# Osmolyte Mediation of T7 DNA Polymerase and Plasmid DNA Stability†

Manjusha Thakar, Arkady Bilenko, and Wayne J. Becktel*

*Department of Biochemistry, The Ohio State University, 484 West 12th, Columbus, Ohio 43210*

*Received April 25, 1994; Revised Manuscript Received August 5, 1994®*

ABSTRACT: The thermal stability of T7 DNA polymerase and pGEM4Z plasmid DNA in the presence and absence of the osmolyte *N*-methylglycine (sarcosine) was determined by means of UV spectroscopy. The decrease in melting temperature observed upon addition of sarcosine to solutions containing the plasmid DNA is linear with the concentration of sarcosine present. The enthalpy of the transition is also linear in its relationship to the melting temperature, and the entropy of the transition is linear in the natural log of the melting temperature. The slopes of both the entropic and enthalpic plots are equal. Destabilization of the plasmid DNA is observed to be entropically driven. The melting temperature of the T7 DNA polymerase complex is increased from 41 °C by addition of sarcosine to the solution. The relationship between the amount of sarcosine added and the melting temperature is linear, with a temperature of 61 °C observed for a 6 M solution. No clear trend of the effect of sarcosine on the enthalpy or entropy of the transitions could be observed.

Adverse effects such as extreme heat or salt challenge the ability of organisms to maintain optimum cytosolic conditions (Santoro et al., 1992). Such conditions can cause denaturation, chemical modification, or other processes leading to a total loss of biological activity. To overcome these problems, some organisms intracellularly incorporate low molecular weight solutes which are collectively known as osmolytes (Yancey et al., 1982). Osmolytes are capable of protecting proteins from heat or solvent inactivation without altering their activity (Santoro et al., 1992). In addition to protecting their catalytic activity, osmolytes have also been shown to be efficient in stabilizing proteins against thermal inactivation (Lee et al., 1981).

Naturally occurring osmolytes broadly consist of (1) inorganic ions which stabilize [$NH_4^+$, $(CH_3)_2NH_2^+$, $F^-$, $SO_4^{2-}$] (Yancey et al., 1982) and destabilize ($Na^+$, $K^+$, $Cl^-$) (Lee & Timasheff, 1981; Hand & Somero, 1982), (2) polyols (Gekko et al., 1981a; Lee et al., 1981), and (3) some commonly occurring free amino acids such as glycine (Santoro et al., 1992), proline (Stewart et al., 1974), taurine, and β-alanine (Yancey et al., 1979). These amino acids show no effects on the catalytic activity of enzymes.

Urea is found in large concentrations in the cells of a few cartilaginous, marine fishes. High concentrations of methylamine derivatives such as trimethylamine *N*-oxide, sarcosine, and betaine are also found in these organisms. These methylamines are the derivatives of amino acids like glycine. Methylamines, present intracellularly in a molar ratio of 1:2 with urea, have been shown to counteract the cytotoxic effects of urea (Yancey et al., 1979; Hand et al., 1982). Several types of bacteria also have significant concentrations of osmolytes such as sarcosine in the cytosol. In this instance, their presence contributes to the thermal stability of intracellular proteins. The effect of osmolytes is seen *in vitro*, in bacteria, and in higher organisms, and is thought to be general in scope.

DNA polymerases are employed in a wide variety of methods such as sequencing (T7 DNA polymerase), the polymerase chain reaction (Taq polymerase), and end-labeling of DNA template (the Klenow fragment). T7 DNA polymerase and the Klenow fragment are derived from mesophilic organisms while Taq polymerase is derived from a thermophile. An important consequence of this is that the first two enzymes cannot be successfully employed at temperatures much above 40 °C, while Taq polymerase remains active above 70–80 °C. T7 DNA polymerase like the Klenow fragment is optimally active around 37 °C. Above this temperature, the protein is known to be thermally inactivated. It is possible that addition of osmolytes to polymerase solutions will increase their thermal stability as it has with other proteins. If osmolytes stabilize these enzymes, then it is possible that polymerases thus treated may exhibit activity at elevated temperatures. This paper reports the study of the thermal stabilization of T7 DNA polymerase in the presence of sarcosine, the destabilization of plasmid DNA under the same conditions, and the relevance of the effects of osmolytes to processes involving nucleotides and proteins.

