The Stabilization of Proteins by Sucrose*

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The interactions between proteins and solvent components have been investigated for the sucrose/water system. Thermodynamic and kinetic measurements of the thermal unfolding of α-chymotrypsin, chymotrypsinogen, and ribonuclease were performed as a function of sucrose concentration. The alteration in protein-solvent interactions in the presence of sucrose was also studied by density measurements and analyzed by multicomponent thermodynamic theory. Sucrose does not induce a conformational change in three proteins studied, although it does induce a small change in the circular dichroism spectrum of ribonuclease. The enthalpy of thermal unfolding shows little dependence on the concentration of sucrose, while the apparent activation energy of the unfolding process is increased by the addition of sucrose. The results from the protein-solvent interaction study indicate that sucrose is preferentially excluded from the protein domain, increasing the free energy of the system. Thermodynamically this leads to protein stabilization since the unfolded state of the protein becomes thermodynamically even less favorable in the presence of sucrose. The exclusion of sucrose from the protein domain seems to be related to the higher cohesive force of the sucrose water solvent system since all the experimental observations can be correlated with the effect of sucrose on the surface tension of water.

The conformation adopted by a biological macromolecule is a sensitive function of residue composition, the sequence of these residues, and solvent environment. At equilibrium, macromolecules tend to cluster around the conformation of lowest free energy. The conformation of the lowest free energy may represent the native state of the macromolecule, although the native conformation need not correspond to the lowest free energy state but may correspond to a relative free energy minimum, with extremely slow rates of conversion to the absolute minimum due to high energy barriers. The particular conformation which represents that of the lowest free energy, as well as the distribution of other conformational energy levels about it, is a strong function of the solvent environment. It is a widely recognized phenomenon that some biological macromolecules in a purified state are unstable and may lose their structural integrity and biological activity. The introduction of polyhydric alcohols and sugars into the solvent medium has been found to stabilize biological macromolecules in solution (Ball et al., 1943; Boyer, 1945; Bradbury and Jakoby, 1972; Frigon and Lee, 1972; Kirkpatrick et al., 1970; Kane, 1965; Utter et al., 1964; and Tanford et al., 1962). The basic observations are that these additives prevent the loss of enzymic activities (Bradbury and Jakoby, 1972), inhibit irreversible aggregation (Frigon and Lee, 1972) or increase the thermal transition temperature of macromolecules (Gerlisa, 1968; Neucere and St. Angelo, 1972). We were particularly drawn to this question by our finding that high concentrations of sucrose stabilize brain tubulin in solution (Frigon and Lee, 1972). The stabilizing effect of these additives has been attributed in the past to a lessened hydrogen bond-rupturing capacity of the medium (Gerlisa, 1968) or to an induced change in topography (Hinton et al., 1969). An alteration of the kinetic properties of an enzyme, however, should not be interpreted as a reflection of a conformational change without further independent experimental observations (Hinton et al., 1969). These alterations could also be due to a change in the chemical potential of the substrate, product, co-factors, or indeed the enzyme itself in the presence of polyhydric alcohols (Pitz et al., 1972; Lee et al., 1979). While the observed increase in the thermal transition temperatures of enzymes or other proteins indicates that the polyhydric alcohols hinder the unfolding of the proteins, it does not reveal the mechanism of the stabilizing effect. Thus, at present, the mechanism of protein stabilization by these additives is essentially not understood.

Solvent additives can affect macromolecular structure by direct interaction with the macromolecule, by indirect action through effects on the structure and properties of the solvent, or by a combination of both of these mechanisms. In order to arrive at an understanding of the stabilizing effects of sucrose on proteins, a detailed study of the thermal transition of several proteins, namely, α-chymotrypsin, chymotrypsinogen, and ribonuclease, in the presence and absence of sucrose, was carried out and the results are reported in this paper. Preliminary reports of these studies have been presented earlier (Lee et al., 1975; Timasheff et al., 1975).

MATERIALS AND METHODS

Ultrapure grade sucrose (lot 3597) was purchased from Schwarz/Mann. Bovine pancreas ribonuclease was obtained from Sigma; chymotrypsinogen and α-chymotrypsin were from Worthington; α-chymotrypsin was purified further by the procedures of Nakagawa and Bender (1970).

Protein Concentration Determination—The concentrations of proteins were determined by UV absorbance using extinction coefficients obtained by dry weight measurements. Lyophilized proteins were weighed into predried and tared 2.0-ml volumetric flasks. The protein samples and the flasks were dried in a vacuum oven for 24 h at 40 °C over phosphorus pentoxide. After attainment of a constant weight, solvents were added to make a 2.0-ml solution of the protein. The spectra of such solutions or of gravimetrically diluted samples were measured with a Cary 14 or 118 spectrophotometer. The extinction coefficients for proteins in various concentrations of sucrose are listed.

