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EFFECT OF ETHANOL ON THE THERMAL STABILITY OF tRNA MOLECULES

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The thermal denaturation of *E. coli* unfractionated tRNA in ethanol/water mixtures has been studied as a function of alcohol concentration in the water-rich region (mole fraction of co-solvent $x_2 < 0.2$). The results show that with increasing alcohol concentration the melting temperature of tRNA first reaches a minimum at an intermediate composition $x_2^* \approx 0.055$ and then increases with increasing x_2. The value of x_2^* is close to that at which structural changes in the mixture occur as inferred from compressibility and optical absorption measurements. The present experimental data support the assumptions that the dominant mechanism by which ethanol affects the thermal stability of tRNA molecules is through its effect on the structure of water.

1. Introduction

It is known that the presence of monohydric alcohols in the solution medium has an appreciable effect on the conformational properties of biological macromolecues [1]. In particular, thermal denaturation studies of proteins [2] and nucleic acids [3] show that alcohol decreases the melting temperature when added at moderate concentrations. These types of study acquire significance in relation to the problem of the type and magnitude of the forces involved in the stabilization of nucleic acid and protein structures.

Two general mechanisms have been suggested for interpreting the effect of alcohols on the conformation and stability of biopolymers. We may hypothesize either a direct binding of alcohol molecules to groups in macromolecules or, alternatively, an indirect mechanism through the effect of alcohol on the solvent structure. In support of the second hypothesis, one can observe that the effects of alcohols on the conformation of aqueous solutions of very different macromolecular systems are strikingly similar and, sometimes, are closely paralleled in simpler physicochemical processes of alcohol/water mixtures themselves [1,3].

The solvent structure may influence the conformation and stability of macromolecules if it has an effect on any of the intramolecular or solvent-macromolecular non-covalent bonds, such as hydrogen bonds, the charge-charge interactions or hydrophobic bonding, all of which stabilize the conformation of large molecules. In this context, it is generally believed that an important role in the structural stabilization of secondary and tertiary structures of nucleic acids and proteins is played by the interaction with the water molecules [1,3,4].

Much effort has been put in over recent years to establish which contribution predominates in any given range of alcohol concentration, the results often being discussed in terms of hydrophobic interactions and changes in the dielectric constant of the medium [3,5,6].

However, despite interest in this problem, as demonstrated by a great number of publications and several hypothetical models, the role played by the solvent in aqueous solutions of proteins

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and nucleic acids is, in many aspects, far from clear and the molecular nature of the influence of water on macromolecules remains uncertain [1]. This situation stems in part from the fact that no satisfactory quantitative description even of the structure of liquid water and of water/alcohol mixtures is yet available and, as a rule, studies on these subjects refer to qualitative models in some limited co-solvent concentration range [7,8]. It is beyond doubt that advances as regards our knowledge of water and of water solutions lead to a better understanding of the problem of macromolecular hydration.

Most recent studies on alcohol/water mixtures strongly suggest the existence of time-average clathrate hydrates in the water-rich region, this structure collapsing when the ratio of alcohol to water exceeds that of clathrates [9–12]. In effect, many properties of alcohol/water mixtures show extremes which occur in the water-rich region and which, for each alcohol, single out the critical solvent composition that corresponds to maximum structuring in the solvent caused by the presence of alcohol [7,13].

This behaviour may play a key role in determining some of the changes observed in the conformational properties of proteins and nucleic acids in alcohol/water mixtures. It is in fact likely that, for the water/macromolecule/alcohol system, the existence of a critical solvent composition will be reflected in the conformational behaviour of the macromolecules. Even if there exist indications in the literature in this sense [1], the measurements are not in general detailed enough in the water-rich region to allow one to establish a precise correlation between certain effects of alcohols on the conformational properties of protein and nucleic acids and the observed changes in the structure of the solvent.

The main object of the present investigation is to extend information about the influence of the solvent on the thermal stability of tRNA molecules due to alteration in water structure by the gradual addition of ethanol in very small amounts.

The water/ethanol system was examined in considerably greater detail with regard to its concentration and temperature dependence than other systems examined previously [7]. The existence of time-average clathrate hydrate-like structures of the ethanol/17H_2O type ($x_2^* = 1/18$) in the ethanol/water mixtures has been proposed by Glew [14] to account for an anomalous molar volume at about 0.06 mole fraction of ethanol in aqueous ethanol solutions; further support for this hypothesis has recently come from compressibility and optical absorption measurements of the ethanol/water mixtures in the water-rich region [15].