## METHODS

The double-stranded plasmid DNA pGEM4Z (reference Promega catalog no. P2161) was the gift of Dr. Caroline Breitenberger. The plasmid DNA was transformed into competent DH5α cells by a standard electroporation method in a cell porator (GIBCO BRL). After transformation, the plasmid was purified using the standard alkaline lysis method. *N*-Methylglycine (sarcosine) was purchased from Sigma. Sarcosine was further purified by fractional crystallization from methanol/water (95:5 v/v), and then was dried under vacuum. Buffers contained 10 mM Tris-HCl (pH 8.3, 25 °C), 50 mM KCl, and 4.5 mM $MgCl_2$. Gene 5 protein and thioredoxin used in the thermal denaturation were gifts of Dr. Smita S. Patel.

Ultraviolet spectroscopy was carried out on a Hewlett Packard 8452 diode array spectrophotometer. Temperature control was achieved with a HP89090A solid-state, thermionic controller. A probe placed inside the cuvette was used to monitor the temperature. A 4-mm Teflon-coated stirbar was used to mix the solutions. Helma QS cuvettes were used

† This research was supported, in part, by a grant from the National Science Foundation (DMB9009402).

* To whom correspondence should be addressed.

® Abstract published in *Advance ACS Abstracts*, September 15, 1994.

0006-2960/94/0433-12255$04.50/0 

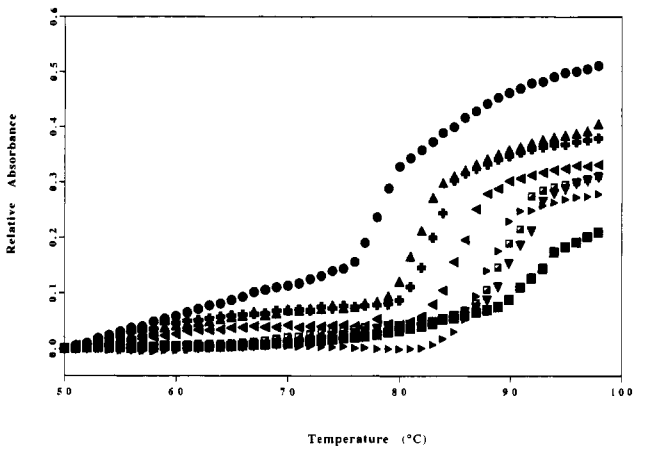


FIGURE 1: Thermal denaturation of pGEM4Z plasmid DNA. pGEM4Z plasmid DNA was thermally denatured in the presence of 0–6 M sarcosine. The relative absorbance was calculated by designating the absorbance at room temperature as unity. The other readings were then calculated with respect to the first reading by dividing the individual absorbance by this initial value. The different melting curves were (■) 0, (▼) 0.9, (◧) 1.7, (▲) 2.6, (▲) 3.4, (+) 4.3, (▲) 5.2, and (●) 6 M.

throughout. Thermally scanned spectra were obtained using TEMPCO, a program made available from Hewlett Packard which interfaces with the HP89090A Peltier temperature control accessory.

*Thermal Denaturation.* For the plasmid DNA thermal scans, an aliquot of 0.3 mL of the DNA (2 μg/μL stock concentration) was added to 2.7 mL of the buffer and mixed in the cuvette. The concentration of the plasmid DNA in the cuvette was determined from the absorbance at 260 nm before scanning and was generally 0.3–0.5 ODU. The scan was then carried out from 25 to 98 °C at the rate of 1 °C/min. The data were gathered at 0.5 °C intervals.

For the T7 DNA polymerase thermal scans, an aliquot of 10 μL of thioredoxin (23 μmol/L) was first mixed with 6.5 μL of gene 5 protein (36 μmol/L). This mixture was incubated on ice for a few minutes. In each denaturation experiment, DTT was present at a concentration of 10 mM. The spectrophotometer was blanked with this solution mixture, and individual temperature points were measured at 280 nm. The OD was usually 0.02–0.04. The temperature range used for scanning T7 DNA polymerase was 25–70 °C. Each data point was gathered at 0.5 °C intervals. The concentration of sarcosine in the thermal melts varied from 0 to 6 M.

## RESULTS

*Thermal Denaturation of Plasmid DNA.* Thermal denaturation of the plasmid DNA pGEM4Z was carried out in the presence of 0–6 M sarcosine. The resulting variation of $OD_{260}$ with temperature is shown in Figure 1. The experiments indicate cooperative transitions both in the presence and in the absence of sarcosine. There is a gradual, practically linear increase in absorbance with temperature both below and above the transition region. In each instance, denaturation was completely reversible, allowing a complete thermodynamic analysis. The observed melting temperatures and other thermodynamic parameters are given in Table 1.