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in Table I. In all concentration determinations, the spectrum was obtained from 400 to 240 nm, and the contribution of light scattering to absorptivity was corrected for by the method of Leach and Scheraga (1960).

Circular Dichroism—Circular dichroism spectra were recorded at 23-25°C on a Cary 60 spectropolarimeter with a Model 6001 circular dichroism attachment. The spectra were recorded from 350 to 190 nm, and overlapping spectra were obtained with the use of 0.01-, 0.1-, and 1.0-cm fused silica cells. Mean residue ellipticities (θ) were calculated with the aid of a Wang 370 calculator.

Density Measurements—the densities of the solvents and of the protein solutions, a series of protein concentrations for each set of conditions, were measured with a Precision Density Meter DMA-02 (Anton Paar, Grazt). All measurements were made at 20°C and the general procedures used were similar to those published previously (Lee and Timasheff, 1974; Lee et al., 1979). The partial specific volumes were obtained from the density measurements by (Cassada and Eisenberg, 1961, 1964).

\[ \phi = \frac{1}{\rho_0} \left( 1 - \frac{\rho - \rho_0}{c} \right) \]  

(1)

where \( \phi \) is the apparent partial specific volume, \( \rho \) and \( \rho_0 \) are the density of solution and solvent, respectively, and \( c \) is the concentration of protein in grams/ml.

The partial specific volumes of the proteins were measured as a function of sucrose concentration at conditions both of constant chemical potential and constant molality using previously described techniques (Lee et al., 1979).

By combining density measurements, carried out at conditions at which either the chemical potential or the solvent composition is kept identical in the protein solution and in the reference solvent, it is possible to determine the extent of preferential interaction (\( \frac{\partial \gamma_i}{\partial x_j} \)) as \( \xi_i \) of the solvent components with the macromolecule since (Eisenberg and Reisler, 1969)

\[ \xi_i = \frac{\partial \rho_i}{\partial x_j} \text{ at } x_j = 0 \]

(2)

where \( \rho_i \) is the concentration of component \( i \) in grams/g of water, \( \mu_i \) is the chemical potential of component \( i \) (\( \mu_i = \mu_i^0 + RT \ln m_i + RT \ln \gamma_i \)), \( m \) is the molality of component \( i \), \( \gamma_i \) is its activity coefficient, \( T \) is the thermodynamic (Kelvin) temperature, and \( P \) is pressure. From the definition of the partial specific volume at infinite dilution, we have:

\[ \left( 1 - \phi_i^0 \rho_0 \right) + \xi_i \left( 1 - \phi_i \rho_0 \right) \]

(3)

where \( \phi_i^0 \) and \( \phi_i \) are the partial specific volumes of the protein at constant chemical potential and constant molality, respectively, extrapolated to zero macromolecule concentration and \( \xi_i \) is the partial specific volume of sucrose, using the convention of Scatchard (1946) and Stockmayer (1950) that component 1 denotes H2O, component 2 denotes macromolecule, and component 3 denotes sucrose. The values of \( \xi_i \) measured as a function of sucrose concentration were: 0.613, 0.05  

<table>
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<th>( \text{g/cm}^3 )</th>
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<td>2.06</td>
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</table>

\[ \frac{\partial \gamma_i}{\partial x_j} \text{ at } x_j = 0 \]

(4)

Thermal Denaturation Equilibrium—for the study of the thermal transition equilibrium, difference spectra of protein solutions at various temperatures were recorded relative to a reference at 25 °C, in 1-cm fused silica cuvettes using a Cary 14 or 118 spectrophotometer. The protein solutions in both the sample and reference compartments were aliquots of the same stock solutions. The base-line was determined before and after each experiment by comparison of two identical protein solutions. The reference compartment was maintained at 25 ± 0.1 °C by a Forma temperature regulator-water bath and the sample compartment was controlled by a Haake constant temperature bath. The temperature of the sample solution was measured with a calibrated Tele-thermometer Thermister probe (Silver Springs Instruments). After reaching thermal equilibrium, a spectrum was taken between 360 and 240 nm. The difference in absorbance at the wavelength of maximum change was then plotted against temperature to obtain the thermal denaturation profiles of the proteins. No light-scattering corrections were necessary.

Kinetic Measurements of Thermal Denaturation—the rate of thermal denaturation was determined by monitoring the rate of change of difference spectra. For each experiment, a concentrated protein solution (usually at 5 mg/ml) was brought to 30 °C, and its solvent was brought to a temperature 2 °C higher than the temperature at which the experiment was to be carried out. At zero time, 0.2 ml of protein and 1.0 ml of solvent were transferred into automatic pipettes into a jacketed cuvette which was kept at the experimental temperature by a Haake regulator. The cell was covered with a Teflon stopper and the solution was mixed by several rapid, but gentle, inversions. The reference was an identical solution kept at 25 °C. The rate of change in the absorbance of the heated solution was then monitored.

