A Proposed Method for the Quantitative Comparison of the Effects of Denaturants on the Stability of Proteins

by

James A. Thomson

A Thesis Submitted to
the Faculty of Graduate Studies and Research
of the University of Manitoba
in partial fulfillment
of the requirements of the degree
Master of Science

Winnipeg, Manitoba

May, 1985

A PROPOSED METHOD FOR THE QUANTITATIVE COMPARISON OF THE EFFECTS OF DENATURANTS ON THE STABILITY OF PROTEINS

BY

JAMES A. THOMSON

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

ABSTRACT

A novel quantitative comparison of denaturants involving the complete reversible unfolding of protein is presented. Ribonuclease A was denatured with guanidinium chloride in the presence of low fixed concentrations of various partial denaturants with the unfolding process being followed by CD and difference spectroscopy. The major advantage of this method is that it allows a direct quantitative comparison of the effects of denaturants on the stability of proteins in physiologically relevant terms. The effect on the stability of ribonuclease A was shown to be linearly dependent upon the denaturant concentration. An in depth investigation of the constitutive ions of salts revealed that their effects were additive only in the case of salts with no specific binding capability. This method was also proven useful in detecting the specific binding of salts.

ACKNOWLEDGEMENTS

I would like to express my appreciation to Dr. Charles Bigelow for his patience, understanding and continued support throughout the last five years.

I would also like to thank Dr. F. Ahmad for many intense discussions that allowed me to express and develop ideas pertinent to this field of endeavor and Dr. H. Duckworth for his assistance during the preparation of this manuscript.

TABLE OF CONTENTS

	Page
Abstract	i
Acknowledgements	ii
List of Tables	iv
List of Figures	v
I. Introduction	1
II. Materials and Methods	17
III. Results	29
A) Preliminary Results	29
B) Refitted Results (Non-Binding Salts)	54
1) Non-effectors	54
2) Effectors	62
C) Verification	65
D) Refitted Results (Binding Salts)	74
IV. Discussion	
References	101

LIST OF TABLES

Table		Page
1	Preliminary results characterizing ribonuclease A denaturation	46
2	"Fitted" results characterizing ribonuclease A denaturation	63
3	Predicted midpoint of transition at zero molar ion/salt concentration	70
4	"Fitted" results characterizing ribonuclease A denaturation in the presence of binding salts	77
5	Predicted midpoint of transition at 0.5 molar ion/salt concentration	79
6	Observed and predicted midpoint of transition values	80

LIST OF FIGURES

Figure		Page
1	The difference spectra of ribonuclease A produced upon denaturation by guanidinium chloride in the presence of NaBr	31
2	Changes in $\Delta\varepsilon_{287}$ of ribonuclease A produced upon denaturation by guanidinium chloride in the presence of NaBr	32
3	CD spectra of ribonuclease A produced upon denaturation by guanidinium chloride in the presence of $NaClO_4$	34
4	Changes in the ellipticity of ribonuclease A upon denaturation by guanidinium chloride in the presence of $NaClO_4$	35
5	Normalized transition curve of the denaturation of ribonuclease A by guanidinium chloride in the presence of NaBr	37
6	Normalized transition curve of the denaturation of ribonuclease A by guanidinium chloride in the presence of $NaClO_4$	38
7	Changes in $\Delta\varepsilon_{287}$ of ribonuclease A produced upon denaturation by guanidinium chloride in the presence of NaCl	40

LIST OF FIGURES (CONTINUED)

Figure		Page
8	Changes in the ellipticity of ribonuclease A upon denaturation by guanidinium chloride in the presence of NaCl	41
9	Normalized transition curve of the denaturation of ribonuclease A by guanidinium chloride in the presence of NaCl	42
10	Variation of ΔG_D as a function of the guanidinium chloride concentration in the presence of NaCl and KCl	44
11	Variation of ΔG_D as a function of the guanidinium chloride concentration in the presence of NaCl	49
12	Variation of ΔG_D as a function of the guanidinium chloride concentration in the presence of NaCl	49
13	Variation of ΔG_D as a function of the guanidinium chloride concentration in the presence of CsCl	49
14	Variation of ΔG_D as a function of the guanidinium chloride concentration in the presence of RbCl	49
15	Variation of ΔG_D as a function of the guanidinium chloride concentration in the presence of NH_4Cl	50

LIST OF FIGURES (CONTINUED)

Figure		Page
16	Variation of ΔG_D as a function of the guanidinium chloride concentration in the presence of NH_4Cl	50
17	Variation of ΔG_D as a function of the guanidinium chloride concentration in the presence of LiCl	50
18	Variation of ΔG_D as a function of the guanidinium chloride concentration in the presence of Phosphate	50
19	Variation of ΔG_D as a function of the guanidinium chloride concentration in the presence of Na_2SO_4	51
20	Variation of ΔG_D as a function of the guanidinium chloride concentration in the presence of $(NH_4)_2SO_4$	51
21	Variation of ΔG_D as a function of the guanidinium chloride concentration in the presence of Li_2SO_4	51
22	Variation of ΔG_D as a function of the guanidinium chloride concentration in the presence of NaAc	51
23	Variation of ΔG_D as a function of the guanidinium chloride concentration in the presence of NH_4Ac	52

LIST OF FIGURES (CONTINUED)

Figure		Page
24	Variation of ΔG_D as a function of the guanidinium chloride concentration in the presence of NaBr	52
25	Variation of ΔG_D as a function of the guanidinium chloride concentration in the presence of LiBr	52
26	Variation of ΔG_D as a function of the guanidinium chloride concentration in the presence of NaSCN	52
27	Variation of ΔG_D as a function of the guanidinium chloride concentration in the presence of LiSCN	53
28	Variation of ΔG_D as a function of the guanidinium chloride concentration in the presence of $NaClO_4$	53
29	Variation of ΔG_D as a function of the guanidinium chloride concentration in the presence of $NaClO_4$	53
30	Variation of ΔG_D as a function of the guanidinium chloride concentration in the presence of $LiClO_4$	53
31	Normalized transition curve of the denaturation of ribonuclease A by guanidinium chloride in the presence of NaCl	55

LIST OF FIGURES (CONTINUED)

Figure		Page
32	Normalized transition curve of the denaturation of ribonuclease A by guanidinium chloride in the presence of NH_4Cl	58
33	Normalized transition curve of the denaturation of ribonuclease A by guanidinium chloride in the presence of NaAc	59
34	Normalized transition curve of the denaturation of ribonuclease A by guanidinium chloride in the presence of NH_4Ac	60
35	Transition curve and normalized transition of the denaturation of ribonuclease A by guanidinium thiocyanate	66
36	Variation of ΔG_D as a function of the guanidinium thiocyanate concentration and the guanidinium chloride concentration in the presence of NaSCN	68
37	Variation of the midpoint of transition as a function of the non-binding salt concentration	71
38	Variation of the midpoint of transition as a function of the binding salt concentration	72
39	Variation of ΔG_D as a function of the concentration of a hypothetical denaturant	89

I. INTRODUCTION

In this dissertation a modification to an old approach used to compare the characteristic stabilizing and destabilizing effects of salts against the denaturing of proteins is presented. The resulting improved approach to this old problem affords the usual rank ordering of the molar effectiveness of each salt along with a never before reported physiologically pertinent direct quantitative comparison of their effects. The results obtained from this study will be used to verify and/or clarify some previously reported conclusions regarding the linearity and additivity of the effects of individual ions. This new approach will also be shown to be useful in detecting any specific binding that may occur between the constitutive ions of the salt in question and the protein being investigated.

The use of salts and other denaturants is quite common in biochemical and biophysical research. Molecular weight determinations, preparation of samples for X-ray crystallographic studies and isolation and purification of proteins and deoxyribonucleic acid are but a few of the commonly used biologically orientated procedures that utilize denaturants such as sodium dodecyl sulfate, ammonium sulfate and guanidinium hydrochloride (GdnHCl). Denaturants are predominantly employed in studies involving the

determination of the structure, function and physicochemical aspects of proteins. The investigation of the overall stability, the stabilizing and destabilizing intermolecular interactions contributing to this stability and the folding and unfolding processes of proteins can only be achieved via the controlled denaturation of these proteins. As the number, variety and characterization of reagents of known denaturing capability increase the potential for development in this field of study also increases.

Many studies have been undertaken with the sole purpose of characterizing the capabilities and modes of action of denaturants. The vast majority of these studies involve qualitative comparisons of various denaturants while very few attempts have been made to quantitatively characterize and compare denaturants. Quantitative comparisons are difficult because different denaturants are commonly known to yield different denatured states (1-8). The difficulty of a direct quantitative comparison lies in finding a suitable criterion that is common to all denaturants. The obvious comparison that should be made is the comparison of the extent to which each denaturant disrupts the native structure of the protein. This is quite difficult in most cases as the structure of the denatured state usually cannot be fully elucidated. X-ray crystallography cannot be employed as in most cases it would be impossible to obtain crystals of the denatured state. Spectroscopic analysis is very useful in distinguishing between folded and unfolded

protein but it cannot be used to successfully identify and/or distinguish structure that remains undisrupted and structure, if any, that is newly formed.

The next most obvious way to compare the capabilities of various denaturants is to compare their effect on the Gibbs free energy of stability of several different proteins. Although Gibbs free energy changes for the denaturation of numerous proteins by a plethora of different denaturants have been reported elsewhere the majority of these values represent the ability of the protein to withstand a particular denaturing action and should not be confused with the Gibbs free energy of stability of the protein (8). Only the free energy change that accompanies the complete and reversible unfolding of a protein should be considered as a measure of the Gibbs free energy of stability for that particular protein. Again, the difficulty in this task lies in the fact that not all denaturants completely unfold proteins and thus their effects on the Gibbs free energy of stability of a protein has up to this time been unattainable.

The main purpose of this study is to propose a means by which the stabilizing and destabilizing capabilities of various denaturants, with specific emphasis on inorganic neutral salts, can be quantitatively compared. With this accomplished the widely accepted, although inadequately proven theory that the effects of the constitutive ions of salts are additive and linearly dependent upon the salt

concentration will be further examined. To appreciate the true significance of the contribution of this investigation to the overall accumulated knowledge of protein stability and denaturation a fair understanding of the current status of this field of endeavor is required. As a lengthy review is beyond the scope of this dissertation and many excellent reviews are available that have been published with this as their sole intent the theory presented here will be the absolute minimum required to convey the relevance of the results.

The predominant configuration that a protein assumes in aqueous solution is called the native state, usually denoted N. For most proteins the native state has a compact globular configuration. However, some proteins such as myosin are rodlike and some are even completely unfolded, such as casein. Tanford has defined the denaturation of proteins as "simply a major change from the original native structure, without alteration of the amino acid sequence, i.e., without severance of any of the primary chemical bonds which join one amino acid to another" (9).

If the product of denaturation can best be described as a random coil, that is, devoid of all residual structure, or best described as a cross-linked random coil, that is, a random coil possessing disulfide bridges, then it is referred to as the fully denatured state, usually denoted D or U. The denaturation process in which all structure is destroyed is also known as "unfolding", hence, the origin of

the term U state. If the product of denaturation contains some residual structure, either original or newly formed, then it is referred to as a partially denatured state or as an intermediate state, denoted I.

The native state is only marginally more stable than the fully denatured state and under physiological conditions these states exist in equilibrium. The Gibbs free energy change for the reversible reaction

$$N \rightleftharpoons D \qquad (1)$$

in the absence of denaturants is used as a measure of the stability of the protein. For most proteins this Gibbs free energy of stability, $\Delta G^{H_2O}_D$, is of the order of magnitude of 5 to 15 kcal mol^{-1}. At room temperature the equilibrium constant, K^{H_2O}, in the absence of denaturants is of the order of magnitude of 10^{-4} to 10^{-11}. Since under physiological conditions the concentration of the denatured state is very much smaller than the concentration of the native state, denaturants must be employed to shift the equilibrium if the reaction is to be investigated.