The variation of the melting temperature of pGEM4Z plasmid DNA with sarcosine concentration is linear with a slope of −2.5 °C/mol and a zero point intercept of 94 °C. The implication of this result is that this plasmid DNA may be reduced in melting temperature by as much as 20 °C in an 8 M sarcosine solution. This observation is in agreement with those of Rees et al. (1993).

Table 1: Plasmid pGEM4Z Thermodynamics

| [S][a] | ln[S] | $T_m$[b] | $\Delta H$[c] | $\Delta S$[d] | $\Delta G(85)$[e] |
|---|---|---|---|---|---|
| 0.0 | | 366.2 | 136.0 | 372.7 | 5.2 |
| 0.9 | (0.105) | 364.5 | 88.4 | 242.5 | 3.2 |
| 1.7 | 0.531 | 363.2 | 92.0 | 253.3 | 2.6 |
| 2.6 | 0.956 | 360.5 | 95.8 | 265.7 | 1.4 |
| 3.4 | 1.224 | 359.2 | 102.0 | 284.0 | 0.6 |
| 4.3 | 1.459 | 356.2 | 114.3 | 320.9 | −1.2 |
| 5.2 | 1.649 | 354.2 | 125.0 | 352.9 | −2.6 |
| 6.0 | 1.792 | 351.4 | 134.3 | 382.2 | −5.0 |

[a] Sarcosine concentration. [b] Melting temperature in degrees kelvin. [c] Enthalpy in kilocalories per mole. [d] Entropy in calories per degree per mole. [e] Free energy calculated from $\Delta H$ and $\Delta S$ with the assumption that there is no temperature dependence of either.

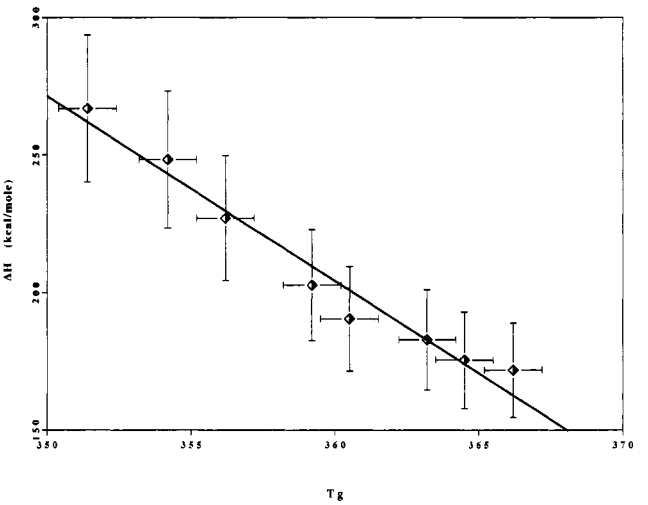


FIGURE 2: Dependence of the enthalpy of denaturation of pGEM4Z plasmid DNA upon melting temperature. The enthalpies of denaturation were extracted from a van't Hoff analysis of the data from Figure 1. Errors in the value of the enthalpy were assumed to be 10% of the determined enthalpy. Errors in the melting temperature were assumed to be ±1 °C.

The enthalpy and entropy of each denaturation were determined by means of van't Hoff analysis and are also given in Table 1. Although the melting temperature of the transition decreases with increasing sarcosine concentration, both the entropy and the enthalpy increase. The change in entropy outpaces that of the enthalpy. The percentage increase from 0 to 6 M sarcosine is 55% for the enthalpy and 62% for the entropy of denaturation. This implies that the destabilization of plasmid pGEM4Z DNA by sarcosine is an entropically driven process.