The solvents used in the thermal denaturation measurements were 10⁻³ M HCl for chymotrypsinogen and α-chymotrypsin and 4 × 10⁻³ M glycine pH 2.9 for ribonuclease. The density measurements of chymotrypsinogen and α-chymotrypsin were performed in 10⁻³ M HCl, 0.1 M KCl.

RESULTS

The effect of sucrose on the structural integrity of the three proteins was first examined by CD since it had been proposed that polyhydric alcohols stabilize proteins by inducing a conformational change in the protein (Bradbury and Jakoby, 1972). Fig. 1, A and B, shows the spectra of α-chymotrypsin and chymotrypsinogen, respectively, in the presence and absence of 1.0 M sucrose. Within the resolving power of CD, there was no difference in the structure of these proteins whether sucrose is present or not. A similar observation had been made with calf brain tubulin (Lee et al., 1975). Fig. 1C, however, shows that sucrose does perturb the CD spectrum of RNase, the intensity of all bands being increased without shifts in band position. Since the structure stabilizing effect of sucrose may be manifested by a change in the energy profile of the protein, a thermal denaturation study in its presence was initiated.

The validity of the two-state analysis in the determination of the thermodynamic parameters of a thermal transition requires that the process be reversible. The complete reversibility of the thermal denaturation of the three enzymes in varying amounts of sucrose was deduced from the total recovery of enzymic activity after incubation of the enzyme solution at 90 °C for 10 min and from the fact that the ultraviolet difference spectra of all the proteins remained constant through the base-line when the samples were cooled following thermal denaturation, as well as by the reversibility of the CD spectra after heating. Fig. 2 shows the thermal denaturation profiles of α-chymo-
midpoint of the transition were observed for chymotrypsinogen and thermal transition temperature and sucrose concentration for it can be seen that there is a linear relationship between the transition temperature of α-chymotrypsin. Similar profiles trypsin in different concentrations of sucrose. It is evident that the presence of sucrose causes an elevation of the thermal transition temperature of α-chymotrypsin. Similar profiles were observed for chymotrypsinogen and RNase. Taking the midpoint of the transition as the transition temperature (Tm), it can be seen that there is a linear relationship between the thermal transition temperature and sucrose concentration for all three proteins, as shown in Fig. 3.

The enthalpy of unfolding as a function of solvent com-

\[
\frac{\Delta \ln K}{\Delta (1/T)} = -\frac{\Delta H^0}{R}
\]

where \(\Delta H^0\) is the standard enthalpy change for thermal unfolding. \(K = \alpha/(1 - \alpha)\) is the equilibrium constant, and \(\alpha\) is the degree of conversion from the initial to the final state in the two-state process. In the process of determining \(\alpha\), the relative amounts of the protein in its native and unfolded states were estimated from the UV absorbance. The variations of the UV absorbances of the pure native and unfolded states as a function of temperature were extrapolated into the transition zone, as shown in Fig. 2. Such a procedure had been employed successfully by Biltonen and Lumry (1969) in their studies on the thermal unfolding of α-chymotrypsin. Within experimental uncertainties, the values of \(\Delta H^0\) for α-chymotrypsin appears to be independent of sucrose concentration, as shown in Fig. 4A, and there is no detectable curvature in the van't Hoff plots. This latter observation is consistent with that of Biltonen and Lumry (1969), for α-chymotrypsin at pH 3.0. Similar results were obtained for chymotrypsinogen, although for this protein there might be an increase in the observed value of \(\Delta H^0\) with increasing sucrose concentration. This increase, however, is within the experimental uncertainty of ±10 kcal/mol and no significance can be attributed to it.

The resulting thermodynamic parameters for α-chymotrypsin and chymotrypsinogen are summarized in Table II. The re-

\[
\text{experimental data.}
\]

<table>
<thead>
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<th>Temperature in °C</th>
<th>Transition Temperature, Tm, °C</th>
</tr>
</thead>
<tbody>
<tr>
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<td>55.0</td>
</tr>
<tr>
<td>30</td>
<td>55.5</td>
</tr>
<tr>
<td>40</td>
<td>56.0</td>
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<td>56.5</td>
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<tr>
<td>60</td>
<td>57.0</td>
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...
The denaturation process was monitored by following the change in absorbance as a function of time and the results were expressed in terms of a pseudo-first order reaction:

\[ \ln K = a + b(1/T) + c \ln T \]  
(6)

which is a truncated form of the integrated van't Hoff equation (Glasstone, 1947). The values of the standard free energy, \( \Delta G^\circ \), the standard enthalpy \( \Delta H^\circ \), the standard entropy \( \Delta S^\circ \), and the heat capacity \( \Delta C_p \) changes in the reaction are given by

\[ \Delta G^\circ = -RT \ln K \]
\[ \Delta H^\circ = R(cT - b) \]
\[ \Delta S^\circ = (\Delta H^\circ - \Delta G^\circ)/T \]
\[ \Delta C_p = R \]

