546 ± 0.007	21700 ± 3100
0.4	0.719 ± 0.001	0.753 ± 0.002	0.0998 ± 0.0088	19.8 ± 1.8	0.665 ± 0.059	26400 ± 2400
			β-Lactoglobulin, pH 4.65			
0	0.745	0.745				
0.4[a]	0.745 ± 0.001	0.753 ± 0.002	0.0241 ± 0.0088	1.29 ± 0.48	0.161 ± 0.0059	1700 ± 600

[a] $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ and $(\partial \mu_2/\partial m_3)_{T,P,m_2}$ were calculated per monomeric subunit.

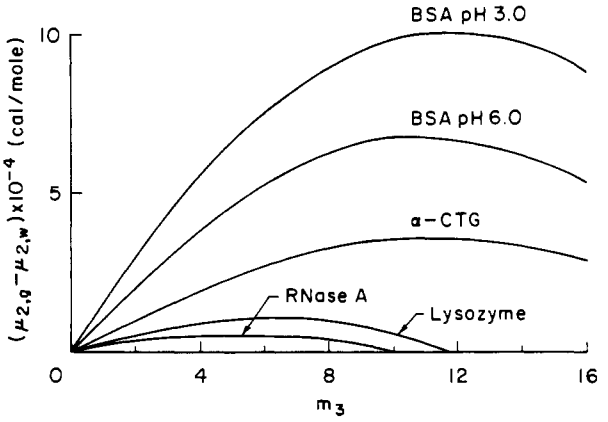
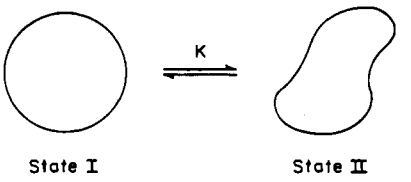


FIGURE 5: Glucose concentration dependence of the transfer free energy of proteins from water to glucose solution at 20 °C.

hand, Figure 4 shows that, for the glucose system, $(\partial \mu_2/\partial m_3)_{T,P,m_2}$ decreases with m_3 in all cases, including BSA at pH 3.0. Integration of $(\partial \mu_2/\partial m_3)_{T,P,m_2}$ with respect to m_3 for each curve of Figure 4 gives the values, plotted in Figure 5, of the free energy of transfer of the protein from water to an aqueous glucose solution, $\mu_{2,g} - \mu_{2,w}$, as a function of m_3. The free energy of transfer is positive and increases with glucose concentration within the measurable concentration range. Lakshmi & Nandi (1976) have reported that the free energy of transfer of small model peptides from water to aqueous sugar solutions was positive and that it increased with sugar concentration up to 2.0 M, suggesting that the activities of proteins and of small peptides increase in sugar solutions by the same mechanism. The apparent difference in the dependence of $(\partial \mu_2/\partial m_3)_{T,P,m_2}$ on m_3 between the lactose and glucose systems may be ascribed best to the limited concentration range of lactose solubility.

All of the results described above were obtained at conditions at which the proteins are in the native and fully dispersed state. It seemed of interest to examine whether a change in protein conformation, and hence in its molecular size, would affect the preferential interaction or not. This was done with BSA, since this protein is known to expand when the pH of the solvent is decreased below 4.5 (Yang & Foster, 1954; Tanford et al., 1955; Kronman et al., 1956; Timasheff & Gibbs, 1957; Laskowski, 1966; Vigai & Foster, 1967). Therefore, the partial specific volume and the preferential interaction were determined at pH 3.0. The results obtained for both sugar systems are given in Tables III and VII, respectively. The decrease in the absorbance value of BSA (Table I) and in its partial specific volume from 0.730 mL/g at pH 6.0 to 0.720 mL/g at pH 3.0 is to be expected when a protein is denatured. The preferential hydration values and, hence, the chemical potential

Scheme I



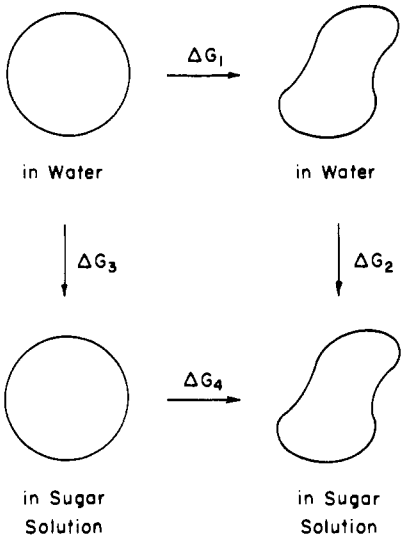
changes of BSA in the sugar solutions were much larger at pH 3.0 than at pH 6.0, indicating that the system is thermodynamically more unfavorable at the low pH where the protein is expanded. For β-LG, the preferential interaction was also measured at pH 4.65, i.e., at conditions at which this protein is known to undergo a reversible self-association (Timasheff & Townend, 1964). In the 0.4 M lactose solution, the enhanced aggregation led to some difficulties in density measurements, in particular at high protein concentrations. Nevertheless, the plot of the apparent specific volume vs. the protein concentration showed no scattering, two separate measurements giving identical results. The results, shown in Table VII, indicate that the preferential hydration of β-LG is much smaller at pH 4.65 than that at pH 2.0, where this protein exists in monomeric form (Townend et al., 1960a,b). These results with both BSA and β-LG suggest that, in sugar solutions, the preferential interaction of proteins with solvent components is a function of the protein molecular dimensions.