There exist two different methods by which reasonable estimates of $\Delta G^{H_2O}_D$ can be obtained, a thermodynamic method and a pseudothermodynamic method. The former requires a sensitive differential scanning microcalorimeter and is therefore not widely employed. The latter method does not require specialized equipment. Commonly available optical

instruments such as UV spectrophotometers can be employed making this method popular by choice if not by necessity. The term "pseudothermodynamic" was coined by Lapanje to signify that estimates of the stability of proteins that are obtained by this method are based on non-thermodynamical measurements, unlike the method employing the differential scanning microcalorimeters (10). In the latter method denaturants are used to shift the equilibrium and thereby alter the relative concentrations of the native and denatured states so that the difference in their concentrations is not so considerable.

The induced transition from native state to denatured state produces a change in many observable parameters. These include considerable changes in the optical rotation and absorption of the solution observable throughout a broad range of wavelengths. The transition can be monitored by plotting the observed value of the parameter, Y_{obs}, versus the concentration of the denaturant used to induce the transition. The resulting plot is called a transition curve. This curve can be used to calculate the fraction of protein that is unfolded if the denaturation process involved is an "all-or-none" process, otherwise known as a two-state transition. The protein molecules in solution must either be in the native state or the denatured state. This is usually assumed to be the case and if this assumption is correct or if the fraction of protein molecules that are in some partially unfolded state is

negligible then

$$f_N + f_D = 1 \qquad (2)$$

where f_N and f_D represent the fraction of protein in the N and D states respectively. The value of Y_{obs} is directly related to the fractions of native and denatured protein

$$Y_{obs} = f_N Y_N + f_D Y_D \qquad (3)$$

where Y_N and Y_D represent the values of the observable parameter Y obtained for pure native protein and pure denatured protein respectively. These values need not be constant and are usually best described as functions of the denaturant concentration. The variation of these values with change in denaturant concentration, outside the transition region, is known as solvent perturbation and the functions are commonly referred to as solvent effects.

All denaturation curves can be divided into three distinct regions. The predominant portion is a sigmoidal (although not necessarily symmetric) function resulting from the denaturation changes. The concentration range corresponding to this change is called the transition region. The other two functions are minor in comparison. They are located before and after the transition region and are a result of the solvent effects on the native and

denatured protein respectively. The corresponding concentration ranges over which these two functions can clearly be resolved are called the pre- and post-transition regions respectively.

If the denaturation process under investigation truly proceeds via a two-state mechanism and is reversible then

$$f_D = \frac{Y_N - Y_{obs}}{Y_N - Y_D} \qquad (4)$$

a plot of the f_D values against the concentrations at which they were calculated yields what is referred to as a normalized transition curve. A normalized curve is simply a denaturation transition curve with the solvent effects removed.

A simple method of determining whether the process involved does not proceed via a two-state mechanism is to monitor the transition using several different techniques, for example the transition could be monitored by plotting the reduced viscosity of the protein and by plotting the reduced mean residue rotation of the protein both as functions of the denaturant concentration. The normalized curves produced by the results obtained using both methods will be coincident if the denaturation truly proceeds by a two-state mechanism and should not be coincident if noticeable concentrations of intermediates are produced.

The extent to which the equilibrium constant has been altered can confidently be calculated from the data

contained in the normalized transition curve. For each observed point within the transition region an equilibrium constant, K_D, can be calculated using the equation

$$K_D = f_D/(1 - f_D) \tag{5}$$

Induced changes in the concentration of the denatured protein that take place when f_D is less than 0.1 are hard to detect with any accuracy. Similarly when f_D is greater than 0.9 the induced changes in the native protein concentration are difficult to detect. For this reason usually only those f_D values that lie between these limits are used in Equation 5.

From these K_D values the Gibbs free energy change, ΔG_D, for the reaction can be calculated using the equation

$$\Delta G_D = -RT \ln K_D \tag{6}$$

where R is the gas constant and T (=298 K) is the temperature at which the denaturation was carried out. The K_D and ΔG_D values obtained in this manner are calculated from non-thermodynamic parameters and therefore should be referred to as "apparent" equilibrium constants and "apparent" Gibbs free energy changes. This is to distinguish them from values obtained using activities to calculate the K_D and ΔG_D values. Hence the distinction between the pseudothermodynamic and the thermodynamic

methods. This is not to say that these apparent values and the true values are not equivalent. Their equality depends upon the validity of the assumption that the denaturation proceeds via a two-state mechanism and to some extent on the accuracy of the functions used to describe the solvent effects.

Several different methods have been suggested whereby the "apparent" Gibbs free energy of stability of a protein can be estimated from the $\triangle G_D$ values obtained in the above described manner. Although the exact mechanism for the denaturation of proteins is not known model compound studies have revealed that most of the constituent parts of a protein are more soluble in denaturants than in aqueous solution. Tanford has developed a method for estimating the Gibbs free energy of stability of a protein utilizing the free energies of transfer of model compounds of these constituent parts (11). His approach is based on the relationship

$$\triangle G_D = \triangle G_D^{H_2O} + \sum_{n=1}^{i} \alpha_i \, n_i \, \delta g_{tr,i} \qquad (7)$$

where α_i is the average fractional change in the degree of exposure of groups of type i, n_i is the number of groups type i in the protein and $\delta g_{tr,i}$ is the free energy of transfer of a group of type i from aqueous solution to denaturant. This latter term varies with denaturant concentration. The α_i term in the above equation is

difficult to assign on an individual group type basis. For this reason this term is usually replaced with a single average term, α. A second modification usually employed is that of assuming that not all amino acid residues contribute significantly to the value of ΔG_D and therefore only the major contributors need be included in the analysis. Although theoretically this method is applicable to this investigation the design of the experiment precludes the use of Tanford's model in the analysis of the obtained Gibbs free energy changes. The reasons for this will be presented with the results.

A second method of determining the Gibbs free energy of stability of a protein results by assuming that the denaturant binds to the peptide and/or amino acid residue side chain groups of the protein (11). If there are a greater number of identical non-specific binding sites accessible on an unfolded protein than there are on a folded protein then the observed free energy change can be related to Δn, this difference in the number of denaturant binding sites.

$$\Delta G_D = \Delta G_D^{H_2O} - \Delta n \; RT \; \ln(1 + ka) \qquad (8)$$

where R is the gas constant, T is the temperature at which the experiment was carried out, k is the equilibrium constant for the binding at each site and a is the activity of the denaturant. Generally the binding constant is

assigned a value between 0.1 and 1.2 when either urea or GdnHCl is used as the denaturant. The difference in the number of binding sites is varied as is the value of k to produce the best fit of the observed data. Knowledge of the relationship between the molarity and the activity of the denaturant in aqueous solution is essential when employing this approach. Empirical equations relating molarity and activity are available for both urea and GdnHCl (12,13). As with Tanford's model this method of analysis does not lend itself to the design of this experiment. The reasons for this will also be made apparent later in the dissertation.

The simplest method and only one applicable to this investigation is the linear extrapolation procedure. For this method it is assumed that the observed linear dependence of the ΔG_D values on the denaturant concentration continues to zero denaturant concentration. That is

$$\Delta G_D = \Delta G_D^{H_2O} + m\,[\text{denaturant}] \qquad (9)$$

where m is a measure of the effectiveness of the denaturant. The denaturant concentration at the point where ΔG_D is equal to zero is referred to as the midpoint of transition.

The other two methods both predict that the function relating the ΔG_D values and denaturant concentration will deviate from linearity. The original arguments favoring this third method were that the observable portion of these

functions do not give any indication of departing from linearity and the estimates of $\Delta G_D^{H_2O}$ obtained separately using urea, GdnHCl and guanidinium thiocyanate data agree quite well with each other when using this method but not when using either of the other two (7,14). A theoretical basis and additional experimental evidence have both been presented lending further credence to this particular method (15,16).

Both the value of the slope and the value of the midpoint of transition are useful in comparing the relative effectiveness of various denaturants. However, such a comparison is only meaningful when the same transition is involved, that is, only denaturants producing the same denatured state should be compared in this manner. Also, only the extrapolated values of ΔG_D that are obtained when the denaturation is both complete and reversible can be considered as estimates of the stability of the protein. The extrapolated values of ΔG_D obtained when partial denaturants such as $NaClO_4$ or LiBr are used are only indicative of the protein's ability to withstand that particular form of denaturation and therefore should not be assumed to be indicative of or in any way be compared to the Gibbs free energy of stability of the protein. Thus it is difficult to directly compare the effects of partial denaturants to one another or to other denaturants such as urea and GdnHCl.

In the approach presented here the problem of the

production of different end-products of denaturation is avoided. The concentration of the denaturant is kept to a minimum thereby preventing any detectable production of these unwanted end-products. The actual denaturing is accomplished by adding increasingly more concentrated aliquots of GdnHCl and treating the results as described above. In this way regardless of the denaturant being investigated the same end-product, that is, the fully unfolded state, is obtained in each case. The only difference is that the extrapolated value of ΔG_D predicted at zero GdnHCl concentration is no longer the $\Delta G_D^{H_2O}$ value. Instead, the Gibbs free energy change that will be obtained, denoted as ΔG_D^{salt}, represents the change in the free energy of stability for that protein that arises from the addition of a fixed low concentration of that particular denaturant. By repeating this procedure using several different concentrations of the denaturant the relationship between the effect of this denaturant on the true stability of a protein and the concentration of denaturant can be determined. With this procedure the effects of many different denaturants can be directly compared.

This procedure is very similar to one used by von Hippel and Wong in their attempt to quantitatively compare the effects of neutral salts on the stability of proteins (17,19). The only difference is that they used heat instead of GdnHCl to carry out the denaturation. In retrospect their choice of denaturant was inappropriate and as a result

led to several improper conclusions and cast unwarranted doubt on some otherwise sound conclusions.

Most other studies of this nature have been restricted to the comparison of denaturants that are known to fully unfold the protein used in the study (20-24). The only other known unrestricted approach to this problem is the one proposed by Pace and Marshall (25). They virtually performed the reverse of the procedure reported here. The concentration of the denaturant under investigation was varied while the other denaturant involved, urea in their case, was held constant at a concentration just high enough to initiate the complete unfolding of the protein used. Although their method allowed for a direct quantitative comparison of the effectiveness of the various denaturants the criterion upon which the comparison was based was not as immediately relevant as that used in this study. It also did not lend itself to an analysis of the effectiveness of individual ions and their additivity as do the results obtained from this study.

Ahmad has published a preliminary study using the method outlined in this dissertation (26). Based on the results obtained using LiBr, NaBr and LiCl he concluded that the midpoint of transition of the GdnHCl induced unfolding was linearly dependent upon the concentration of the salt present during denaturation. Also, he concluded that these effects were additive. That is, the magnitude of the shift in the midpoint of transition arising from the addition of a

fixed concentration of LiBr could successfully be predicted from the individual shifts in the midpoint of transition arising separately from the addition of NaBr and LiCl. These conclusions were as expected.

Ahmad also concluded that the presence of salts altered the effectiveness of the denaturing capability of GdnHCl and that this phenomenon was concentration dependent and unrelated to specific binding. He also concluded that the free energy of stability of ribonuclease A (RNase A) was unaffected by the presence of pre-transition region concentrations of denaturants such as LiBr and NaBr. Both these conclusions were rather startling and deserving of further scrutiny.

It is the expectation of this investigation to prove that the presence of pre-transition region concentrations of denaturants does affect the free energy of stability of proteins and that the presence of these denaturants does not alter the effectiveness of GdnHCl to denature proteins unless they specifically bind to either the GdnHCl or the native state of the protein.