(7)

The temperature dependence of the thermodynamic parameters for the thermal unfolding of RNase is summarized in Fig. 5 where \( \Delta H^\circ \) is plotted as a function of \( T \) and \( \Delta S^\circ \) is plotted as that of \( \log T \). From the slopes of the straight lines it is evident that the unfolding of RNase is characterized by an apparent positive change in heat capacity, while the enthalpy and entropy changes are positive above 35 °C. The heat capacity change \( (\Delta C_p) \) seems to decrease from 4,300 ± 600 cal/degree mol in the absence of sucrose to 3,600 ± 600 cal/degree mol in 1 M sucrose. These changes in heat capacity are consistent in sign with, but higher than the values reported by Tsong et al. (1970) and Privalov and Khechinashvili (1974).

The possibility that sucrose increases the energy barrier between the native and denatured states of these proteins was examined by kinetic measurements of their thermal unfolding. The denaturation process was monitored by following the change in absorbance as a function of time and the results were expressed in terms of a pseudo-first order reaction:

\[ (\Delta A)_n = (\Delta A)_i = (\Delta A)_e \exp(-k_f t) \]  
(8)

where \( (\Delta A)_e \) is the maximum change in absorbance, \( (\Delta A)_i \) is the change in absorbance at time \( t \), and \( k_f \) is the first order rate constant. Although according to the experimental procedures used in these kinetic measurements the results should be analyzed in terms of relaxation kinetics, these data can be treated as first order reactions since the proteins are essentially fully in their native states before the thermal perturbation. Fig. 6 shows typical first order plots of the kinetic data. A linear relationship was observed in all cases, namely, for all three proteins studied, at all concentrations of sucrose, and at all temperatures examined. It is evident from Fig. 6 that \( k_f \) is a function of the concentration of sucrose since, at any fixed temperature, the value of \( k_f \) decreases with increasing sucrose concentration. The apparent activation energies of denaturation \( (E_{ac}) \) determined from Arrhenius plots are listed in Table II. The addition of sucrose induces a small, but consistent, increase in the apparent activation energy for the thermal perturbation of all proteins tested, i.e. it increases the energy barrier between their end states. In the case of ribonuclease the situation may be somewhat more complicated since addition of sucrose at low temperature per se affects the conformation of that protein (see Fig. 1C). Therefore, the

![Fig. 5. Temperature dependence of the enthalpy and entropy changes of ribonuclease. The \( \Delta S^\circ \) temperature dependence is plotted semilogarithmically.](image)

**Table II**

<table>
<thead>
<tr>
<th>Su-</th>
<th>a-Chymotrypsin</th>
<th>Chymotrypsinogen</th>
<th>Ribonuclease</th>
<th>E&lt;sub&gt;ac&lt;/sub&gt;</th>
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</thead>
<tbody>
<tr>
<td>crose</td>
<td>( \Delta H^\circ )</td>
<td>( \Delta S^\circ )</td>
<td>E&lt;sub&gt;ac&lt;/sub&gt;</td>
<td>E&lt;sub&gt;ac&lt;/sub&gt;</td>
</tr>
<tr>
<td>M</td>
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<td>kcal/mol</td>
<td>kcal/mol</td>
<td>kcal/mol</td>
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<tr>
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<td>90 ± 10</td>
<td>280 ± 30</td>
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<td>290</td>
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<td>290</td>
<td>118</td>
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**Fig. 6.** First order plots of the kinetic data for the thermal perturbation of a-chymotrypsin in \( 10^{-3} \) M HC1, at 56 °C. Sucrose concentrations: ○, 0; ●, 0.3 M; △, 0.7 M; ▲, 1.0 M.