In the present paper the thermal denaturation of *E. coli* unfractionated tRNA in water/ethanol mixtures has been investigated through ultraviolet difference absorption spectroscopy as a function of alcohol concentration in the water-rich region ($0 \le x \le 0.2$). In order to obtain information about the properties of the solvent and gain some insight into their action on tRNA stability, we also measured the adiabatic compressibility and the ultraviolet absorption spectra of water/ethanol mixtures themselves in the same concentration range.

The present data show that tRNA stability in water/ethanol mixtures is closely linked to the properties of the solvent itself, thus strongly supporting the hypothesis that the dominant mechanism by which ethanol affects the thermal stability of tRNA is through its effect on the structure of water.

2. Materials and methods

2.1. Preparation of tRNA samples

To remove divalent cations, *E. coli* MRE 600 unfractionated tRNA (Boehringer, Mannheim) was exhaustively dialyzed against 4 l of 0.1 M NaCl, 4×10^{-3} M EDTA (disodium salt), pH 6.5, and then against three changes of 0.1 M NaCl, pH 6.5. The samples were stored at −20°C until use. Before use, all tRNA samples were checked for divalent ion content. They were found to have a residual Mg^{2+} concentration of about 10^{-5} M or less. To remove this residue, an equivalent amount of EDTA was added. As a rule, the tRNA absorption at 260 nm was 1.5; a rough estimate of the tRNA nucleotide concentration was made by assuming a molar extinction coefficient of 7500 [16].

The desired pH (6.5) was obtained by an appropiate addition of NaOH. The solutions were not buffered.

2.2. *Spectrophotometric measurements*

The ultraviolet absorption spectra were recorded by means of a Shimadzu model 360 spectrophotometer. 2 ml cuvets, 1 cm long, were used. The samples were divided into two parts, one of which was maintained at 20 °C and used as a reference while the other was used for filling the cuvet to be heated. In this way we were able to record directly the thermal difference spectra with an absorbance sensitivity of 0.001. The melting profiles were drawn by measuring the absorbance at 260 nm as a function of temperature. All the absorbance values were corrected for thermal expansion of water.

2.3. *Adiabatic compressibility measurements*

Ultrasonic velocities were measured by means of a sing-around velocimeter, model 6080, available from Nusonic Corp., NJ, U.S.A. The temperature control was better then 0.1 °C and the measurement of the sound velocity was reproducible within ± 0.1 m/s. From the values of sound velocity and density ρ, the adiabatic compressibility β_s was calculated by means of the relation $\beta_s = 1/v^2\rho$. The densities used to calculate β_s from velocity measurements were taken from the literature [14]. Ethanol (J.T. Baker, analytical grade) was used without any further purification. The water used was distilled and all mixtures studied were prepared by weighing the components.

Water is designated as component 1 and the co-solvent as component 2. Thus, in the water/ethanol mixture, x_1 is the mole fraction of water and $x_2 = 1-x_1$ that of alcohol.

3. Results and discussion

3.1. *Thermal difference spectra*

The ultraviolet absorption spectrum of a nucleic acid depends on the interaction of the base residues with their neighbours and can, therefore, be considered to be a suitable probe for revealing even minor conformational changes that entail modifications in the base-base 'stacking' interaction.

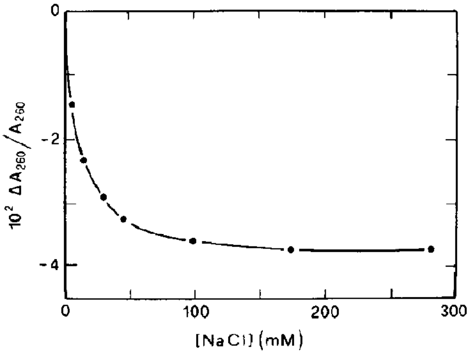

Fig. 1. Absorption changes, ΔA_{260}, at 260 nm of an *E. coli* unfractionated tRNA sample at pH 6.5 and 15 °C as a function of NaCl concentration. ΔA_{260} was normalized with respect the sample absorption at 260 nm in ion-free conditions.

The conformation of tRNA in solution is a function of temperature, pH, salt concentration and solvent. It is known that in an ion-free solution the tRNA molecules display an 'extended' structure which is mainly controlled by electrostatic repulsion between the negatively charged adjacent residues [18]; this structure appears to be significantly different from the 'native' one.

Fig. 1 shows the absorption changes at 260 nm of an *E. coli* unfractionated tRNA sample at pH 6.5 and 15 °C as a function of Na^+ concentration. The absorption at 260 nm decreased as soon as Na^+ was added, reaching a maximum at a concentration of about 100 mM. The drop in absorption observed on adding Na^+ (fig. 1) may be attributed to an increase in the stacking interactions due to a refolding of the molecules. It is likely that this high-salt ($[Na^+] = 0.1$ M), low-temperature ($T = 15$ °C) form, is the native conformation with tertiary structure [18].