II. MATERIALS AND METHODS

RNase A (bovine pancreas) phosphate free was obtained from Worthington Diagnostic Systems. Ultrapure GdnHCl was purchased from Schwarz/Mann. Cacodylic acid was obtained from Sigma. The salts used as denaturants were obtained from the following sources: Fisher Scientific Company, KCl, RbCl, CsCl, NaH_2PO_4, $(NH_4)_2SO_4$, $NaC_2H_3O_2$ (NaAc) and $NH_4C_2H_3O_2$ (NH_4Ac). J. T. Baker Chemical Company, LiCl, NaCl, NaBr, Na_2SO_4, Na_2HPO_4, Li_2SO_4, NH_4Cl and GdnHSCN. Matheson Coleman & Bell, $NaClO_4$, $LiClO_4$ and NaSCN. Aldrich, LiBr. ICN K&K Laboratories, LiSCN. These and other analytical grade chemicals were used without further purification with the exception of the LiSCN and GdnHSCN.

The LiSCN was no longer anhydrous and contained a yellow colored impurity. The following procedure was used to remove this impurity: Roughly 20 grams of impure LiSCN was dissolved in about 50 mL of deionized water. Approximately 4 grams of activated charcoal was added. The mixture was then gently heated and stirred for one hour. The bulk of the charcoal was removed by filtration under suction using Whatman 42 filter paper. The remaining charcoal was removed by filtration using a 0.45μ Millipore filter. This procedure resulted in a clear colorless

solution.

The GdnHSCN was purified using the following method: 500 grams of GdnHSCN was dissolved in ethanol at 55°C. The solution was filtered and the GdnHSCN was precipitated from the solution by the addition of hexane. The mixture was cooled to about -20°C and the GdnHSCN was filtered off. The sample obtained was redissolved in near-boiling methanol, cooled in a Dry Ice-acetone bath for several hours and filtered (27).

In most cases the buffered stock salt solutions were prepared as follows: An appropriate amount of the salt was precisely weighed and dissolved in deionized water. Enough NaCl and cacodylic acid were added to the solution to yield final concentrations of 0.1 M and 0.013 M, respectively. The pH of the solution was then adjusted to 7.0 using concentrated aqueous NaOH. This solution was then quantitatively transferred to a volumetric flask and the volume adjusted accordingly. The final solution was then filtered through a Millipore filter. All pH measurements were made using a Radiometer type TTT1C pH meter.

Deviations from the above procedure were as follows: HCl, rather than NaOH, was required to adjust the pH of the NaAc and NH_4Ac stock solutions.

The stock KCl solution was made without the addition of NaCl.

The 1.0 M stock phosphate solution was prepared by titrating a 1.0 M Na_2HPO_4, 0.013 M cacodylate, 0.1 M

NaCl solution with a 1.0 M NaH_2PO_4, 0.013 M cacodylate, 0.1 M NaCl solution to obtain a solution of pH 7.0.

The LiSCN stock solution was prepared by adding 0.0449 grams of cacodylic acid and 0.146 grams of NaCl to 23 mL of the filtered aqueous LiSCN, adjusting the pH to 7.0 using NaOH and adjusting the volume to 25.00 mL with water.

The concentrations of the stock salt solutions of RbCl, $NaClO_4$ and $LiClO_4$, as determined by weight, were assumed to be correct. For all other denaturants the concentrations as determined by weight were verified by measuring refractive indices. Refractive index measurements were made using a Spencer 1070 refractometer.

The concentrations of the GdnHCl stock solutions were verified using the refractive index data reported by Nozaki (28).

The concentration of the stock GdnHSCN solution was verified using the equation

$$n_D^{25} = 1.3327 + 0.02475\ [\text{GdnHSCN}] \qquad (10)$$

where n_D^{25} is the refractive index at 25°C and [GdnHSCN] is the molar concentration of the GdnHSCN (29).

The refractive index data found in the International Critical Tables were used when dealing with the stock solutions of Li_2SO_4, LiBr, LiSCN and NH_4Ac (30). For the rest of the salts the refractive index data were taken from the CRC Handbook (31). In all cases the presence of

buffer was taken into account.

As previously mentioned, the stock buffered LiSCN solution was not prepared from the anhydrous salt. Because of this the concentration of the stock solution could not be determined by weight. The concentration of the SCN^- ion in the purified aqueous LiSCN was determined by the Volhard argentometric precipitation method. The standard $AgNO_3$ solution was prepared by transferring 4.2555 grams of $AgNO_3$ to a 250 mL volumetric flask and diluting to the mark with deionized water. The titration was carried out with a vigorous swirling of the flask. The titration procedure was repeated several times and the results were averaged.

The concentration of the LiSCN solution as determined by the titration technique was found to be less than that determined by refractive index measurements. This discrepancy was attributed to the presence of non-titratable impurities which contributed to the observed refractive index. To verify that the LiSCN concentration as determined by the titration technique was correct, or if titratable impurities were also present, the concentration of the Li^+ ion in the aqueous LiSCN solution was determined by use of an Evans flame photometer.

Aqueous LiCl solutions ranging from 0 ppm to 24.9 ppm, in terms of grams Li^+ per mL solution, were used as standards. The latter standard was prepared by dissolving 0.304 grams of LiCl in water and diluting the solution to

2000.00 mL. All subsequent solutions were prepared by dilution of this standard. A calibration curve was prepared by plotting the galvanometer reading obtained for each solution against the Li^+ concentration of the standard. Exactly 25.00 mL of the previously diluted aqueous LiSCN was further diluted to 1000.00 mL. The Li^+ concentration of this sample was then determined and used to calculate the concentration of the buffered LiSCN solution.

The cacodylate and NaCl concentrations as determined by weight were never verified by other methods. The experimental results suggested that even 10-fold deviations in either concentration had little to no effect on the stability of the protein. It was therefore considered inconsequential to know the precise concentration of either one.

Separate buffered RNase A stock solutions were prepared for the CD experiments and the difference spectroscopy experiments. For the CD experiments 1.8 mg/mL to 2.2 mg/mL buffered RNase A stock solutions were prepared and for the difference spectroscopy experiments 10 mg/mL buffered stock solutions were prepared. In both cases an appropriate amount of protein was dissolved in 10 to 15 mL of deionized water and dialysed against a total volume of 2L of 0.1 M NaCl for 24 hours. The protein solution was then dialysed against a total volume of 1L of buffer again for 24 hours. Hereafter, unless otherwise specified, "buffer" refers to a solution consisting of 0.1 M NaCl and 0.013 M cacodylate

with a pH of 7.0. The dialysis tubing used was permeable to compounds under a molecular weight of 12,000.

The protein solution was then filtered through a Millipore filter. The final protein concentration was determined spectrophotometrically using a value of 9800 for the molar extinction coefficient of RNase A at 277.5 nm (1).

To ensure that each sample being analysed had the same protein concentration as the control each set of samples was prepared as follows: For the CD experiments the control sample was prepared by delivering 0.1 mL of buffered stock RNase A solution to a 1 mL volumetric flask and the volume was then adjusted to 1.00 mL using buffer. To a series of 1 mL flasks 0.1 mL of protein was similarly delivered using the same lambda pipet. To each of these flasks a specific fixed volume of a buffered stock solution of the salt in question and varying amounts of buffered stock GdnHCl solution were added to the flasks. Buffer was used to adjust the volume of each sample to exactly 1.00 mL.

Protein was added first followed by slightly less than the required amount of buffer. The final volume was adjusted with buffer after the stock salt solution and stock GdnHCl solution were added. The stock solutions were mixed in this order to prevent the protein from coming in direct contact with either a too concentrated GdnHCl solution or a too concentrated salt solution. The samples were gently shaken and allowed to sit overnight at room temperature to reach equilibrium. The samples were then incubated in a

25°C water bath for at least one hour prior to the recording of the spectra.

The samples used in the difference spectroscopy experiments were prepared in the same way except that a more concentrated stock protein solution had to be used. For each sample a reference solution containing an identical concentration of salt and GdnHCl but lacking the 0.1 mL of stock protein solution was required.

Direct contact between the stock protein solution and each stock salt solution was avoided to prevent the production of intermediates. The presence of large amounts of intermediates during the measuring of the ellipticity or absorbance values would lead to erroneous results. Direct contact between the stock protein solution and the stock GdnHCl solution was avoided to prevent the production of excessive amounts of the fully denatured protein.

Production of intermediates or excessive amounts of the fully denatured state of the protein would not affect the experiment if the denaturation process is fully reversible within the imposed time constraints, both in terms of intermediate production and complete unfolding. Reversibility is essential if the ΔG_D calculations are to be meaningful. Ahmad has previously shown the reversibilty in the cases of LiCl, LiBr and NaBr mixed with GdnHCl (26). In all other cases the reversibility has not yet been investigated and therefore had to be assumed.

This order of mixing was employed for kinetic

considerations only. As kinetic studies have not yet been applied to these mixed denaturants the rates of the forward and reverse reactions are not known. Preliminary studies suggest that an overnight incubation time is more than sufficient to allow the process to reach equilibrium via the forward reaction. It is reasonable to believe that an equilibrium could be reached via the reverse reaction given this length of time. However, as no attempt was made to confirm this belief the described order of mixing was used throughout the experiment as a precautionary measure and to ensure complete reproduciblity.

The CD measurements were made using a Jasco 500A spectropolarimeter. A thermostated quartz cell of 0.1 cm path length was used and maintained at a temperature of 25.0^{o}C with the aid of a circulating water pump. Initially the instrument was periodically calibrated with 0.05% (W/V) androsterone in dioxane using the procedure recommended by the manufacturer. The instrument was later calibrated with d-camphor sulfonic acid following the procedure of Chen and Yang (32).

Full spectra were recorded from 240 nm to 200 nm using a buffer spectrum as the baseline. However, full spectra were not recorded for all samples. Only the value of the ellipticity at 220 nm was required for the calculations. To minimize the uncertainty associated with this measurement a time averaged value was obtained by running a spectrum of each sample over a period of 3 to 5 minutes with the

wavelength fixed at 220 nm. A time averaged value of the ellipticity of the buffer measured at 220 nm was used as a reference.

The same cell was used when recording the spectrum of each sample. The volume of the sample required to fill the cell was less than 0.1 mL. Because such small volumes were used any trace of the previous sample still left behind in the cell could significantly alter the observed ellipticity. To avoid such contamination the cell was always thoroughly rinsed and dried before each sample was transferred to the cell. Rinsing the cell with a small volume of the next sample to be used was considered an inefficient means of removing all traces of the previous sample because of the narrowness of the cell and the small opening through which the samples had to be transferred.

Because of instrumental instability the observed value of the ellipticity of the buffer would occasionally drift over the course of the day. To prevent this from being a problem a buffer spectrum could have been run prior to running the spectrum of each sample. As this would have meant doubling the number of times the cell had to be washed and dried a different method for dealing with the drifting was used. The time averaged buffer spectrum was recorded once at the start of the day. Immediately after this a spectrum was recorded with the spectropolarimeter chamber empty. This "air" spectrum was then used as a reference throughout the course of the day. Prior to the running of

the spectrum of each sample the ellipticity of the empty chamber was measured. If drifting was detected an appropriate baseline adjustment was made to correct for it.

CD data were reduced to the concentration-independent parameter $[\theta]$, the mean molar residue ellipticity, defined as:

$$[\theta] = \frac{M\,\theta}{10\,c\,l} \qquad (11)$$

where θ is the observed ellipticity in millidegrees at a fixed wavelength, M is the mean molar residue weight of RNase A (M = 110), c is the protein concentration in milligrams/cm^3 and l is the path length of the cell in centimeters. All CD results are presented in the units of degrees cm^2 $dmol^{-1}$.

The denaturation of proteins is accompanied by CD changes, indicating the loss of helical and β-structure and a corresponding increase in the random coil content. A CD spectrum of a protein represents the sum of the spectra of its components. Studies with model compounds have shown that the ellipticity at 220 nm for both helical structure and β-structure is negative. The contribution of random coil at this wavelength is almost zero and positive. A decrease in the magnitude of the value of $-[\theta]$ calculated at this wavelength results when denaturation takes place. By plotting the ellipticity (or $-[\theta]$ value) observed at this wavelength as a function of the denaturant concentration the

denaturation process can easily be followed.

Some of the salts used in this study were opaque at 220 nm and therefore this technique could not always be used to follow the induced unfolding. In these cases difference spectroscopy was employed.