Discussion

Since all of the proteins examined were found to be preferentially hydrated in all of the aqueous lactose and glucose solutions, just as in the aqueous sucrose systems (Lee & Timasheff, 1981), it seems reasonable to conclude that protein preferential hydration is a common feature of aqueous sugar systems within the sugar concentration range ordinarily employed, regardless of the kind of protein and the solvent conditions used. In a three-component system, preferential hydration of a protein is a good indication that the third component is a stabilizer of the structure of the macromolecule, as shown for MPD (Pittz & Timasheff, 1978), sucrose (Lee & Timasheff, 1981), glycerol (Gekko & Timasheff, 1981), and some salts (Arakawa & Timasheff, 1982). Lactose and glucose should therefore be stabilizers of proteins in aqueous solutions. Back et al. (1979) have shown that glucose, as well as other sugars, has a stabilizing action against the thermal denaturation of proteins. Similar observations were made for some sugars and polyalcohols by Gerlsma & Stuur (1972, 1974).

The stabilizing effect of the third solution component on proteins can be explained in terms of the positive change in the chemical potential of the proteins induced by the addition

Scheme II



of these substances. The thermodynamic stabilization of macromolecules in these systems can be understood intuitively by examining Scheme I where state I is the native form, state II is the denatured one, and K is the equilibrium constant of the denaturation reaction. Since the addition of sugars results in a positive free-energy change, and this change is assumed to increase with an increase in the surface area of the macromolecule (Timasheff et al., 1976), the macromolecule in state II will experience a greater increase in chemical potential when sugar is added than that in state I; i.e., contact of sugar with state II is thermodynamically more unfavorable than with state I. This must be reflected in the equilibrium constant, K, which, in sugar solutions, will shift to a value favoring the native conditions, state I.

If the surface area of proteins increases upon denaturation, then the denatured protein will have a larger value of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ than one in the native state, and this, in turn, will be reflected in a greater preferential hydration. This was tested with BSA in both the lactose and glucose systems. In the absence of sugar, BSA is known to have a compact form above pH 5.0 and to expand as the pH decreases, leading to a larger contact area between the protein molecule and solvent. That this expansion persists in the presence of sugar is indicated by the constant decrease in BSA absorptivity when the pH decreases, whether sugar is present or not. The results shown in Tables III and VII and Figure 4 clearly confirm this deduction; namely, $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ at pH 3.0 is significantly greater than that at pH 6.0.

The free-energy change for transferring BSA from water to a lactose solution, $\mu_{2,1} - \mu_{2,w}$, is $12m_3$ (kcal/mol) at pH 6.0 and $27m_3$ (kcal/mol) at pH 3.0. For the glucose system, as shown in Figure 5, the same free energy of transfer is about 1.5 times larger at pH 3.0 than at pH 6.0 at any glucose concentration. Thus, the free energy required to transfer expanded BSA from water to the sugar solution is 1.5–2 times larger than that for the native one. Let us consider Scheme II in which we assume identical conformations of the expanded BSA in both the absence and presence of sugars. Now, $\Delta G_1 + \Delta G_2 = \Delta G_3 + \Delta G_4$, where ΔG_i is the free-energy change of each step shown in Scheme II. Since $\Delta G_2 > \Delta G_3$, $\Delta G_1 < \Delta G_4$; i.e., in the presence of sugars, the native state is thermodynamically more favorable. When the values of ΔG_2 and ΔG_3, measured in 0.4 M lactose or 1.0 M glucose, are used, for example, the difference $\Delta G_2 - \Delta G_3 \simeq 4000$ cal/mol. As a consequence, the equilibrium constant of the denaturation reaction in the sugar solution decreases by a factor of 10^3 relative to that in water.