Heating is accompanied by a progressive change in the interactions between neighbouring bases which are reflected in parallel changes in the ultraviolet absorption spectra of tRNA molecules. Fig. 2 shows a series of thermal difference spectra obtained by heating a solution of tRNA molecules

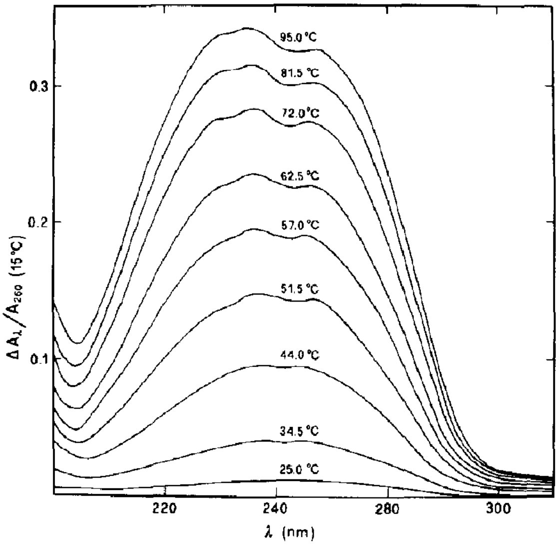

Fig. 2. Thermal difference spectra for the denaturation of *E. coli* tRNA in 0.1 M NaCl.

that were initially in the native conformation (0.1 M Na^+, 15 °C). The thermal difference spectra were collected over the range 15–95 °C, the transition being complete at the upper temperature. Similar measurements to those shown in fig. 2 have been performed in the presence of ethanol in the water-rich region.

From an analysis of these difference spectra we can extract a series of data relative to the thermal denaturing process including information on the base residues taking part in the transition [19].

In fig. 3 we compare the melting profile at 260 nm, obtained from the recorded thermal difference spectra, of a solution of tRNA in water and in water/ethanol mixtures at two different ethanol concentrations. It is obvious from this figure that ethanol affects the thermal stability of tRNA molecules.

From the melting profiles, such as those shown

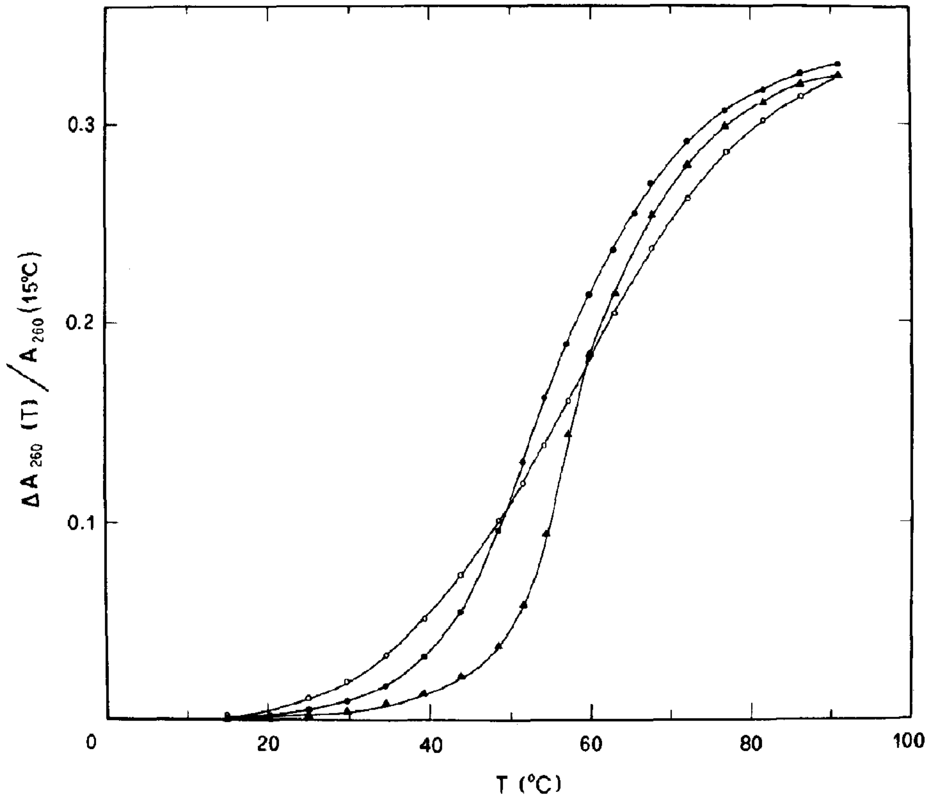

Fig. 3. Melting profiles of *E. coli* tRNA at various ethanol concentrations: (○) $x_2 = 0$, (●) $x_2 = 0.040$, (▲) $x_2 = 0.107$.