RNase A has 6 tyrosines. In the native state three of these tyrosines are buried and the other three are exposed. Because of the strong absorption of tyrosine the ultraviolet absorption spectrum of RNase A in an aqueous solution has a maximum at 277.5 nm. Upon denaturation the three buried tyrosines are removed from their non-polar environment and placed in the aqueous polar environment. This causes a blue shift in the protein spectrum. This shift is most noticeable at 287 nm.

Difference spectral measurements were made in a Cary 219 spectrophotometer using matched tandem cells. Thermostated cell holders were used to maintain the temperature of the cells at 25.0°C. The sample cell contained buffer in one compartment and protein plus denaturant in the other. The reference cell contained denaturant in one compartment and native protein in the other. The protein and denaturant concentrations were identical in both cells. The difference spectral measurements, ΔAbs, obtained at 287 nm were converted to difference molar extinction coefficients, $\Delta\varepsilon_{287}$. The difference molar extinction coefficient is defined as the difference between the molar extinction coefficient of the

native protein at 287 nm and the molar extinction coefficient of the protein in denaturant at 287 nm. By plotting the observed ΔAbs value or $-\Delta\varepsilon_{287}$ value as a function of the denaturant concentration the denaturation process can easily be followed.

Typical plots, as obtained by both CD measurements and difference spectroscopy, are presented in the next section.

III. RESULTS

The results will be presented in several sub-sections each dealing with one aspect of the analysis. To ensure that the proposed method receives proper scrutiny no prior knowledge of the stabilizing or destabilizing effects of the salts and no prior knowledge of the binding will be utilized in the analysis.

A. PRELIMINARY RESULTS

In this section the data will be converted from their raw form into transition curves that will eventually yield preliminary midpoints of transition and changes in the Gibbs free energies of stability of RNase A.

The $[\theta]_{220}$ value obtained for native RNase A was initially -8350 deg cm^2 $dmol^{-1}$. Because of a faulty crystal in the modulator of the spectropolarimeter this value, which should be a constant, gradually changed over a period of six months. Once the crystal was replaced with a properly functioning unit the value of -8350 deg cm^2 $dmol^{-1}$ was consistently obtained for native RNase A. These values were obtained when the spectropolarimeter was calibrated with androsterone. When the spectropolarimeter was calibrated with d-camphor sulfonic acid a $[\theta]_{220}$ value of -8940 deg cm^2 $dmol^{-1}$ was consistently obtained for native RNase A. This latter value is more in keeping with

the value of -9060 obtained by Chen et al (33).

All values obtained when the spectropolarimeter was calibrated with d-camphor sulfonic acid were found to be roughly 1.1 times larger than those obtained when the spectropolarimeter was calibrated with androsterone. As the difference was found to be uniform and as only the relative changes and not absolute changes were required in the analysis the method of calibration used had no effect on the results.

On the other hand the problem with the crystal was much more serious. Most of the data obtained while the first crystal was in use had to be discarded. As can be seen in later figures, the data that were salvaged, although obviously of poorer quality, were considered sufficiently reproducible and yielded enough information to warrant reporting.

The unfolding transition of RNase A induced by the addition of GdnHCl in the presence of 0.99 M NaBr was followed by observing the changes in the $\Delta\varepsilon_{287}$ value. Buffer and 0.1 M NaCl were present in these and most other solutions. Their presence should always be assumed unless specifically stated otherwise. The data obtained for this experiment are typical. Figure 1 presents some difference spectra of RNase A obtained under these conditions. To maintain clarity only a few selected spectra have been presented here. The spectra shown clearly demonstrate their concentration dependence.

FIGURE 1

The difference spectra of RNase A produced upon denaturation by 0.0 M (1), 1.04 M (2), 1.74 M (3), 2.08 M (4), 2.26 M (5), and 2.78 M (6) GdnHCl in the presence of 0.99 M NaBr.

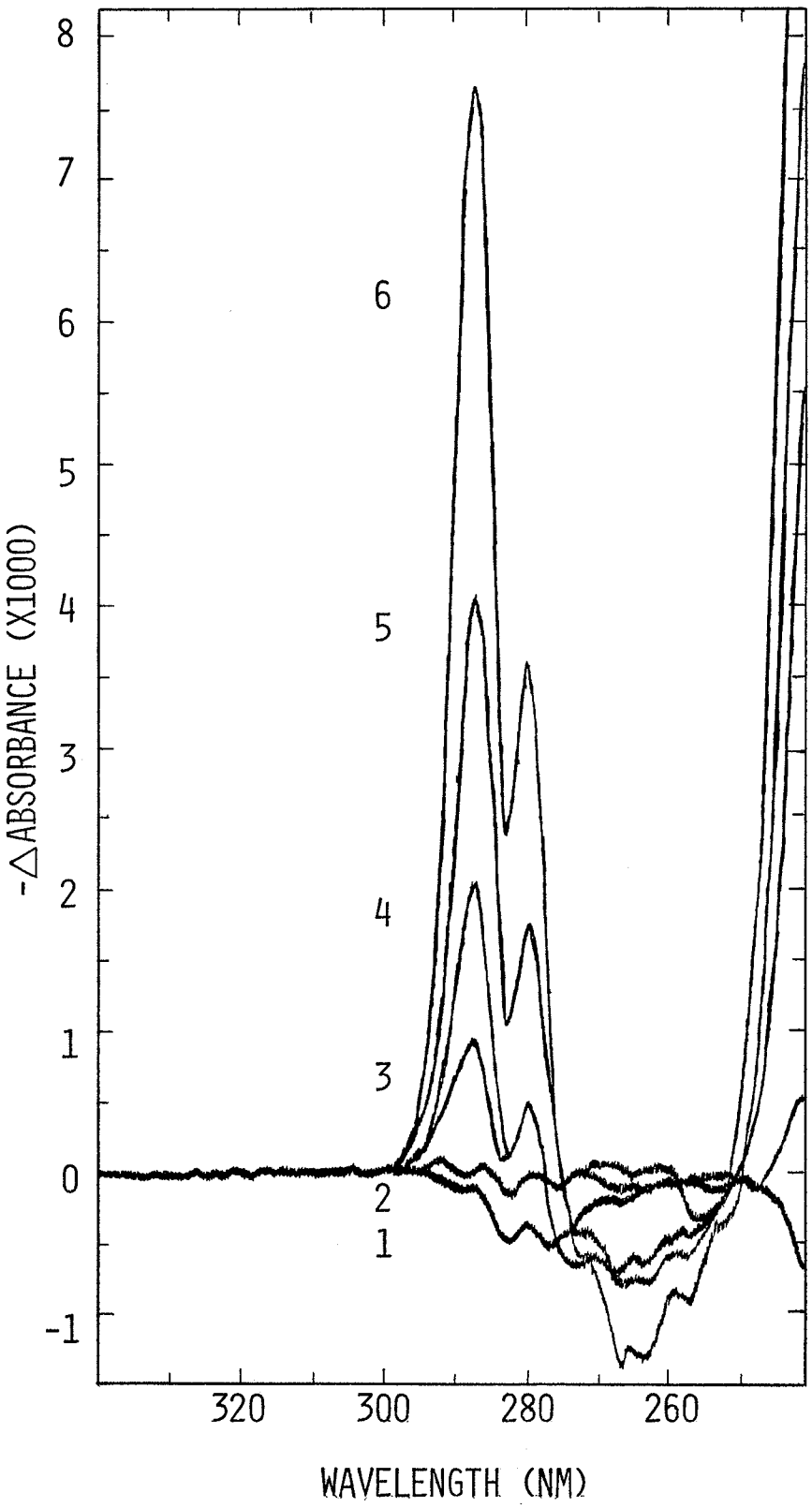

8
7
6
5
4
3
2
1
0
-1
-△ABSORBANCE (X1000)
6
5
4
3
2
1
320
300
280
260
WAVELENGTH (NM)

FIGURE 2

Changes in $\Delta\varepsilon_{287}$ of RNase A on denaturation by GdnHCl in the presence of 0.99 M NaBr.

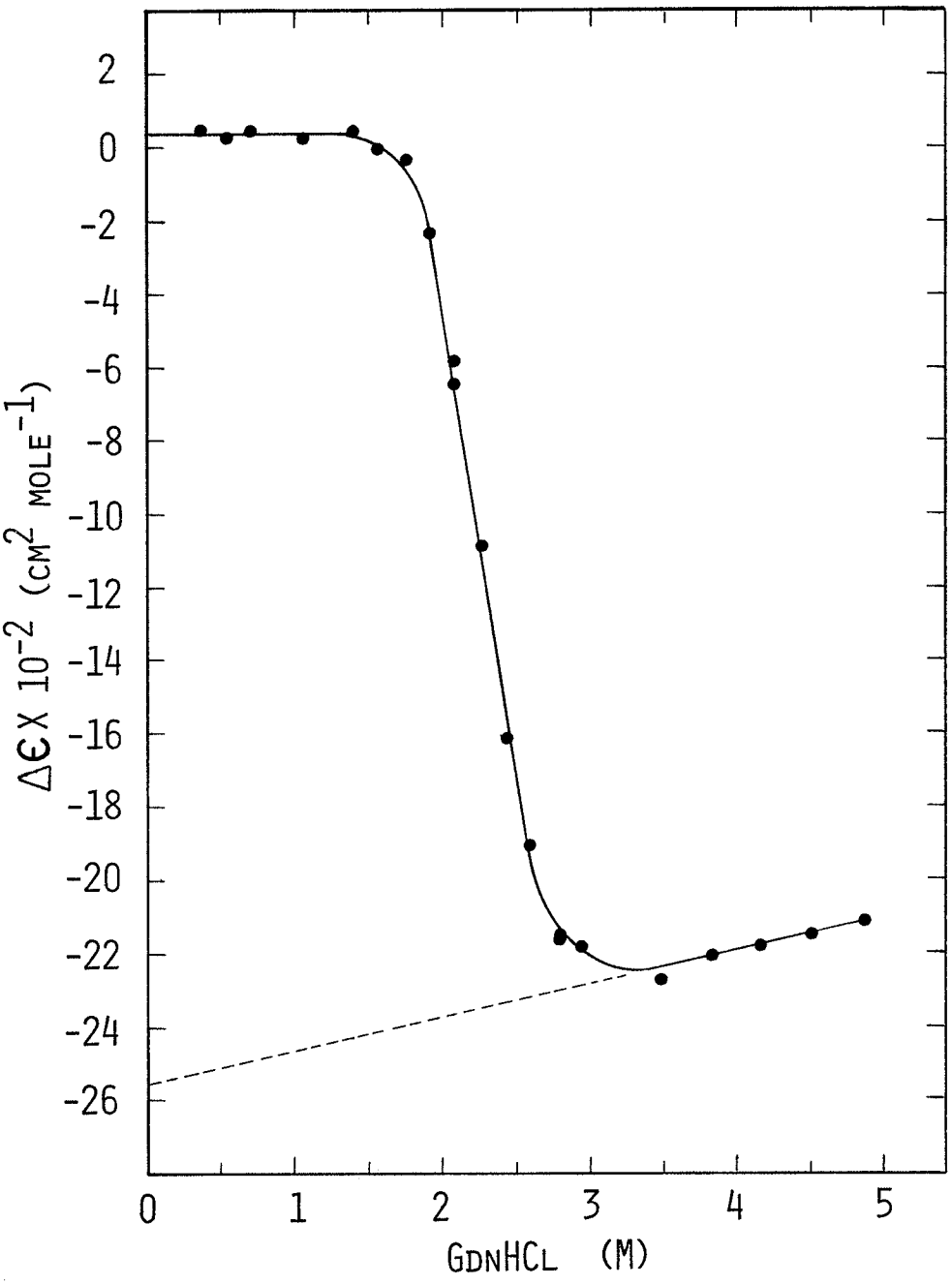

2
0
-2
-4
-6
-8
-10
-12
-14
-16
-18
-20
-22
-24
-26
ΔεX 10⁻² (cm² mole⁻¹)
0
1
2
3
4
5
GdnHCl (M)

Figure 2 presents the transition curve obtained by plotting the $\Delta\varepsilon_{287}$ values against the concentration of the GdnHCl used to induce the unfolding. This profile has all the general characteristics described previously. There is a small concentration region where the change in absorption is most pronounced. The changes below this region are minor in comparison and the changes above this region are so minor as to appear negligible.