Another important consequence of preferential hydration is its effect on the solubility and self-assembly reactions of macromolecules. This has been discussed previously for the MPD–RNase (Pittz & Timasheff, 1978) and tubulin–glycerol systems (Na & Timasheff, 1981). For a system which undergoes self-assembly, the positive change in chemical potential induced by the addition of the sugar should favor the assembled state, since the formation of contacts between the protein molecules decreases the total surface area of the protein and hence their chemical potential change per monomeric unit. In fact, the results obtained with β-LG showed a large decrease in $(\partial\mu_2/\partial m_3)_{T,P,m_2}$, from 6000 cal/mol at pH 2.0 to 1700 cal/mol at pH 4.65, in 0.4 M lactose. If it is assumed that the surface area of an associated protein increases by factor of $n^{2/3}$ over that of the monomer (where n is the degree of association) and that $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ is proportional to the surface area in any associated state, $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ for a β-LG dimer should be ~4750 cal/mol of monomer, when estimated from the monomer value at pH 2.0. The observed value, however, was only 1700 cal/mol, indicating that, in the 0.4 M lactose solution, aggregates greater than dimer are formed at pH 4.65 as suggested by the turbidity of the β-LG in this medium. It is evident, therefore, that lactose strongly enhances the self-association of β-LG at pH 4.65. At that pH, this protein is known to exist predominantly in the dimeric form at 20 °C and in an octameric form at 0 °C (Townend & Timasheff, 1960; Timasheff & Townend, 1964). The value of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ calculated by the above method for the octamer is ~3000 cal/mol of monomer. This suggests that the aggregates are even larger. The same problem can be explained formally and in a more quantitative manner in terms of the Wyman linked functions (Wyman, 1964):

$$\frac{\mathrm{d}\ln K_a}{\mathrm{d}\ln a_3} = n\left[\frac{1}{n}\left(\frac{\partial m_3}{\partial m_2}\right)^{\text{polymer}}_{T,\mu_1,\mu_3} - \left(\frac{\partial m_3}{\partial m_2}\right)^{\text{monomer}}_{T,\mu_1,\mu_3}\right] = n\Delta\nu_3 \tag{9}$$

Here, K_a is the association constant and $\Delta\nu_3$ is the difference in the number of the ligand molecules bound per monomer in a polymer and in the free state. The first term on the right-hand side is the preferential binding of lactose to β-LG in the polymer state, expressed as moles of lactose per mole of β-LG monomer, i.e., −1.29 mol/mol, while the second term is the binding to β-LG in the monomer state, i.e., −4.51 mol/mol. The result, $\Delta\nu_3 = +3.22$, shows that the association must be strongly enhanced by the addition of lactose.

The final problem that should be discussed is the cause of the preferential exclusion of the sugars from the domain of the proteins. Schachman & Lauffer (1949), following a suggestion by Kauzmann, have interpreted the preferential hydration of virus particles in sucrose solutions in terms of the steric exclusion of sucrose molecules from the surface of the virus due to the difference in the molecular sizes of water and sucrose. According to this model, there must exist a layer of water around the macromolecules, extending from their surface to a distance corresponding to the effective radius of the sucrose molecule. Since the effective radii of lactose, glucose, and water are not well-defined, a calculation of the statistical excess of water around protein molecules is not easy. If the preferential hydration in the sugar solutions is assumed to be solely due to this mechanism, however, the following must be true. First, in this mechanism, the absolute hydration values should be independent of sugar concentration. The results for the glucose system, which was examined over a wide con-

centration range, clearly show that this is not true. Second, the extent of the total hydration should parallel the effective radii of the sugar molecules. If a spherical approximation is used for the lactose and glucose molecules, radii of about 4.4 Å for lactose and about 3.6 Å for glucose can be calculated from their partial specific volumes and molecular weights. The effective radius for lactose should be even larger because of its asymmetric structure. Schachman and Lauffer assumed the effective radius of sucrose to be 5–6 Å. If a protein radius of 20 Å is assumed, this difference in the effective radii of lactose and glucose predicts that the water layer around the protein should be ~1.4 times larger in the lactose system than in the glucose system. The experimental results, however, do not indicate any difference of such magnitude. We may conclude, therefore, that steric exclusion is not the sole factor, and possibly not the predominant one, in the preferential hydration of proteins in the presence of sugars.

An alternate mechanism may stem from the increase in the free energy of cavity formation induced by the introduction of sugars, i.e., the surface tension effect, which was first proposed for pure solvent systems by Sinanoglu & Abdulnur (1964) and applied to three-component systems by Lee & Timasheff (1981) and Timasheff et al. (1976). From the Gibbs adsorption isotherm, we may write

$$-\left(\frac{\partial m_3}{\partial m_2}\right)_{T,\mu_1,\mu_3} = \frac{N_{Av}m_3}{RT}s_2\left(\frac{\partial \sigma}{\partial m_3}\right)_{T,P,m_2} \quad (10)$$

where N_{Av} is Avogadro's number, s_2 is the surface area of the protein molecule, and σ is the surface tension of the solvent. Combining this equation with eq 3 and 6, we have

$$-\left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_2} = \frac{N_{Av}m_3M_3}{RT}\frac{s_2}{M_2}\left(\frac{\partial \sigma}{\partial m_3}\right)_{T,P,m_2} \quad (11)$$

and

$$\left(\frac{\partial \mu_2}{\partial m_3}\right)_{T,P,m_2} = N_{Av}s_2\left(\frac{\partial \sigma}{m_3}\right)_{T,P,m_2}\left[1 + \left(\frac{\partial \ln \gamma_3}{\partial \ln m_3}\right)_{T,P,m_2}\right] \quad (12)$$