in fig. 3, we can define two parameters: the melting temperature T_m, defined as the temperature at which the absorbance has risen halfway between its initial and final values and the width, or degree of cooperativeness, ΔT, of the transition, defined as the temperature difference between the points at which the transition is one-quarter and three-quarters complete. These parameters are plotted vs. the concentration of added alcohol in fig. 4. The experimental data show a linear depression in the melting temperature T_m with alcohol concentration up to a critical concentration $x_2^* \approx 0.055$; for $x_2 > x_2^*$ an inverted trend is observed. At the same time the degree of cooperativeness, ΔT, decreases markedly up to x_2^* and then stays constant for higher alcohol concentrations.

3.2. Evidence for the biphasic nature of thermal melting

It is known that when native tRNA is gradually heated, a number of intermediate states are observed before the randomly coiling single-strand conformation is reached at high temperature [18]. This fact should be shown up in the measured thermal difference spectra shown in fig. 2. In effect, a careful analysis of these spectra shows a change in the form of the spectrum shape as the temperature varies. This is brought out well if one looks (fig. 5) at the observed changes in the absorbance at 260 nm vs. the corresponding changes at 280 nm. The experimental points lie on two straight lines with a break in the gradient at $\Delta A_{260}/A_{260} = 0.195$ and $\Delta A_{280}/A_{260} = 0.120$. This result clearly shows the biphasic nature of thermal melting. As the dispersion of hyperchromism of ribonucleic acids is such that at 280 nm only G-C base-pairs can be followed [19], the structure broken in the first phase of the thermal melting is less G-C-rich. In agreement with Cole et al. [18], we may regard the first transition as involving the loss of all the tertiary structure in tRNA molecules.

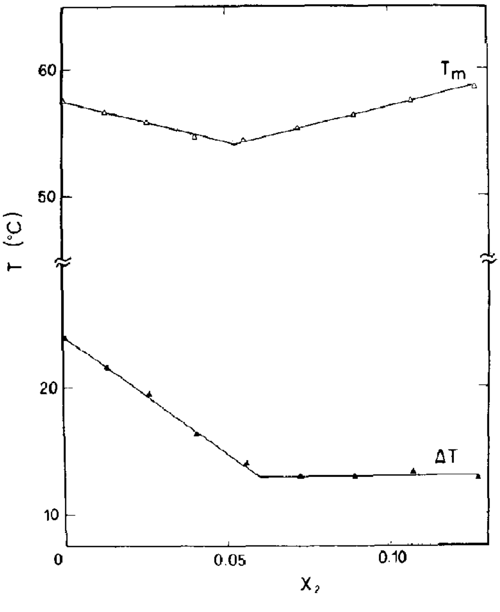

Fig. 4. Variation of the melting temperature, T_m, and of the width, ΔT, of the transition with the ethanol mole fraction. (△) T_m, (▲) ΔT.

Fig. 5 also shows the results for water/ethanol mixtures concerning the same two tRNA samples shown in fig. 2. One can see that all the experimental points fall on the same two straight lines. This indicates that, notwithstanding the observed variations in the melting temperature and in the cooperativeness of the transition (fig. 4), the thermal denaturing process maintains certain similar characteristics as the composition of the solvent varies. In particular, it turns out that the effect of ethanol is to change the stability of the secondary and tertiary structures present in high Na^+ concentrations but it does not produce new melting structures in the tRNA molecules. This is also confirmed by the fact that the addition of ethanol at 15°C to aqueous solutions of tRNA does not produce variations in the absorption other than those connected with the dilution of the sample.

Fig. 6 shows the thermal difference spectra associated with the first, $0 \leq \Delta A_{260}/A_{260} \leq 0.195$, and second, $0.195 \leq \Delta A_{260}/A_{260} \leq 0.330$, phase of the melting. Analysis of these spectral changes may provide information on the base residues taking part in the transition. They can be obtained as the superposition of the 'reference' spectra for the denaturation of A-U and G-C base-pairs in double-helical polyribonucleotides according to