**Fig. 7.** Sedimentation equilibrium data of a-chymotrypsin at 24,000 rpm and 20 °C. A, in \( 10^{-3} \) M HC1, 0.1 M NaCl; initial protein concentration, 0.903 mg/ml. B, in 0.7 M sucrose, \( 10^{-3} \) M HC1, 0.1 M NaCl, initial protein concentrations, 0.639 mg/ml. ●, number average molecular weight; ○, weight average molecular weight.
Stabilization of Proteins by Sucrose

TABLE III
Preferential solvent interactions in sucrose-water systems

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sucrose concentration (M)</th>
<th>$\phi_1^m$</th>
<th>$\phi_2^m$</th>
<th>$(\partial g_1/\partial g_2)<em>T</em>{m,0}$</th>
<th>$(\partial m_1/\partial m_2)<em>T</em>{m,0}$</th>
<th>$g_1$</th>
<th>$(\partial g_1/\partial g_2)<em>T</em>{m,0}$</th>
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<td>-2.554</td>
<td>0.109</td>
<td>0.312</td>
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<td>-2.554</td>
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<td>0.312</td>
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<td>0.753</td>
<td>0.735</td>
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<td>-2.554</td>
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<td>-7.622</td>
<td>0.457</td>
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* Solvent systems include 0.1 M NaCl, 10^-3 M HCl in addition to sucrose.
* Values assumed to be the same as $\phi_1^m$ in the absence of sucrose.

The results presented indicate that it is most unlikely that sucrose stabilizes $\alpha$-chymotrypsin, chymotrypsinogen, and tubulin (Lee et al., 1975) by inducing these proteins to adopt a more stable conformation. The similarity between the CD spectra of these proteins (see Fig. 1 and Lee et al. (1975)) in 1 M sucrose and dilute aqueous buffer indicates strongly that there is no conformational change, although in the case of RNase a small structural perturbation may take place. Hence, sucrose must exert its stabilizing effect through another mechanism for at least three of the proteins studied. Kinetic experiments have indicated that the presence of sucrose increases the apparent energy of activation of the unfolding reaction. In this paper, the denaturation process is treated in terms of the two state model. It is known, however, that kinetically the process is much more complex, our experiments measuring only the slow process to the neglect of one or more rapid reactions (Brandts et al., 1975; Tsong et al., 1970). Nevertheless, our results indicate that sucrose has a definite effect on the kinetics of the denaturation-renaturation transition and that it induces an increase in the energy barrier between the final states, even though we do not know where in the process this barrier is imposed.

The increase in $E_{act}^{app}$ in the presence of sucrose could be a reflection of changes in the physicochemical properties of the system, e.g. solvent structure. If such a change affected the composition of the solvent associated with the protein it should also affect the protein stability. It has been found that, in all four protein sucrose systems examined, the solvent composition in the immediate domain of the protein is differ-
dent from that of the bulk solvent and that this difference is a function of the concentration of sucrose present, as shown in Fig. 8. All four proteins are preferentially hydrated, meaning either (a) that the solvent around the proteins contains less sucrose than the bulk solvent or (b) that the solvent in the immediate vicinity of the protein may be approximated as not to include any sucrose at all, water being the only solvent component present within the protein domain, as suggested by Kauzmann (Schachman and Lauffer, 1949).

Preferential interactions measured as binding are actually only a reflection of activity coefficient changes of the various components of the solution induced by the introduction of the other components. In fact, since at equilibrium the activities of the solvent components must be the same within the domain of the protein and in the bulk, a change in the activity coefficient of any solvent component induced by the presence of the protein must necessarily result in a change in the concentration of that solvent component within the domain of the protein. This gives rise to what is normally referred to as binding. The thermodynamic relation between preferential potential of component 3 to protein and changes in the chemical potential of component 3 is

\[
\frac{\partial \mu_3}{\partial m_3} = RT \left( \frac{1}{m_3} + \frac{\partial \ln y_3}{\partial m_3} \right)
\]

where

\[
\frac{(\partial \mu_3/\partial m_3)}{y_3} = RT \left( \frac{1}{m_3} + \frac{\partial \ln y_3}{\partial m_3} \right)
\]

Then

\[
(\partial \mu_3/\partial m_3)_{T,P,m_3} = \frac{M_3}{m_3} RT \left( \frac{1}{m_3} + \frac{\partial \ln y_3}{\partial m_3} \right)
\]

The term \( \partial \ln y_3/\partial m_3 \) can be calculated from osmotic coefficient values, by

\[
\ln y_3 = (\phi_3 - 1) + \frac{1}{3} (\phi_3 - 1) d \ln m_3
\]

where \( \phi \) is the osmotic coefficient and \( m \) is the molar concentration. Use of the osmotic coefficients of sucrose reported by Scatchard et al. (1938) results in the values of \( \partial \mu_3/\partial m_3 \) and \( \partial \mu_3/\partial m_3 \) given in Table IV. Positive values of the last parameter signify that introduction of the protein into the sucrose solution is thermodynamically unfavorable and the system is destabilized. The source of the thermodynamic destabilization may be either a specific repulsion between the protein and the sugar or a solvent structure effect which excludes the sugar from the surface of the cavity that contains the macromolecule. In either case, introduction of sucrose into the protein solution would exert pressure to reduce the surface of contact between the protein and the solvent. Unfolding of the protein is generally accompanied by an increase in its radius of gyration and hence in the surface of the protein domain. This leads either to greater contact between protein and sugar or to a higher volume from which sugar is preferentially excluded, an even more unfavorable situation. That this is true in the present case can be demonstrated by analysis of the thermal unfolding equilibrium data in terms of the Wyman (1964) linkage equation. Expressing the degree of unfolding at any given temperature in terms of an equilibrium constant, we have:

\[
\frac{\partial \ln K}{\partial \ln a_3} = \frac{(\partial \ln m_3)}{(\partial \ln m_3)}_{T,P,m_3} = \Delta \tau
\]

where the superscripts \( U \) and \( N \) refer to unfolded and native states of the protein. Thus, the slope of a plot of \( \ln K \) versus \( a_3 \) gives the change in the thermodynamic interaction of solvent components with proteins which accompanies the unfolding reaction. The values of \( \Delta \tau \) derived in this way are listed in Table V. In all three cases, unfolding is accompanied by an increase in the exclusion of sucrose from the domain of the protein. By Equation 9b this means that the chemical potential of the sucrose becomes even more positive in the presence of unfolded protein than of native protein, rendering contact between these two components thermodynamically even more unfavorable. In fact, unfolding is accompanied by an increase in the chemical potential of the protein of +1.4, +1.4, and +0.7 kcal/mol of protein/mol of sugar added in 1000 g of water for \( \alpha \)-chymotrypsin, chymotrypsinogen, and RNase, respectively. According to the LeChatelier Principle, addition of sucrose to the solvent system must displace the equilibrium toward the native state, i.e. it must stabilize the native structure. In fact, repulsive interactions such as described here are known to result in phase separation and even in the crystallization of proteins, such as the crystallization of ribonuclease from the water 2-methyl-2,4-pentanediol solvent system (Pittz and Timasheff, 1978).

At this point, it seems of interest to examine in greater detail the nature of the solvent interactions which lead to the observed phenomena. The preferential interaction parameter is related to the total binding of the solvent components to protein by the following relationship (Inoue and Timasheff, 1972):

\[
A_3 = (\partial g_3/\partial g_3)_{T,P,a_3} + g g A_1
\]

where \( A_3 \) and \( A_1 \) are grams of component 3 and component 1, respectively, located within the protein domain/g of protein and \( g \) is the solvent composition in g of sucrose/g of \( H_2O \). If \( A_3 \) is set equal to zero, i.e. in the approximation that component 3 is totally excluded, Equation 11 becomes

\[
(\partial g_3/\partial g_3)_{T,P,a_3} = -g g A_1
\]

and \( A_1 \) is equal to \( (\partial g_1/\partial g_1)_{T,P,a_3} \).

The last column of Table III, therefore, gives the values of \( A_1 \), for \( A_3 = 0 \). These are similar to hydration values determined by Bull and Breese (1968) and Kuntz (1971) for a variety of proteins. The fact that \( A_1 \) approaches normal hydration values indicates that sucrose is probably near to fully excluded from the domain of the protein. This may be tested more rigorously by introducing an exclusion term (\( E_3 \) into
In a series of solvents they concluded that a major factor in that system is the free energy of cavity formation. Since a change in the size of the cavity must be accompanied by a change in cavity surface area, the change in free energy which accompanies this process should be reflected in a change in the surface free energy ($dG_s$). The last quantity may be equated to the work ($du'$) required to increase the area of a surface by an increment ($ds$). At constant pressure, this may be expressed as

$$du' = -dG_s = S'dT - nds - \mu' d\nu'_s$$

Equation 11 (Reisler et al., 1977; Kupke, 1973) which becomes

$$\left(\frac{\partial \gamma_i}{\partial \gamma_s}\right)_{T,P} = A_1 - E_2 - \gamma A_1$$

where $E_2$ is the number of g of sucrose excluded/g of protein. If $A_1$, $A_2$, and $E_2$ are independent of solvent composition, a plot of $(\partial \gamma_i/\partial \gamma_s)_{T,P}$ as a function of $\gamma_i$ should give the hydration as the slope and the balance between binding and exclusion of co-solvent as the intercept. Such an analysis of the results of protein-sucrose interactions is presented in Fig. 9. The slopes of these plots yield $A_1$ values of 0.43, 0.33, and 0.25 g/g for RNase, chymotrypsinogen, and $\alpha$-chymotrypsin respectively, which are in good agreement with the values of $A_1$ obtained by Equation 11a (see Table III, last column). The intercepts yield ($A_2 - E_2$) values of $-0.004, +0.0007,$ and 0 for RNase, chymotrypsinogen, and $\alpha$-chymotrypsin respectively, indicating that if any sucrose is bound to protein, at least an equal amount is excluded from the protein domain, although both $A_2$ and $E_2$ may be equal to zero (Reisler et al., 1977).