The denaturation curves obtained by plotting the change in ellipticity against the concentration of GdnHCl used to induce the unfolding have the same general appearance. The unfolding transition of RNase A induced by the addition of GdnHCl in the presence of 0.35 M $NaClO_4$ was followed by observing the changes in the ellipticity. Figure 3 presents some of the CD spectra obtained. Again, only a few examples are shown. Figure 4 presents the transition curve obtained by plotting the $[\theta]_{220}$ values against the GdnHCl concentration. It too has the general appearance of all transition curves. The only major difference between the two transition curves presented is that the former possesses a positive post-transition region solvent effect while the latter possesses a negative post-transition region solvent effect.

For the quantitative analysis of the denaturation data the solvent effect functions were defined and removed. The equations used to define the solvent effect were derived with the assumption that the solvent effects were linearly

FIGURE 3

The CD spectra of RNase A produced upon denaturation by 0.74 M (1), 2.23 M (2), 2.60 M (3), 2.97 M (4), and 5.20 M (5) GdnHCl in the presence of 0.35 M $NaClO_4$.

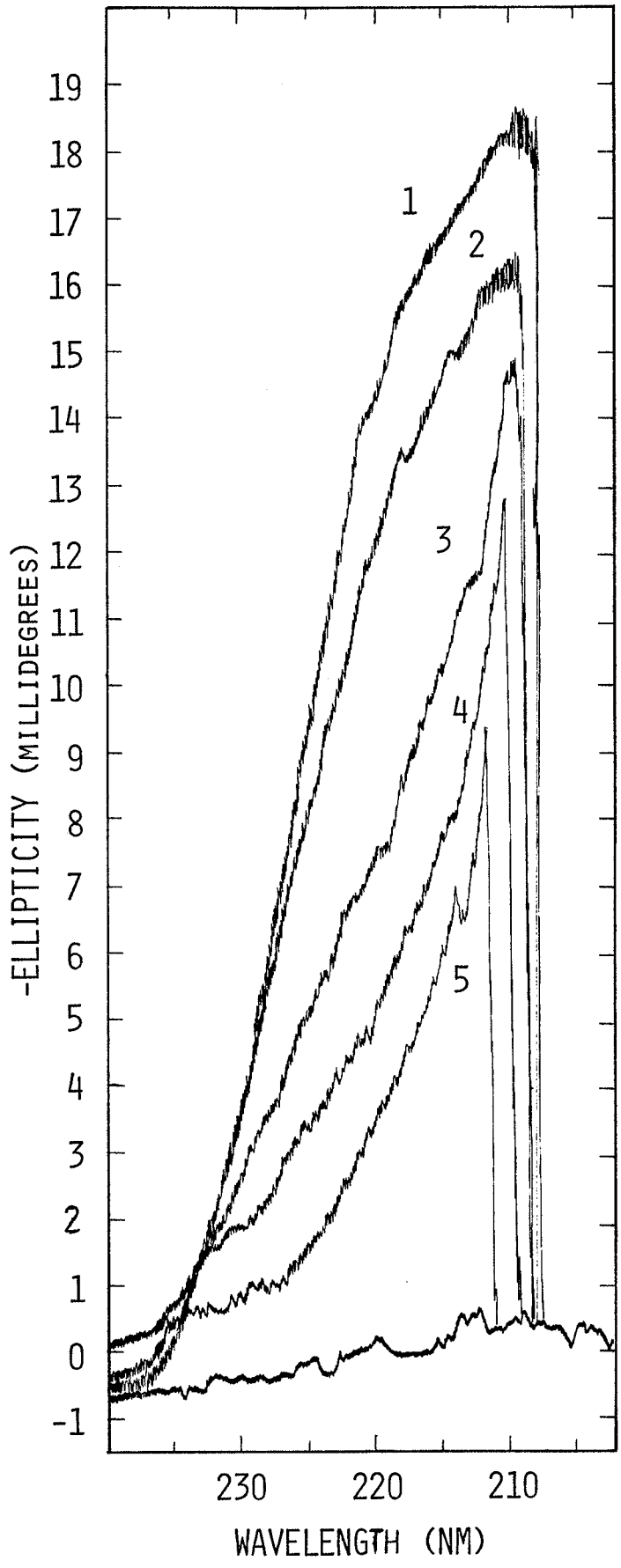
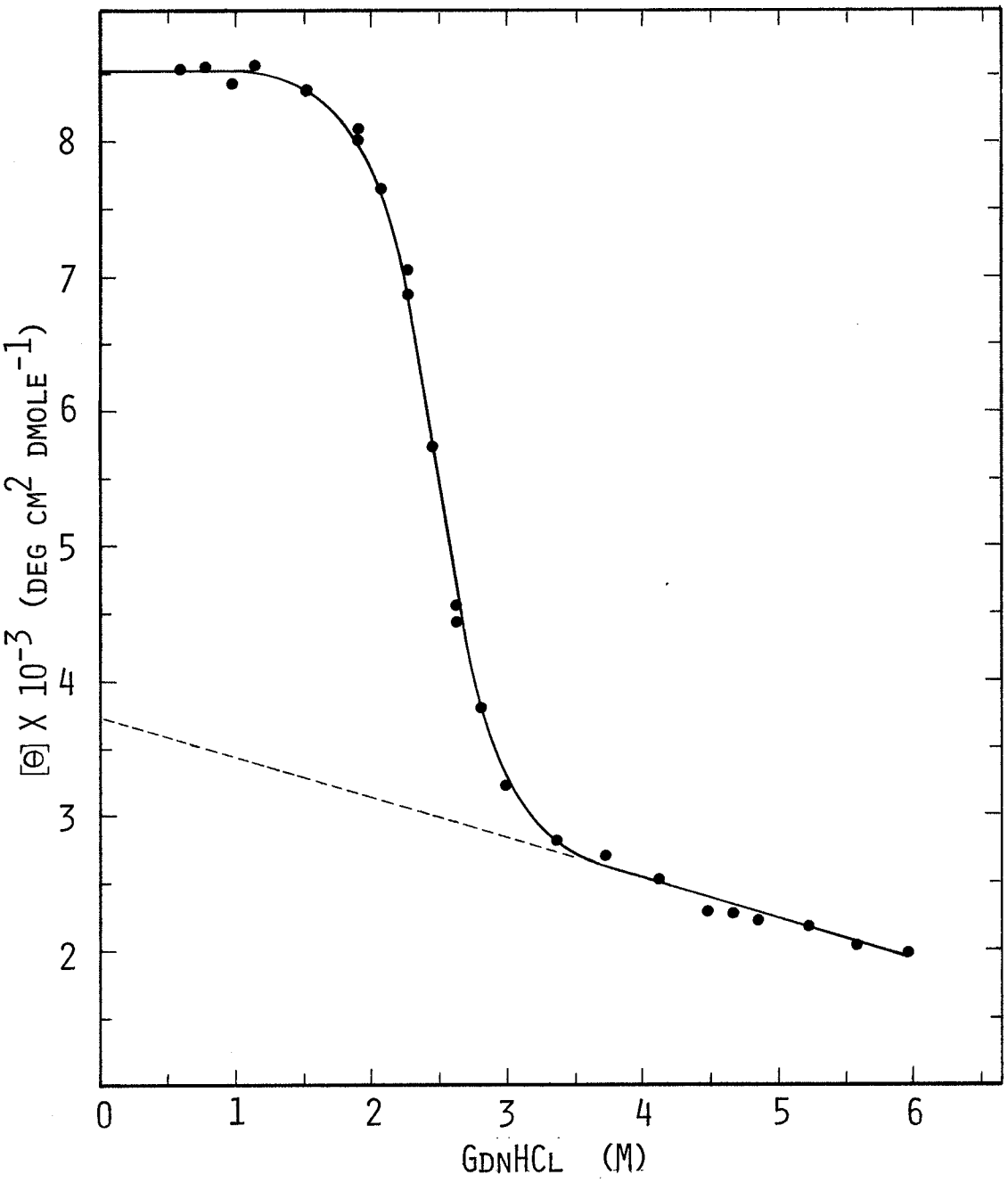

19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
-1
-ELLIPTICITY (MILLIDEGREES)
1
2
3
4
5
230
220
210
WAVELENGTH (NM)

FIGURE 4

Changes in ellipticity of RNase A on denaturation by GdnHCl in the presence of 0.35 M $NaClO_4$.


[Θ] X 10^-3 (DEG CM^2 DMOLE^-1)
8
7
6
5
4
3
2
0
1
2
3
4
5
6
GDNHCL (M)

dependent upon the GdnHCl concentration. The points clearly outside the transition region were used in a least mean squares analysis to define the post-transition region solvent effects in both examples. For the 0.99 M NaBr transition the equation used to define the post-transition solvent effect, Y_D, was

$$Y_D = -2537(\pm 28) + 87(\pm 7)\ [\text{GdnHCl}] \qquad (12)$$

This equation predicts the value of $\Delta\varepsilon_{287}$, Y_D, that would be observed at any concentration of GdnHCl, [GdnHCl], if denatured RNase A could be investigated in the absence of native RNase A.

Similarly, the equation

$$Y_D = 3300(\pm 90) - 222(\pm 17)\ [\text{GdnHCl}] \qquad (13)$$

defines the solvent effect obtained from a least mean squares treatment of the post-transition region data presented for $NaClO_4$.

In both cases the pre-transition region data indicated that the solvent effect for the native protein was negligible. To obtain the pre-transition region solvent effect the points clearly belonging to this region were averaged. Thus the pre-transition region solvent effects, Y_N, as determined for the 0.99 M NaBr and the 0.35 M $NaClO_4$ data were simply

FIGURE 5

Normalized transition of the denaturation of RNase A by GdnHCl in the presence of 0.99 M NaBr.