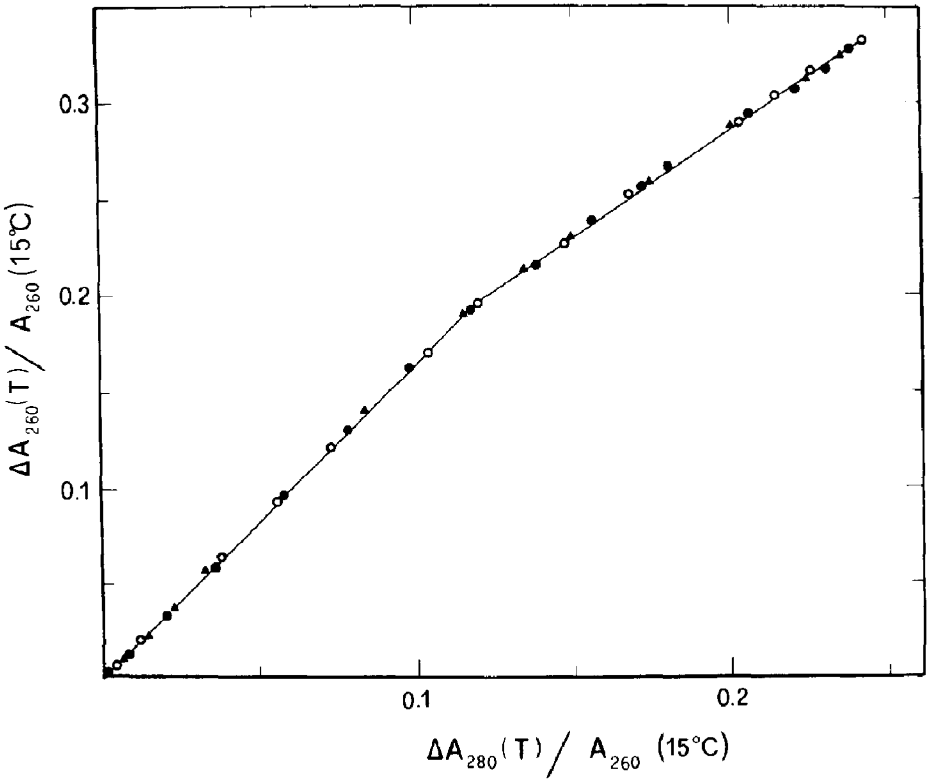

Fig. 5. Absorption changes, ΔA_{260}, at 260 nm vs. the corresponding changes, ΔA_{280}, at 280 nm observed on heating *E. coli* tRNA samples at various ethanol concentrations. ΔA_{260} and ΔA_{28} were normalized with respect to the sample absorption at 260 nm at 15°C. All the experimental points lie on two straight lines with a break in the gradient at $\Delta A_{260}/A_{260} = 0.195$ and $\Delta A_{280}/A_{260} = 0.120$. (○) $x_2 = 0$, (●) $x_2 = 0.040$, (▲) $x_2 = 0.107$.

the equation:

$$\Delta\epsilon(\lambda) = f_{AU}\Delta\epsilon_{AU}(\lambda) + f_{GC}\Delta\epsilon_{GC}(\lambda) \qquad (1)$$

in which $\Delta\epsilon(\lambda)$ is the change in molar extinction coefficient observed on melting, f_{AU} and f_{GC} the mole fractions of the total number of nucleotides which are in the A-T and G-C base-pairs, and $\Delta\epsilon_{AU}(\lambda)$ and $\Delta\epsilon_{GC}(\lambda)$ the reference spectra on a molar nucleotide basis for the denaturation of the A-U and G-C pairs, respectively [19]. The circles shown in fig. 6 are the values drawn for the best fits.

This analysis gives only approximate values, as the contribution coming from the stacking in the structured single-stranded regions is neglected [20]. Bearing this in mind, our analysis provides the values $f_{AU} = 0.22$ and $f_{GC} = 0.28$ for the first phase of the transition and $f_{AU} = 0.13$ and $f_{GC} = 0.27$ for the second.

Having thus identified the limits of the first and second phases of the melting, limits which turn out to be be the same for all the samples studied, we can associate with each phase a melting temperature (T_{m1} and T_{m2}) and a transition width (ΔT_1 and ΔT_2) defined according to the same criteria used for T_m and ΔT.