The variation of the enthalpy with melting temperature is shown in Figure 2. It has been previously shown that plots of $\Delta H$ vs $T_m$ for globular protein denaturations as a function of pH often yield straight lines whose slopes equal the difference in heat capacity between the native and denatured states ($\Delta C_p$; Privalov, 1979). The relationship between $\Delta C_p$ and $\Delta H(T)$ has been shown to be given by the equation (Becktel & Schellman, 1987):

$$\Delta H(T) = \Delta C_p(T - T_h)$$

where $T_h$ is the temperature where $\Delta H(T)$ passes through zero. From Figure 2, we see that there is a similar linear dependence of the enthalpy of denaturation of this DNA but that, unlike proteins under differing pH conditions, the slope

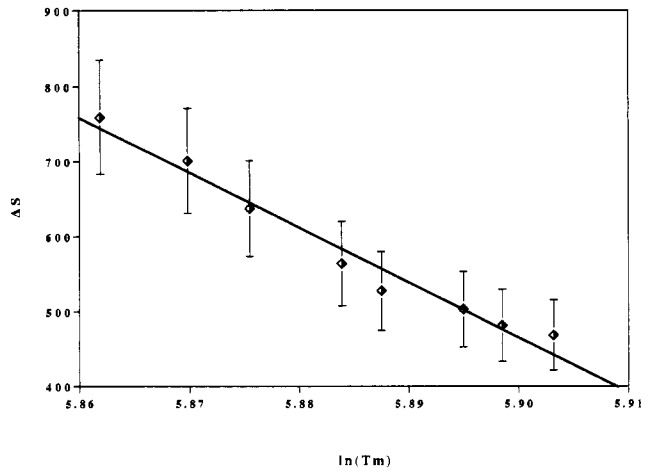


FIGURE 3: Variation of the entropy of denaturation of pGEM4Z DNA. The values of the entropy were determined in the same manner as the enthalpy in Figure 2. Errors in the entropy wee assumed to be of the order 10%.

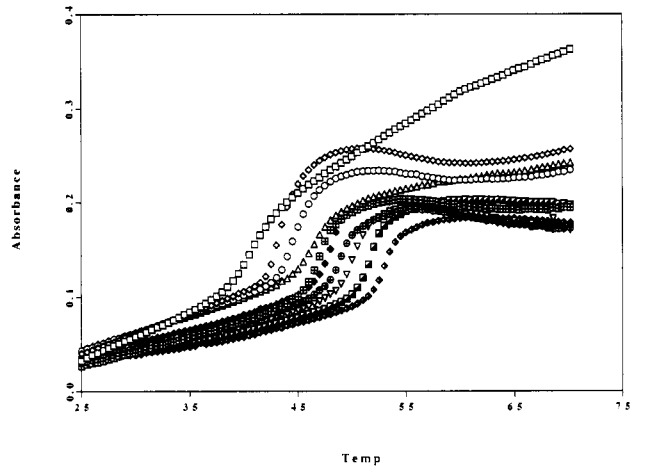


FIGURE 4: Thermally induced transitions in T7 DNA polymerase. T7 DNA polymerase was denatured in the presence of different concentrations of sarcosine. The different curves were at (□) 0, (◇) 0.3, (○) 0.7, (△) 1.0, (□) 1.4, (◇) 1.7, (⊕) 2.1, (▽) 2.4, (◪) 2.7, and (◇) 3.2 M sarcosine concentrations.

is large and negative instead of large and positive. If this is in fact $\Delta C_p$ for this process, it would imply $T_h$ = 117 °C.

A similar analysis can be made for the entropy. In this instance, it has been previously shown that for constant $\Delta C_p$ the relationship between temperature and entropy is given by the equation (Becktel & Schellman, 1987):

$$\Delta S(T) = \Delta C_p \ln\left(\frac{T}{T_s}\right)$$

where $T_s$ is the temperature at which the entropy change passes through zero. Figure 3 is a plot of $\Delta S$ vs $\ln(T_m)$. It is also linear. More importantly, the slope of this line is −7.3 kcal/(deg·mol) as compared to −6.7 kcal/(deg·mol) for the plot in Figure 2. If these systems follow simple two-state thermodynamics, the two slopes must be identical. In fact, they are nearly the same with a mean value of −7.0 ± 0.3 kcal/(deg·mol). This observation is discussed in detail below.

*Thermal Denaturation of T7 DNA Polymerase.* Figure 4 shows the thermal denaturation of T7 DNA polymerase in various concentrations of sarcosine. The curves show an abrupt increase in the absorbance with temperature, and appear to be sigmoidal in shape, suggesting that melting of T7 DNA polymerase is cooperative in nature. After the completion of each melt, the solution was rapidly cooled down to 25 °C. The absorbance did not decrease to the initial value. This indicates that the transition observed was irreversible, which is consistent with thermal denaturations of other proteins in the presence of osmolytes. The midpoint of the transition increases with sarcosine concentration, indicating that sarcosine renders the proteins thermally stable but does not permit refolding (Santoro et al., 1992). The variation of the absorbance at temperatures above that of the denaturation is associated with irreversible denaturation of the complex. Denaturation curves of this sort are associated with reversible denaturation followed by a slow, irreversible step. The thermodynamic analysis of these denaturations is given in Table 2. It is important to note that the thermodynamic parameters given in Table 2 are approximate in nature because it is not possible to carry out a full thermodynamic analysis of any system which is not fully reversible.