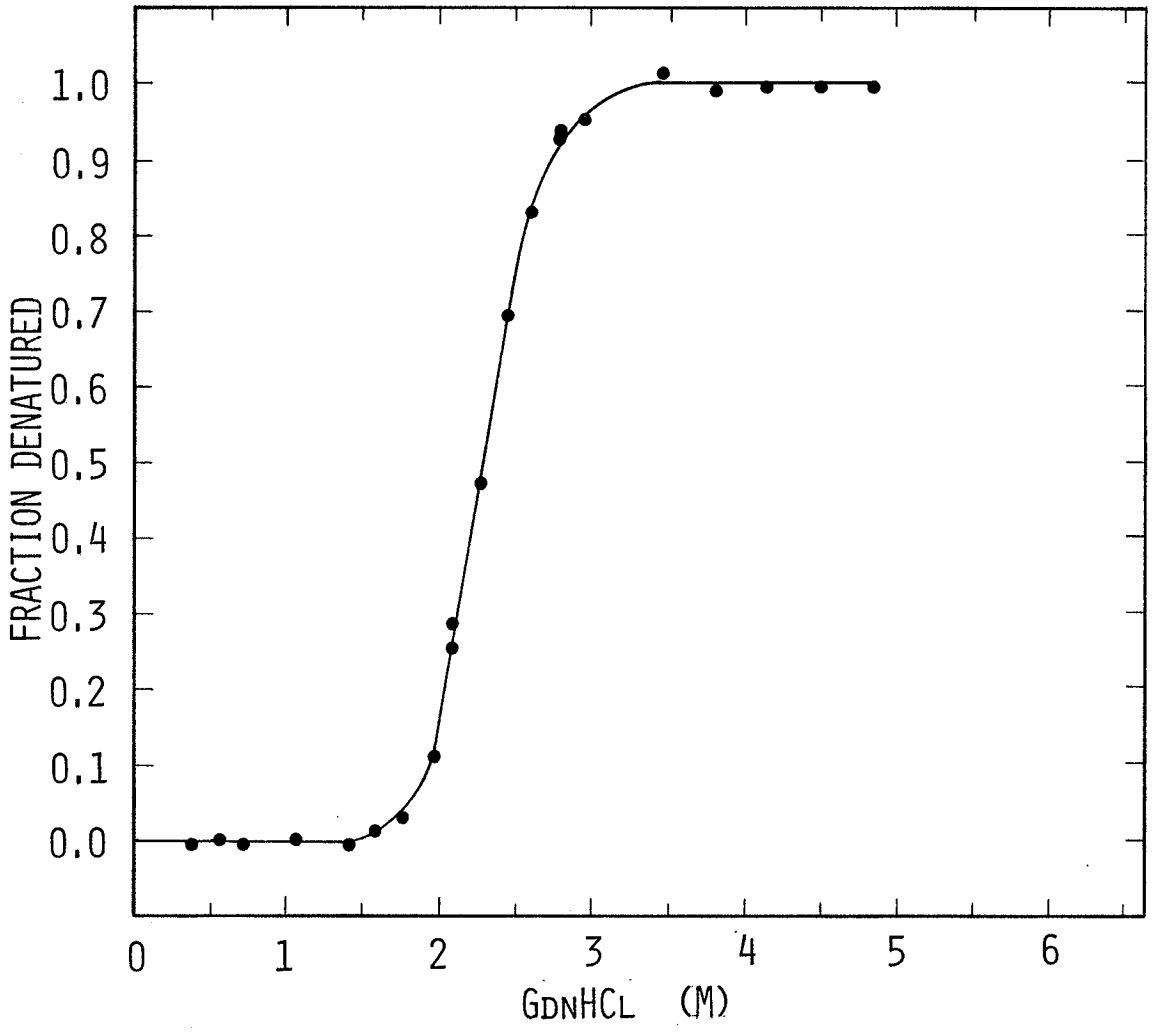
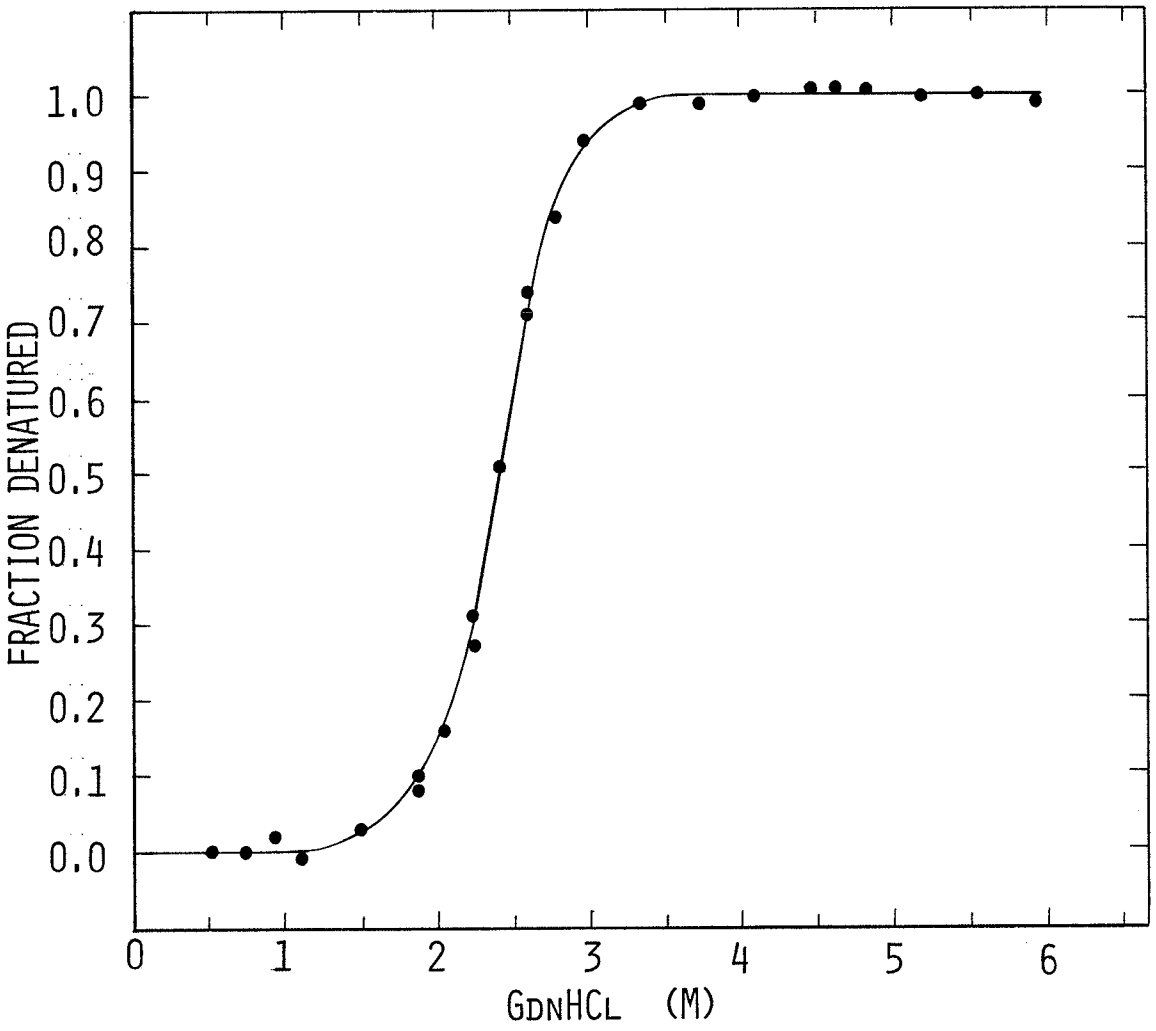

FRACTION DENATURED
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
1
2
3
4
5
6
GDNHCL (M)

FIGURE 6

Normalized transition of the denaturation of RNase A by GdnHCl in the presence of 0.35 M $NaClO_4$.


FRACTION DENATURED
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
1
2
3
4
5
6
GdnHCl (M)

$$Y_N = 44(\pm 5) \qquad (14)$$

and

$$Y_N = 8520(\pm 30) \qquad (15)$$

respectively.

With the solvent effect functions defined normalized curves can be produced. Figures 5 and 6 present the normalized transition curves of the 0.99 M NaBr and 0.35 M $NaClO_4$ data as obtained by plotting the f_D values against the concentrations at which they were calculated. The f_D values were calculated using Equation 4 as outlined in the introduction.

As briefly mentioned in the introduction, if the same unfolding process is observed by several different methods and the denaturation truly proceeds via a two-state mechanism then the resulting normalized curves will be coincident. The GdnHCl denaturation of RNase A was monitored both by observing the changes in the ellipticity and by difference spectroscopy. Figures 7 and 8 present the transition curves as obtained by both methods. The scattering of the points in the pre-transition region of Figure 8 is a result of the previously mentioned instrumental difficulty. This scattering made the task of characterizing the pre-transition region solvent effect difficult but not impossible. The normalized transition

FIGURE 7

Changes in $\Delta\varepsilon_{287}$ of RNase A on denaturation by GdnHCl in the presence of 0.10 M NaCl.

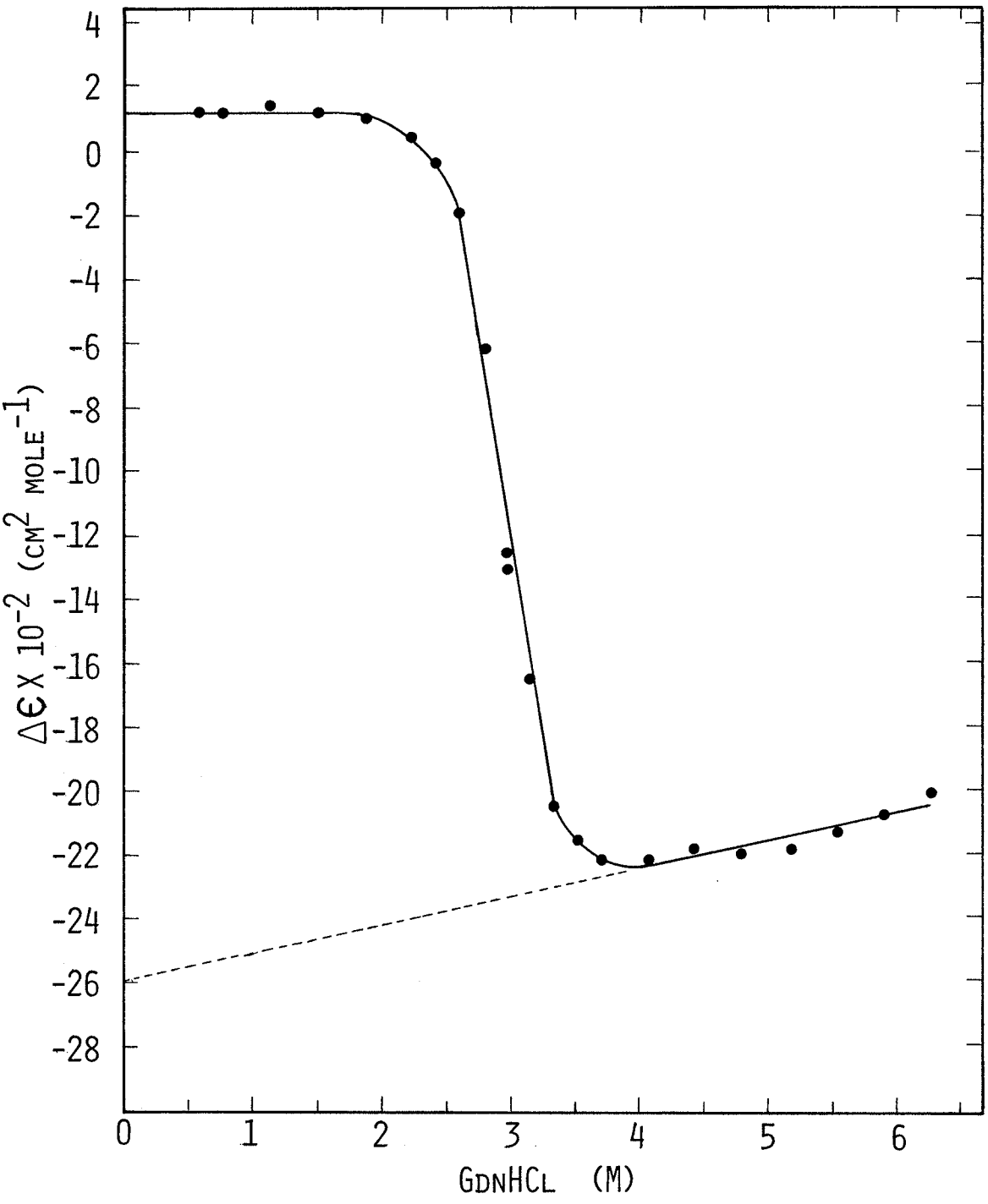

4
2
0
-2
-4
-6
-8
-10
-12
-14
-16
-18
-20
-22
-24
-26
-28
$\Delta\epsilon \times 10^{-2}$ (CM^2 MOLE^{-1})
0
1
2
3
4
5
6
GDNHCL (M)

FIGURE 8

Changes in ellipticity of RNase A on denaturation by GdnHCl in the presence of 0.10 M NaCl.