These parameters are shown in fig. 7 as a function of ethanol concentration. In the very rich water region, T_{m2} decreases linearly with alcohol concentration while T_{m1} remains about constant. This behaviour ceases at the critical concentration x_2^*; for $x_2 > x_2^*$ both T_{m1} and T_{m2} increase lin-

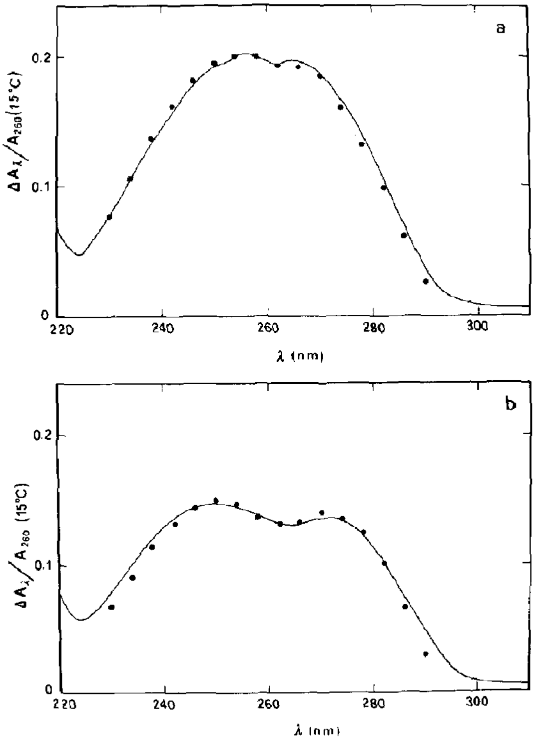

Fig. 6. Comparison of the observed and calculated thermal difference spectra associated with the first and second phases of tRNA thermal melting. (———) Observed values, (●) values calculated by means of eq. 1. (a) Thermal difference spectrum associated with the first phase of the melting; this spectrum corresponds to an absorption change at 260 nm, $\Delta A_{260}/A_{260} = 0.195$ (see fig. 5). (b) Thermal difference spectrum associated with the second phase of the melting; this spectrum was obtained from the difference between the thermal difference spectrum corresponding to $\Delta A_{260}/A_{260} = 0.330$ and that corresponding to $\Delta A_{260}/A_{260} = 0.195$.

early when the alcohol concentration increases. The size ΔT_1 and ΔT_2 of the transitions decreases markedly up to x_2^* and then remains constant for higher ethanol concentrations.

The concentration, x_2^*, at which we observe a change in tendency for the various parameters examined is in the range 0.05–0.06. It should be noted that in this same concentration range one observes a change in tendency in many physical properties of the water/ethanol mixtures, such as sound velocity, sound absorption, compressibility, molar volume, etc. This suggests that the effect of ethanol on the thermal stability of tRNA may depend upon how it affects the structure of water.

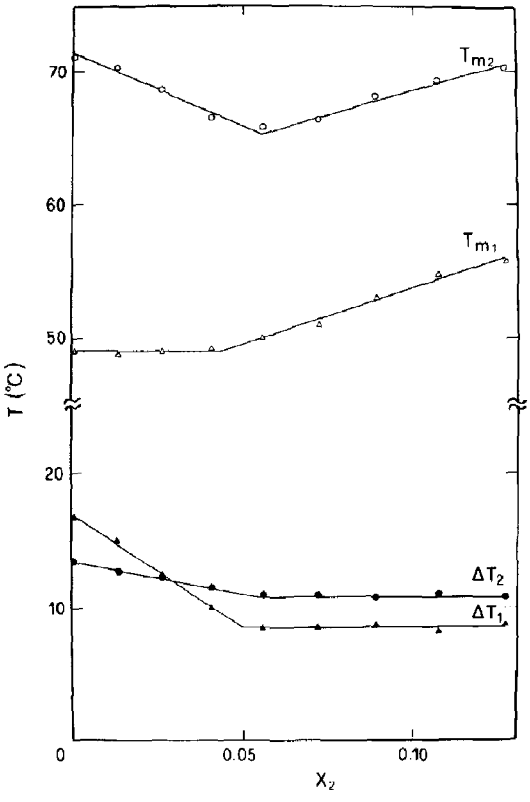

Fig. 7. Variations of the melting temperature and of the width of the transition for the first and second phases of the tRNA thermal melting. (△) T_{m1}, (▲) ΔT_1, (○) T_{m2}, (●) ΔT_2.

3.3. Compressibility and optical absorption measurements

Several physico-chemical properties of ethanol/water mixtures lead one to subdivide the water-rich region $0 \leq x_2 \leq 0.2$ into two, discriminating between the dilute $x_2 > x_2^*$ (with $x_2^* \approx 0.055$) and extremely dilute $x_2 < x_2^*$ aqueous solutions of ethanol [7]. This subdivision appears particularly in evidence from detailed measurements of adiabatic compressibility and ultraviolet optical absorption study of ethanol/water mixtures performed recently in our laboratory [15].