Table 2: T7 DNA Polymerase Thermodynamics

| [S][a] | ln[S] | $T_m$[b] | $\Delta H$[c] | $\Delta S$[d] |
|---|---|---|---|---|
| 0.0 | | 314.7 | 77 | 243 |
| 0.3 | (1.203) | 317.0 | 153 | 481 |
| 0.7 | (0.357) | 317.8 | 130 | 410 |
| 1.0 | 0.000 | 319.9 | 98 | 305 |
| 1.4 | 0.336 | 320.0 | 132 | 412 |
| 1.7 | 0.531 | 321.1 | 147 | 457 |
| 2.1 | 0.741 | 322.1 | 131 | 406 |
| 2.4 | 0.875 | 323.3 | 130 | 402 |
| 2.7 | 0.993 | 325.0 | 134 | 411 |
| 3.2 | 1.163 | 326.0 | 149 | 458 |

[a] Sarcosine concentration. [b] Melting temperature in degrees kelvin. [c] Enthalpy in kilocalories per mole. [d] Entropy in calories per degree per mole.

In the absence of sarcosine, the complex is half-denatured at 42 °C. As sarcosine is added to the solution, the melting temperature increases. At a concentration of 3.2 M sarcosine, the melting temperature increases. At a concentration of 3.2 M sarcosine, the melting temperature increased to 53 °C, and at a concentration of 6 M, it is 61 °C (data not shown). As was the case for pGEM4Z DNA, the dependence of the melting temperature on sarcosine concentration is linear. The slope of this line is 3.1 °C/mol, and the intercept is 42.5 °C. The latter is close to the experimental value in the absence of sarcosine. On the basis of this relationship, it may be possible to measure the T7 DNA polymerase melting temperatures approaching 70 °C. The source of the stabilization is not clear. Examining either the enthalpy or the entropy in Table 2, no clear trend in the data emerges.

## DISCUSSION

*pH Dependence of Tris Buffers.* The buffers used throughout these studies contained Tris. It is well-known that the $pK_a$ of Tris has a very significant dependence upon temperature. Despite this fact, Tris buffers were employed so as to match as exactly as possible the conditions commonly used in PCR and other protocols involving polymerase reactions. The temperature dependence of the $pK_a$ of Tris may be approximated by a linear function with a slope of 0.027 pH unit/°C (ISCO Tables). Since the solutions were originally pH 8.3 at 25 °C, this would be reduced to approximately pH 6.5 at the melting point of the plasmid DNA in the absence of sarcosine. From Table 1, the melting temperature range of the plasmid DNA is from 94 to 74 °C and that of the T7 DNA polymerase from 61 to 42 °C. This implies a pH range in the vicinity of the melting points of 6.5–7.0 for the DNA and 7.4–7.9 for the protein. It is unlikely that a ±0.25 variation, near neutral pH, is responsible for the variation of

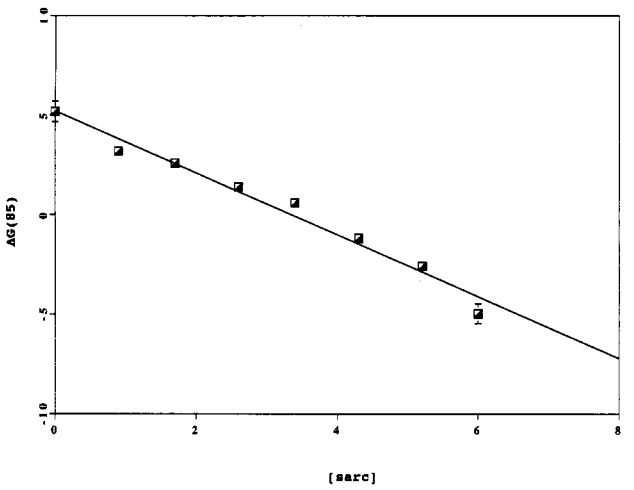


FIGURE 5: Variation of $\Delta G(85)$ of pGEM4Z plasmid DNA with sarcosine concentration. The method of calculation of the free energies is given in detail in the text. The error associated with each energy is assumed to be of the order ±10%.

stability observed for either the plasmid DNA or the polymerase.