If exclusion of sucrose is indeed the thermodynamic source of the protein structure stabilization and the increase in free energy required to unfold the protein in the presence of sucrose is related to the increase in the surface of contact between protein and solvent on unfolding, then protein stabilization could well be related to the sucrose-induced increase in the free energy of enlarging the surface of the solvent cavities which contain the bulky solute molecules. Sinanoglu and co-workers (Sinanoglu et al., 1964; Sinanoglu and Abdul-nur 1964, 1965) have considered this possibility in their studies of the stability of DNA molecules. From an examination of the relative magnitudes of the free-energy contributions of a variety of effects in a series of solvents they concluded that a major factor in that system is the free energy of cavity formation. Since a change in the size of the cavity must be accompanied by a change in cavity surface area, the change in free energy which accompanies this process should be reflected in a change in the surface free energy ($dG_s$). The last quantity may be equated to the work ($du'$) required to increase the area of a surface by an increment ($ds$). At constant pressure, this may be expressed as

$$du' = -dG_s = S'dT - nds - \mu' d\nu'_s$$

where superscript $s$ denotes surface, $S'$ is the surface entropy, $\mu'_s$ is the surface chemical potential of component $i$, and $n'_s$ is the number of moles of the surface excess of that component. $\sigma = (\partial G/\partial s)_{T,P}$ is the surface tension, i.e. the work which must be done/unit increase in the surface area at constant temperature and composition.

The thesis that the stabilizing action of sucrose is related to its effect on the surface free energy of cavity formation was tested in two ways. Sucrose is known to increase the surface tension of water (Landt, 1931) and by equation 13 it should, therefore, increase the free energy of cavity formation at any given temperature. On the other hand, the surface tension of aqueous sucrose solutions is also known to decrease with an increase in temperature (Landt, 1931). Therefore, the surface tension values corresponding to the transition temperatures of the proteins at the various solvent compositions were established. The plots of these values for the three proteins as a function of sucrose concentration are shown in Fig. 10. The results indicate clearly that, for each of the three proteins studied, the transition occurs at a value of surface tension which, within experimental uncertainty, is close to constant. The increase in $T_m$ in the presence of sucrose appears, then, to be closely related to the need of lowering the surface free energy of the cavity to a level at which the free energy change provided by protein expansion is sufficient to overcome the pressure of the solvent that counteracts that process. A second way of testing the involvement of surface free energy, or surface tension, is afforded by the fact that, in binary solutions, the solvent composition in the surface layer is a function of the effect of the solute on the surface tension of the solvent. When the system is at equilibrium, the surface chemical potentials of all components must be equal to their chemical potentials in the bulk phase. If addition of a component perturbs the surface tension of the solvent, then by Equation 13 its concentration in the surface layer must differ.

For the sake of reference, at constant temperature, the surface tension of a 1 M sucrose solution is 1.3 dynes/cm greater than that of water; conversely, at constant composition, the surface tension decreases by 1.0 dyne/cm for a temperature increase of 6 °C.
from its concentration inside the solution. The excess amount of that component (component $i$) found/unit area of surface is given by the Gibbs Adsorption of Isotethone (1878):

$$
\Gamma_i = \frac{n_i}{s} = -\left( \frac{\partial a_i}{\partial n_i} \right)_T = -\frac{a_i}{RT} \left( \frac{\partial a_i}{\partial n_i} \right)_T
$$

(14)

where $a_i$ is the activity of component $i$ and $s$ is the surface area. Defining $(\partial m_i/\partial m_s)_T = n_i$, and assuming that the protein-solvent interface is chemically inert, it should be possible to calculate from surface tension data the contribution of excess co-solvent in the surface layer to preferential interaction of a protein of molar surface area ($s$):

$$
\left( \frac{\partial m_i}{\partial m_s} \right)_T = s \Gamma_i = s \frac{a_0}{RT} \left( \frac{\partial a_0}{\partial m_s} \right)_T
$$