For glucose solutions, $(\partial\sigma/\partial m_3)_{T,P,m_2}$ was obtained from a plot of σ vs. m_3 (Landt, 1931), and the values are given in Table IV. The surface areas, s_2, of the protein molecules, listed in Table VI, were calculated from their partial specific volumes and their radii by using a spherical approximation. The calculated values of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ for the glucose system are given in Table III. Such a calculation could not be carried out for the lactose system, since no surface tension data are available for this system. Although the calculated and experimental values of this parameter are not identical, their ratios are essentially constant within any given protein system, suggesting that the observed decrease of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ with m_3 reflects the corresponding decrease of the solvent surface tension increment. The ratio $(\partial\mu_2/\partial m_3)^{exptl}_{T,P,m_2}/(\partial\mu_2/\partial m_3)^{calcd}_{T,P,m_2}$ has a value of 0.5–0.65 for BSA, CTG, and lysozyme and is much lower for RNase A. Since the surface areas of the first three proteins vary by a factor of 3, the similarity of this ratio suggests that the difference in their surface areas may be a major factor responsible for the observed difference in their values of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$. The only exception, RNase A, shows the importance of the chemical nature of the protein surface, as will be described later. Essentially the same conclusion can be deduced from the result for the lactose system. According to eq 11, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ should decrease linearly with m_3 if we assume no dependence of $(\partial\sigma/\partial m_3)_{T,P,m_2}$ on m_3. The result

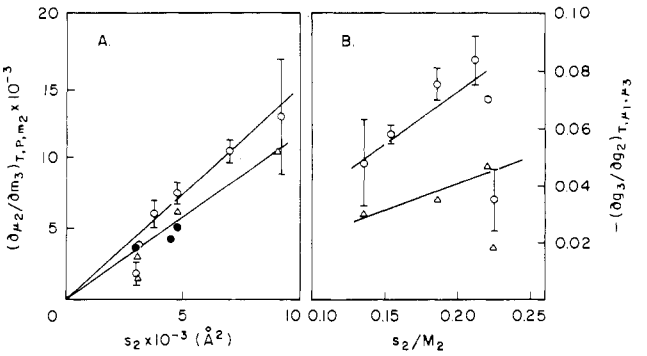


FIGURE 6: Dependence of the preferential interaction on the surface area of proteins in sugar solutions at 20 °C: 0.4 M lactose (○), glucose (△), and sucrose (●). Glucose concentration was 1 M (A) and 0.5 M (B).

shown in Figure 2 agrees with this. Following eq 12, the experimental values of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ were plotted against s_2. The linear plots shown in Figure 6A support again the notion that $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ is a close function of the protein surface area. Similarly, in Figure 6B, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ is shown to vary linearly with s_2/M_2, as expected from eq 11. In both sugar systems, CTG shows a slight upward deviation from the straight line, while the RNase A values are strikingly low. For both proteins, the deviation may be a reflection of their chemical nature. CTG is the least polar of the proteins examined, as shown by the polarity parameter (Bigelow, 1967), i.e., the ratio of the number of polar amino acid residues to the number of nonpolar residues, listed in Table VI. RNase A, on the other hand, has an extraordinarily high value. With such a high polarity, RNase A may be expected to have a very hydrophilic surface which may alter the interaction of the protein with the sugar and reduce $(\partial\mu_2/\partial m_3)_{T,P,m_2}$. It is interesting to note that in the water–glycerol system (Gekko & Timasheff, 1981) interactions with solvent components are related mostly to the protein surface polarity. Glycerol lowers the surface tension of water, and, hence, it cannot act through a cavity surface stabilizing mechanism.

In conclusion, this study and the one on the sucrose system (Lee & Timasheff, 1981) strongly suggest that the cohesive force of sugars responsible for the increase in the surface tension of water is a very important factor governing the preferential interaction of proteins with solvent components in aqueous sugar systems and hence the stabilization of proteins. In Figure 6, the results for the sucrose system are given as an average of data at sucrose concentrations between 0.1 and 1 M for each protein. Since $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ is almost independent of the sugar concentration for both the lactose and sucrose systems, it may be suggested that lactose has a cohesive force similar to or somewhat higher than that of sucrose. This may reflect the same number of hydroxyl groups but slightly different molecular structures.

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