From these measurements it seems that the structural properties of the mixtures change at a critical concentration $x_2^* \approx 0.055$, thus supporting the hypothesis of the existence of clathrate hydrate-like structures of the type $(H_2O)_{17}$/ethanol in ethanol/water mixtures in the water-rich region; x_2^* corresponds to the maximum concentration, $x_2^* = 1/18$, where, on a time average, all the water is held in clathrate-like structures.

In this context, we should like to report, succintly, the results obtained at 20°C relative to the concentration range $0 \leq x_2 \leq 0.15$ (figs. 8 and 9).

3.4. *Adiabatic compressibility*

There is no volume change on mixing two liquids to form a thermodynamically ideal solution at constant temperature and pressure. Thus, letting $\bar{V}^{id}$ be the ideal molar volume and $\bar{V}_1$ and $\bar{V}_2$ the molar volume of the pure components, we have:

$$\bar{V}^{id} = x_1\bar{V}_1 + x_2\bar{V}_2 \tag{2}$$

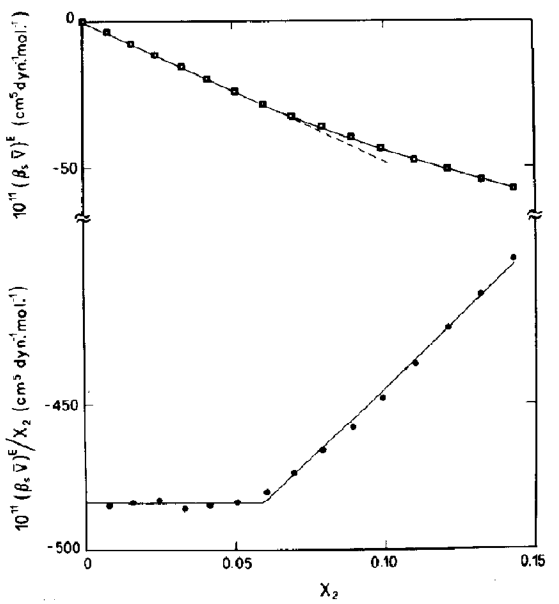

Fig. 8. Plot of the excess quantity $(\beta_s V)^E$ (see eq. 5) and of $(\beta_s V^E/x_2$ as a function of ethanol mole fraction. (□) $(\beta_s \bar{v})^E$, (●) $(\beta_s \bar{V})^E/x_2$. The solid lines were obtained from eq. 6.

for an ideal mixture. Differentiating eq. 2 with respect to pressure, P, at constant entropy,

$$\left(\frac{\partial \bar{V}^{id}}{\partial P}\right)_s = x_1\left(\frac{\partial \bar{V}_1}{\partial P}\right)_s + x_2\left(\frac{\partial \bar{V}_2}{\partial P}\right)_s \tag{3}$$

i.e.

$$(\beta_s \bar{V})^{id} = x_1\beta_{s1}\bar{V}_1 + x_2\beta_{s2}\bar{V}_2 \tag{4}$$

The quantity $\beta_s \bar{V}$ for ethanol/water mixtures shows deviations from a rectilinear dependence on mole fraction and there is evidence that the magnitude of such deviations depends on the strength of the ethanol/water interaction. Deviations of $\beta_s \bar{V}$ from a linear dependence on mole fraction were calculated using the relation:

$$(\beta_s \bar{V})^E = \beta_s \bar{V} - (\beta_s \bar{V})^{id} \tag{5}$$

This quantity is plotted vs. x_2 in fig. 8. The same figure also shows the quantity $(\beta_s \bar{V})^E/x_2$. This quantity is constant at low alcohol concentrations but increases steeply as more alcohol is added to the solution. Two distinct regions becomes evident: the experimental points can be described by the functional form:

$$(\beta_s V)^E = \begin{cases} ax_2 & \text{for } 0 \leq x_2 \leq x_2^* \\ ax_2 + bx_2(x_2 - x_2^*) & \text{for } x_2 > x_2^* \end{cases} \tag{6}$$

with $x_2^* \approx 0.06$. The continuous lines show the result of the fitting.

Since b is the parameter of the second-order term with respect to the concentration in eq. 6, it may be related to the mutual interaction between the solute molecule themselves. These results, taken as a whole, are consistent with the hypothesis that in the $0 \leq x_2 \leq x_2^*$ region the ethanol molecules interact with the water molecules and are kept physically apart by virtue of the clathrate shells; but with increase in co-solvent mole fraction, a point is reached $x_2 = x_2^*$, at which there is insufficient water to accommodate the co-solvent in a clathrate-like structure and, for $x_2 > x_2^*$, two or more alcohol molecules come into contact. Further support for this hypothesis comes from a detailed analysis of the ultraviolet spectra of ethanol/water mixtures.