*Thermodynamic Consequence of Sarcosine Binding to Plasmid DNA.* In the presence of sarcosine, the melting temperature of plasmid pGEM4Z DNA was lowered by as much as 14 °C (from 92 to 78 °C). The same results have been reported using betaine (Rees et al., 1993), which, like sarcosine, is a glycine-based osmolyte. The plasmid pGEM4Z contains approximately 50% guanine and cytosine. This is comparable in GC content to the results for *E. coli* DNA reported by Rees et al. In that study, an approximate 26 °C decrease in melting temperature was observed comparing 0 to 5.6 M betaine. This is twice as large a change as observed for sarcosine destabilization of pGEM4Z plasmid DNA, and may indicate the different ability of betaine and sarcosine to bind to DNA. It should be observed, however, that *E. coli* DNA is linear and the plasmid is circular. In addition, the ionic strengths in the two studies are not exactly the same.

The large increases in $\Delta H(T_m)$ reported here are also consistent with the data of Rees et al. (1992). Calf thymus DNA in the presence of 5 M betaine is reported to exhibit both a lower melting temperature and a sharper slope than in 0 M betaine. The plasmid pGEM4Z DNA also exhibits a lower $T_m$ but has a 50% increase in $\Delta H(T_m)$ in the presence of 6 M sarcosine, and a larger percentage increase in $\Delta S(T_m)$.

The effect on the free energy of the transition as a function of sarcosine concentration is shown in Figure 5. The temperature at which the comparison is carried out was chosen to be 85 °C, which lies in the mid-range of the melting temperatures of the DNA in these experiments. The $\Delta G(85)$ in the absence of sarcosine was calculated by assuming $\Delta H$ and $\Delta S$ are independent of temperature. The free energy values for the remaining data were calculated using the assumption

$$\Delta G(T) = \Delta G(T) - \Delta G(T_m) = \Delta S \Delta T$$

which was proposed as an approximation for protein denaturation under conditions of constant $\Delta C_p$ (Becktel & Schellman, 1987). If the 0 and 6 M points are omitted from a linear fit of the data, a straight line results with a slope of −1.6 kcal/mol[2]. This slope represents the interaction energy, at 85 °C, between sarcosine and the plasmid DNA (Schellman, 1985), or an interaction equilibrium constant of approximately 9. This is typical of weak bonding of a small molecule to a large macromolecule.

*Sarcosine Stabilization of T7 DNA Polymerase May Be either Entropic or Enthalpic.* The actual mechanism in the stabilization of proteins by osmolytes is still unknown. The stabilization by such zwitterions (osmolytes) has been compared to the stabilization by ions of a Hofmeister series (Yancey et al., 1982; Hand et al., 1982; Santoro et al., 1992). Studies carried out with sucrose explain the phenomenon of stabilization of proteins in terms of a stable cohesive force between sucrose and the solvent surrounding the protein (Lee et al., 1981). This force forms a strong cavity around the protein, stabilizing the folded structure. Studies carried out with glycerol explain that glycerol and the water of hydration reorder the protein solvent cage so as to keep the solvent composition constant (Gekko et al., 1981). Glycerol penetrates the solvation sheath of proteins and has been shown to stabilize the delicate balance between repulsion of nonpolar and polar regions of proteins. The stabilization of proteins by methylamines like sarcosine is predicted to have a similar hydration effect as with sucrose and glycerol (Santoro et al., 1992).

Because the thermal denaturation of T7 DNA polymerase is not fully reversible, the thermodynamic analysis presented in Table 2 is approximate in nature. Within this context, the change in stability of T7 DNA polymerase indicates that sarcosine does not, apparently, modify the enthalpy of denaturation. What is not clear is that the mechanism of stabilization in this instance is entropic in nature. The dependence of the entropy upon the log of $T_m$ is not clear from the data in Table 2.