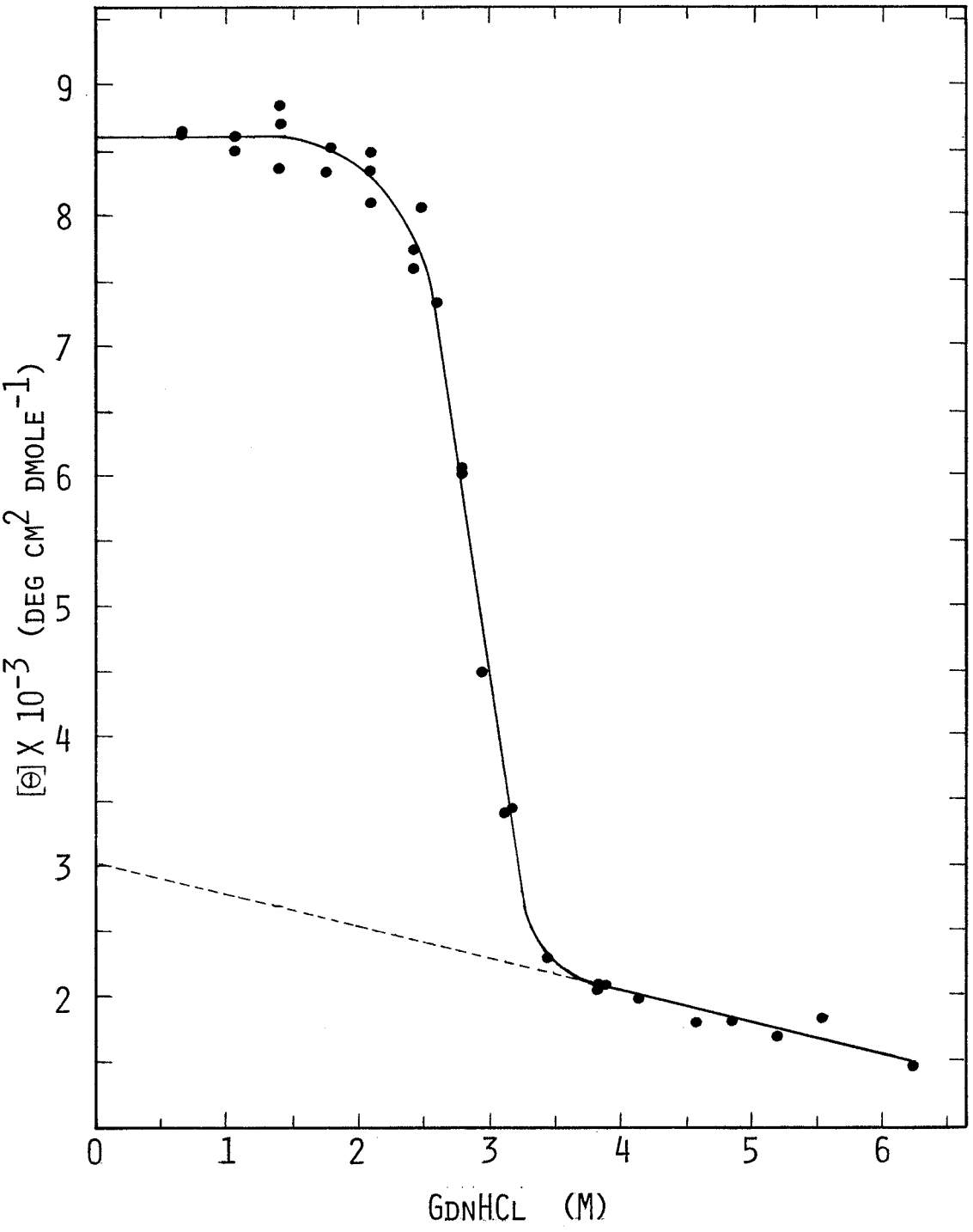
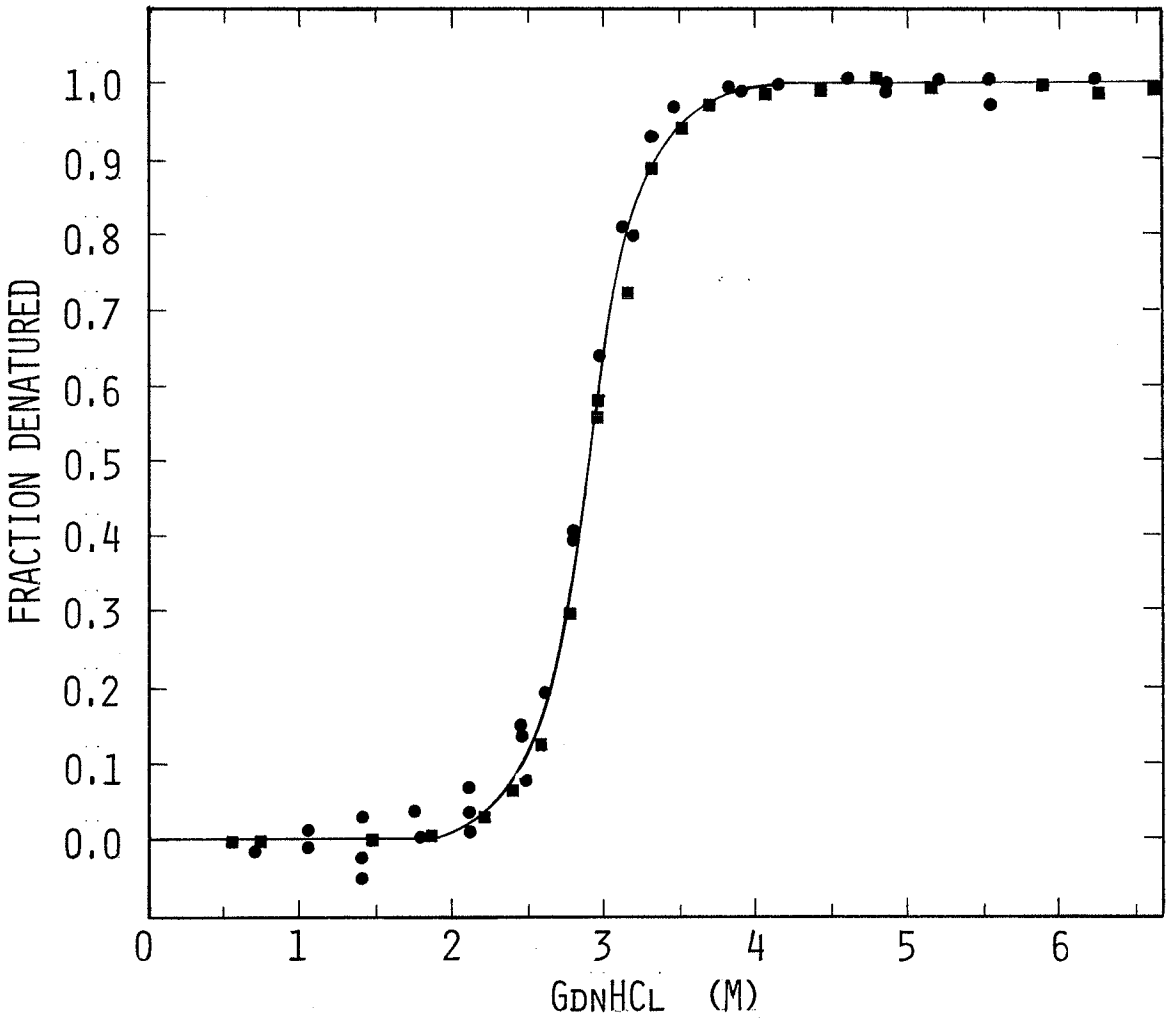

9
8
7
6
5
4
3
2
[Θ] X 10^-3 (DEG CM^2 DMOLE^-1)
0
1
2
3
4
5
6
GDNHCL (M)

FIGURE 9

Normalized transition of the denaturation of RNase A by GdnHCl in the presence of 0.10 M NaCl as determined using both CD results (•) and difference spectroscopy results (■).


FRACTION DENATURED
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
1
2
3
4
5
6
GdnHCl (M)

curves are plotted together in Figure 9. Their coincidence suggested that the characterization of the pre-transition solvent effect was successfully accomplished despite the scattering and that the denaturation does proceed via a two-state process. However, it should be restated that coincidence of the two curves is not conclusive proof that the denaturation proceeds via a two-state process.

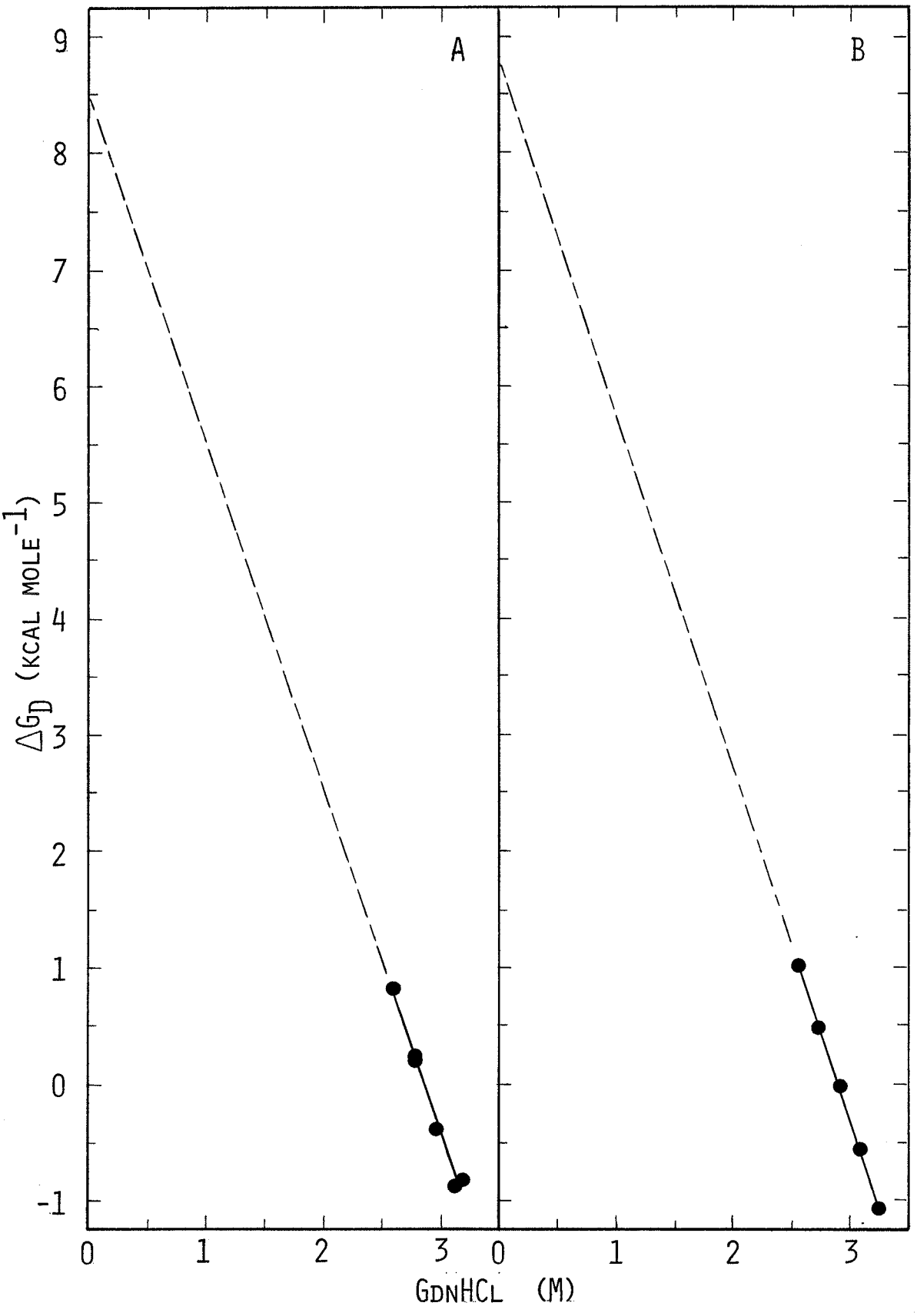
From the normalized transition curves the equilibrium constants and subsequently the Gibbs free energy changes were calculated, using Equations 5 and 6, for all points inside all the transition regions. In the introduction three methods were described for extrapolating these ΔG_D values back to a zero GdnHCl concentration. Of these three approaches only the linear extrapolation method was stated to be practical in light of the given information.