(14a)

where the superscript $s$ indicates that this is the contribution to preferential interaction from the surface free energy effect. For the proteins in this study, the molar surface areas ($s$), listed in Table V, were calculated from known values of their surface-to-volume ratios ($s/V$) determined by small angle x-ray scattering (Pessen et al., 1973; Krigbaum and Godwin, 1968), using the relation

$$
S = (s/V)M\bar{u}
$$

(15)

where $\bar{u}$ and $M$ are the partial specific volume and molecular weight of the protein. The values for $(\partial n_i/\partial n_s)_T$ were obtained from the slopes of a plot of surface tension versus sucrose activity (Landt, 1931). The calculated values of $(\partial m_i/\partial m_s)_T$ are listed in Table VI where they are compared with the values of preferential interaction determined in the solvent binding study. Similar values have been calculated for tubulin and are listed in the last column of Table VI where they are compared with our previously published experimental results (Lee et al., 1975). Since sucrose increases the surface tension of water, Equation 14a predicts a negative adsorption of sucrose molecules to protein. Comparison of the values calculated from the surface free energy considerations with the experimentally determined total amounts of preferential exclusion of sucrose show that surface tension may account for a major part of the measured effect.² In fact, the calculated $\Gamma$ is greater than the total measured one. Actual comparison between these numbers, however, must be approached with the realization that the surface tension data were obtained for a water-air interface rather than water-protein and that no allowance has been made for the curvature of the surface of the cavity, which may reduce

²While the interfacial tension at the protein-water interface is certainly not equal to the surface tension of water, we must stress that the present calculation is concerned only with the difference between interfacial tensions at the protein-water and protein-sucrose solution interfaces in the absence of any protein-sucrose specific interactions.

from the surface tension by a factor of approximately three (Choi et al., 1970; Tanford, 1979). Therefore, the seemingly good agreement between the calculated and experimental values should be regarded with great caution, and no conclusion seems warranted beyond the one that the surface free energy effect can make a significant contribution to protein-solvent component interactions in the water/sucrose system.

If the surface tension is indeed the predominant factor in increasing the free energy of unfolding at any given temperature, then it should be possible to estimate the change in surface of contact between solvent and protein on unfolding ($\Delta s$) since

$$
\Delta s = \frac{a_0 \ln \left( \frac{\partial a_0}{\partial \sigma} \right)}{RT} \left( \frac{\partial n_i}{\partial \sigma} \right)_T
$$

(16)

A plot of the unfolding equilibrium data as a function of surface tension gave the $\Delta s$ values listed in Table V. On unfolding, the protein surface increases by 10 to 20%. Since the exclusion of sucrose dominated by the surface tension effect is non-specific, it can be regarded, within a close approximation, as being statistically distributed over the surface of the protein molecule, and it can be expressed as moles of sucrose excluded/cm² of protein surface for 1 mol of sucrose in the solvent. Such values calculated from the data of Table III for the native proteins are listed in the last column of Table V. A similar calculation for $\Delta s/\Delta s$, i.e., for the change in exclusion/unit increase in surface area on unfolding, is shown in column 5 of Table V. The similarity between the two sets of values fully supports the thesis that the observed phenomena stem in great part from the effect of sucrose on the surface tension of water.

The studies described here have provided at least a qualitative correlation of the experimental observations on the protein structure-stabilizing effect of sucrose and the thermodynamic properties of the sucrose water system. A major factor in the stabilization seems to be the free energy required to form a cavity in the solvent needed for accommodating the protein molecule, the stabilization being conferred on the protein by the increase in the solvent cohesive force when sucrose is added to water in the solvent system.

REFERENCES


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0.3
-1.48
-3.16
-2.55
-3.30

0.5
-3.32
-7.97
-4.13
-6.01

0.7
-5.38
-8.87
-7.14
-9.25

1.0
-7.62
-14.97
-10.35
-15.38

TABLE VI
Comparison of experimental and calculated values of $(\partial m_i/\partial m_s)_T$ in protein-sucrose water systems

<table>
<thead>
<tr>
<th>Sucrose concentration</th>
<th>a-Chymotrypsin</th>
<th>Chymotrypsinogen</th>
<th>Ribonuclease</th>
<th>Tubulin*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experimental</td>
<td>Calculated</td>
<td>Experimental</td>
<td>Calculated</td>
</tr>
<tr>
<td>0.3</td>
<td>-1.48</td>
<td>-3.16</td>
<td>-2.55</td>
<td>-3.30</td>
</tr>
<tr>
<td>0.5</td>
<td>-3.32</td>
<td>-7.97</td>
<td>-4.13</td>
<td>-6.01</td>
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<td>0.7</td>
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<td>-8.87</td>
<td>-7.14</td>
<td>-9.25</td>
</tr>
<tr>
<td>1.0</td>
<td>-7.62</td>
<td>-14.97</td>
<td>-10.35</td>
<td>-15.38</td>
</tr>
</tbody>
</table>

²In the case of tubulin, the molar protein surface area was calculated using expressions for a prolate ellipsoid of revolution, with an axial ratio of 3:1 (Frigon and Timasheff (1975)), with a molecular weight of 110,000 and a $\bar{u}$ value of 0.736 ml/g (Lee and Timasheff (1974)).
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Nakagawa, Y., and Bender, M. L. (1970) *Biochemistry* 9, 259–267