*Dependence of Thermodynamic Parameters upon Solution Perturbants.* The dependence of $\Delta H(T)$ and $\Delta S(T)$ upon $T_m$ shown in Figures 2 and 3 is similar to that of globular proteins in which only the pH of the solution is changed. For proteins, the alteration of stability is caused by a change in the overall charge of the molecule with titration of amino acid side chains. In principle, this results in a series of native and denatured populations. The enthalpies derived from each denaturation result from transitions between these different states and are not comparable. In practice, the temperature dependence of the enthalpy determined by means of calorimetry at a single pH is frequently within experimental error of that determined from enthalpies at many different values of pH (Privalov, 1979). This is particularly true when the pH range is from neutral to acidic. It arises from the fact that some globular proteins melt as single, cooperative units and from the fact that the energy of ionization of acidic groups has a small temperature dependence (Becktel & Schellman, 1987).

Biological macromolecules for which the cooperative domain is smaller than the entire molecule do not exhibit the same enthalpy when determined by calorimetry and van't Hoff methods. This is true for large DNA chains and for multiple domain proteins. If a linear dependence of the enthalpy with melting temperature is still found, one explanation is that the conformational transition consists of a series of transitions of nearly identical cooperative units.

The data in Figures 2 and 3 may result from a set of conformational transitions of this sort. If sarcosine alters the energies of the native and denatured states and not the distribution of conformers in those states, then two-state transitions are taking place. The thermodynamic parameters (derived from van't Hoff analysis, while dependent upon the length of the cooperative unit, are valid. This suggests that the effect of sarcosine is similar to that of changes in pH. Sarcosine acts as a solvent perturbant.

Alternatively, the introduction of sarcosine changes the native and denatured states in such a manner that comparing enthalpies at different sarcosine concentrations is meaningless and the linear dependence coincidental. The difference between these two possible explanations may possibly be examined by means of determining the apparent volume of the native and denatured states as a function of sarcosine concentration in solutions containing DNA.

*Implications to Processes Involving DNA and Polymerases.* T7 DNA polymerase is stabilized by sarcosine while, under the same conditions, plasmid DNA is destabilized. Preliminary results also indicate that the activity of T7 DNA polymerase, in the presence of sarcosine, is retained at temperatures in excess of 45 °C (data not shown). Destabilization of DNA is known to be advantageous in order to help to eliminate sequencing artifacts. Coupled with the stabilization of the polymerase, this suggests that the inclusion of osmolytes in processes involving both DNA and polymerase may improve the overall efficiency of sequencing or other procedures. One important factor which remains to be demonstrated is whether the fluidity of the polymerase is retained at elevated temperature, in the presence of sarcosine.

## ACKNOWLEDGMENT

We gratefully acknowledge the many conversations, assistance, and gifts of DNA and proteins from Dr. Caroline A. Breitenberger and Dr. Smita S. Patel. We also acknowledge the observations and suggestions of one of the reviewers regarding the temperature dependence of Tris buffers in general and in their application to the polymerase chain reaction in particular.

## REFERENCES

Becktel, W. J., & Schellman, J. A. (1987) *Biopolymers 26*, 1859–1877.

Gekko, K., & Timasheff, S. N. (1981a) *Biochemistry 20*, 4667–4676.

Gekko, K., & Timasheff, S. N. (1981b) *Biochemistry 20*, 4677–4686.

Hand, S. C., & Somero, G. N. (1982) *J. Biol. Chem. 257*, 734–741.

Isco Tables (1987) *A handbook of data for biological and physical scientists*, Ninth ed., p 40, Lincoln, NE.

Lee, J. C., & Timasheff, S. N. (1981) *J. Biol. Chem. 256*, 7193–7201.

Privalov, P. L. (1979) *Adv. Protein Chem. 33*, 167–241.

Rees, W. A.; Yager, T. D., Korte, J., & Von Hippel, P. H. (1993) *Biochemistry 32*, 137–144.

Santoro, M. M., Liu, Y., Khan, S. M. A., Hou, L., & Bolen, D. W. (1992) *Biochemistry 31*, 5278–5283.

Schellman, J. A. (1990) *Biopolymers 29*, 215–224.

Stewart, G. R., & Lee, J. A. (1974) *Planta 120*, 279–289.

Tabor, S., & Richardson, C. (1987a) *J. Biol. Chem. 262*, 15330–15333.

Tabor, S., & Richardson, C. (1987b) *J. Biol. Chem. 264*, 6447–6458.

Yancey, P. H., & Somero, G. N. (1979) *Biochem. J. 183*, 317–323.

Yancey, P. H., Clark, M. E., Hand, S. C., Bowlus, D., & Somero, G. N. (1982) *Science 217*, 1214–1222.