3.5. Ultraviolet absorption spectra

It is known that the saturated alcohols begin to absorb near 200 nm, with the maximum of the first band near 185 nm. This band has usually been ascribed to an n → σ^* transition [21], i.e., absorption of a photon in this spectral region results in the excitation of a non-bonding electron of the hydroxyl oxygen to an antibonding σ^* orbital of the alcohol.

When ethanol is added to water some hydrogen bonds must be broken, new hydrogen bonds between water and alcohol being formed. The involvement of the oxygen unshared pair of electrons in hydrogen bonding influences the absorption spectrum greatly. The ethanol absorbance in the range 185–200 nm is therefore a sensitive probe of solute-solute and solute-solvent interactions and as such is a useful parameter in elucidating the structural interactions in the water/ethanol mixtures.

Fig. 9 shows the absorbance at 194 nm (A_{194}) of a water/ethanol mxiture as a function of molar alcohol concentration. The absorbance increases linearly with the molar alcohol concentration, in accordance with Beer's Law, up to a critical concentration of $x_2^* = 0.055$. For $x_2 > x_2^*$, there is evidence that the molar extinction coefficient decreases. This fact, in agreement with what is suggested by adiabatic compressibility measurements, reflects the change in the state of association at $x_2 > x_2^*$ due to hydrogen bonding between alcohol molecules.

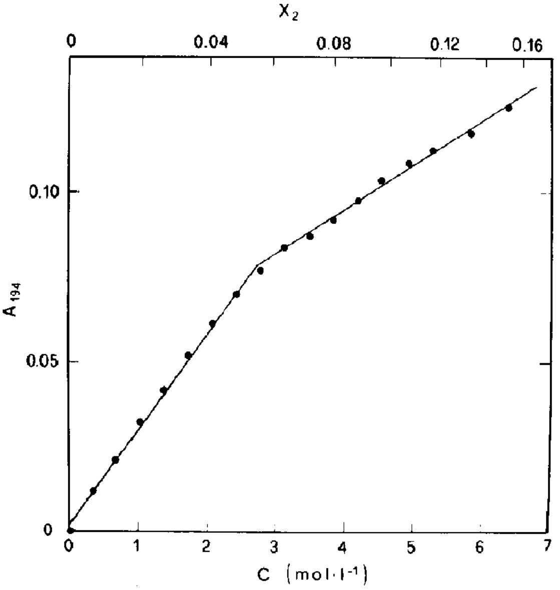

Fig. 9. Absorbance at 194 nm, A_{194}, of a water/ethanol mixture at 20°C as a function of molar alcohol concentration.

4. Conclusions

In this paper we have tried to demonstrate that tRNA conformation stability in water/ethanol mixtures is closely linked with the properties of the solvent system. Our data show that the minimum stability of the native conformation of a tRNA molecule is observed at the ethanol concentration corresponding to maximum structuring in the solvent caused by the presence of alcohol. This fact is strong evidence for the involvement of water in the transition.

One should note that the melting temperatures of proteins [2] and DNA [3] decrease with increasing ethanol concentration even at the alcohol concentration at which the maximum stability of the solvent structure occurs ($x_2 > x_2^*$). Probably, there occurs in these cases a positive deviation of the linear depression of the melting temperature for $x_2 > x_2^*$. More accurate data in the water-rich region are needed to confirm this statement.

It is known that, in apparent contrast with the observed decrease in melting temperature, ethanol has a marked stabilizing effect on the helix conformation of the protein, the maximum stabilizing capability arising over the ethanol concentration range close to x_2^* [22,23]. To reconcile these apparently contradictory facts, it has been suggested [1] that the initial decrease in T_m ($T_m = \Delta H_m / \Delta S_m$) is due to an increase in ΔS_m relative to ΔH_m as the alcohol concentration increases, i.e., a relatively larger entropy change occurs on transition, which would be expected if a greater degree of solvent structuring is involved.

Our data relative to the melting transition of tRNA indicate that a low alcohol concentrations ($x_2 < x_2^*$) the transition becomes more and more cooperative; this fact reflects an increased structural order in the water/alcohol/macromolecule system. Therefore, in this case as well, the greater the extent of structuring in the total system, the more sensitive the system becomes to the effects of temperature.

The anomaly in some physical parameters presented by ethanol in the water-rich region is not limited to ethanol alone, but is generally exhibited in dilute aqueous solutions of many non-electrolytes. More measurements in aquoues solutions of other alcohols in the low-x_2 region may be desirable to confirm the result obtained in the present work for the case of the ethanol/water system. These studies are now in progress; the results will be reported in due course.

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