To employ Tanford's model the Gibbs free energy of transfer for each amino acid residue side chain and for the peptide linkages from each specified concentration of each salt used to numerous different concentrations of GdnHCl would be required. The amount of time and effort needed to gather this essential information is prohibitive. Similarly to use the binding method an equally prohibitive amount of time and effort would have to be spent gathering information regarding the concentration dependence of the activity of GdnHCl in various specified concentrations of all the salts investigated.

Figures 10A and B present the preliminary extrapolated

FIGURE 10

The variation of ΔG_D as a function of GdnHCl concentration in the presence of (A) 0.10 M NaCl and (B) 0.10 M KCl as determined using the CD results.


A
B
9
8
7
6
5
4
3
2
1
0
-1
0
1
2
3
0
1
2
3
ΔG_D (KCAL MOLE^{-1})
GDNHCL (M)

plots of the ΔG_D values, as a function of the GdnHCl concentration used to induce the unfolding in the presence of 0.10 M NaCl and 0.10 M KCl respectively. A least mean squares analysis was used to fit the data and obtain a value of ΔG_D^{salt}.

All the ΔG_D values obtained based on f_D values between 0.1 and 0.9 are shown in the figures. However, the least mean squares analysis was performed only on those ΔG_D values obtained based on f_D values between 0.12 and 0.88. This reduction of the previously discussed limits was deemed necessary in light of the observed scattering displayed in some of the data. Many of the unused points clearly could have been included in the least mean squares analysis. However, to maintain consistency the limits imposed on the data obtained for some salts were applied to all salts throughout the course of the experiment.

Table 1 presents a summary of the ΔG_D^{salt} values and slopes resulting from a least mean squares analysis of all the usable data. Also included in this table are the denaturant concentrations at the midpoint of each transition (mdpt). The uncertainty presented for this value was calculated based upon the uncertainty, $\sigma\Delta\bar{G}_D$, of the average ΔG_D value, $\Delta\bar{G}_D$.

$$\sigma\Delta\bar{G}_D = \left[\frac{\sum(\Delta G_{Di} - \Delta\hat{G}_{Di})^2}{n\,(n-1)}\right]^{1/2} \qquad (16)$$

where ΔG_{Di} represents the calculated ΔG_D value at a

TABLE 1

PRELIMINARY RESULTS CHARACTERIZING RNase A DENATURATION

Salt	[Salt]	ΔG_D^{salt}	Slope	midpoint
	M	kcal/mole	kcal/mole/M	M
NaCl*	0.1	8.6(±0.6)	-3.01(±0.19)	2.86(±0.01)
NaCl (UV)	0.1	9.2(±0.7)	-3.15(±0.24)	2.92(±0.01)
NaCl*	0.5 + 1.0	8.6(±0.7)	-3.00(±0.24)	2.89(±0.01)
KCl*	0.1	8.8(±0.1)	-3.04(±0.02)	2.90(±0.01)
CsCl*	0.49	8.9(±0.6)	-3.18(±0.22)	2.81(±0.01)
RbCl (UV)	0.50	8.4(±0.6)	-2.89(±0.19)	2.89(±0.01)
NH_4Cl	0.45	8.5(±0.4)	-3.02(±0.15)	2.83(±0.01)
NH_4Cl	0.90	9.0(±0.3)	-3.25(±0.11)	2.77(±0.01)
LiCl	0.50	7.4(±0.1)	-2.71(±0.05)	2.74(±0.01)
LiCl	0.99	8.0(±0.2)	-3.13(±0.06)	2.56(±0.01)
Phosphate*	0.10	8.0(±0.6)	-2.59(±0.18)	3.11(±0.01)
Phosphate*	0.20	9.5(±0.6)	-2.91(±0.18)	3.29(±0.01)
Na_2SO_4*	0.15	8.8(±0.5)	-2.83(±0.15)	3.12(±0.01)
Na_2SO_4*	0.30	8.6(±0.9)	-2.59(±0.28)	3.34(±0.02)
$(NH_4)_2SO_4$	0.15	9.0(±0.3)	-2.85(±0.08)	3.16(±0.01)
$(NH_4)_2SO_4$	0.30	9.8(±0.6)	-2.90(±0.18)	3.38(±0.01)
Li_2SO_4	0.20	8.9(±0.2)	-2.82(±0.05)	3.16(±0.01)
Li_2SO_4	0.40	9.5(±0.2)	-2.82(±0.05)	3.35(±0.01)
NaAc	0.25	8.3(±0.3)	-2.79(±0.09)	2.96(±0.01)
NaAc	0.50	9.4(±0.5)	-3.04(±0.16)	3.07(±0.01)
NH_4Ac	0.30	8.0(±0.8)	-2.63(±0.25)	3.02(±0.02)
NH_4Ac	0.60	9.2(±0.2)	-2.91(±0.07)	3.15(±0.01)

TABLE 1 (CONTINUED)

Salt	[Salt]	ΔG_D^{salt}	Slope	midpoint
	M	kcal/mole	kcal/mole/M	M
NaBr (UV)	0.50	8.7(±0.3)	-3.42(±0.10)	2.54(±0.01)
NaBr (UV)	0.99	6.7(±0.2)	-2.97(±0.10)	2.27(±0.01)
LiBr (UV)	0.76	6.8(±0.4)	-3.14(±0.20)	2.15(±0.01)
LiBr (UV)	1.52	5.1(±0.2)	-3.41(±0.10)	1.48(±0.01)
NaSCN (UV)	0.25	7.4(±0.2)	-3.11(±0.08)	2.39(±0.01)
NaSCN (UV)	0.49	5.6(±0.2)	-2.93(±0.08)	1.91(±0.01)
LiSCN (UV)	0.09	8.3(±0.4)	-3.05(±0.14)	2.72(±0.01)
LiSCN (UV)	0.34	5.9(±0.3)	-2.87(±0.14)	2.06(±0.01)
LiSCN (UV)	0.69	4.1(±0.3)	-3.08(±0.21)	1.34(±0.01)
$NaClO_4^*$	0.35	5.7(±0.3)	-2.41(±0.12)	2.39(±0.01)
$NaClO_4^*$	0.70	4.1(±0.7)	-2.23(±0.34)	1.84(±0.03)
$NaClO_4$	0.35	6.4(±0.3)	-2.62(±0.11)	2.43(±0.01)
$NaClO_4$	0.70	5.5(±0.2)	-2.79(±0.07)	1.96(±0.01)
$LiClO_4^*$	0.35	6.8(±0.2)	-2.93(±0.09)	2.31(±0.01)
$LiClO_4^*$	0.70	5.6(±0.5)	-3.41(±0.32)	1.65(±0.02)

given GdnHCl concentration, $\widehat{\Delta G}_{Di}$ represents the expected value of ΔG_D based on the least mean squares fit of the data and n is the number of points used in the analysis. The range of GdnHCl concentrations that produce a ΔG_D value of zero, within this error limit, was determined. The net result was that the uncertainty in the midpoint, σmdpt, could be calculated using the equation

$$\sigma\text{mdpt} = \Delta G_D/m \qquad (17)$$

where m is the value of the slope obtained from the analysis.

The mdpt value should not have a smaller uncertainty than that associated with the GdnHCl concentrations used to denature the RNase A. For this reason when the calculated uncertainty was found to be too low to be in keeping with the limits of the experiment a minimum uncertainty value of 0.01 M GdnHCl was assigned in place of the calculated uncertainty.

Figures 10 to 30 present the ΔG_D plots from which the preliminary results presented in Table 1 were obtained. Two sets of results for $NaClO_4$ are reported in this table. The second set represents data obtained prior to the replacement of the faulty crystal. These and all other data obtained prior to the replacement of the crystal are marked with an asterisk.

The preliminary results are required in order to obtain

FIGURE 11

The variation of ΔG_D as a function of GdnHCl concentration in the presence of 0.10 M NaCl as determined using the difference spectroscopy results.

FIGURE 12

The variation of ΔG_D as a function of GdnHCl concentration in the presence of 0.5 and 1.0 M NaCl.

FIGURE 13

The variation of ΔG_D as a function of GdnHCl concentration in the presence of 0.49 M CsCl. Open faced points not used in regression analysis.

FIGURE 14

The variation of ΔG_D as a function of GdnHCl concentration in the presence of 0.49 M RbCl. Open faced points not used in regression analysis.

FIGURE 11

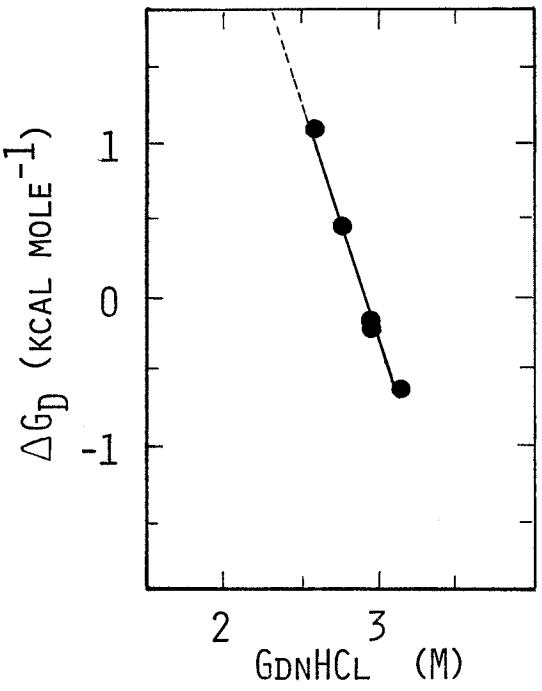


FIGURE 12

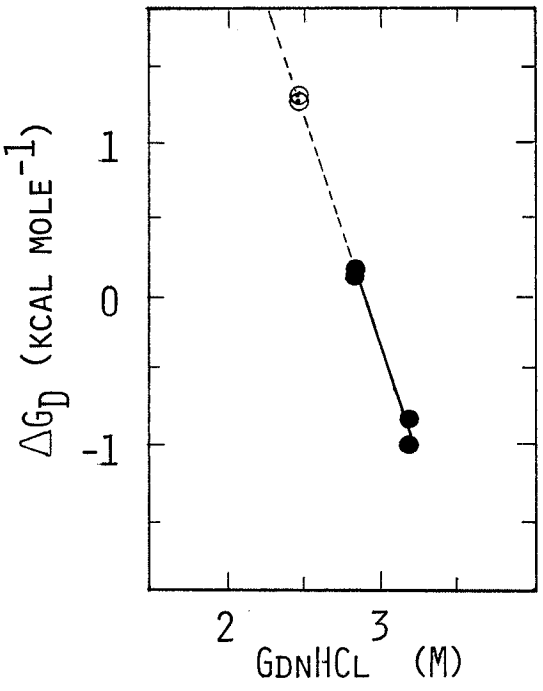


FIGURE 13

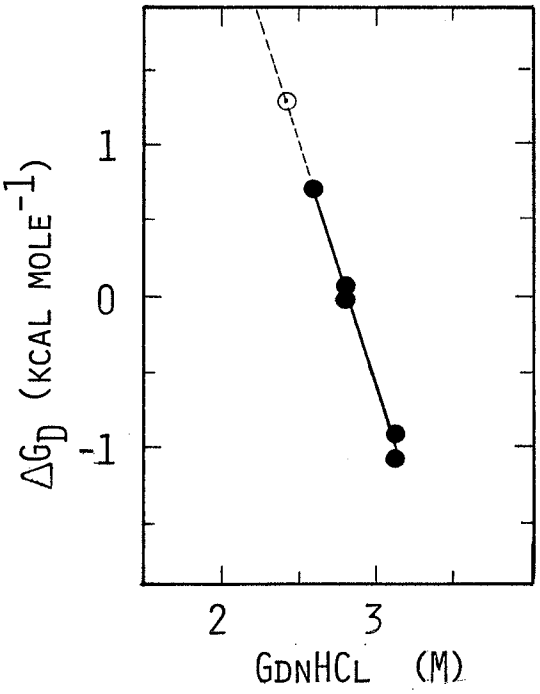


FIGURE 14

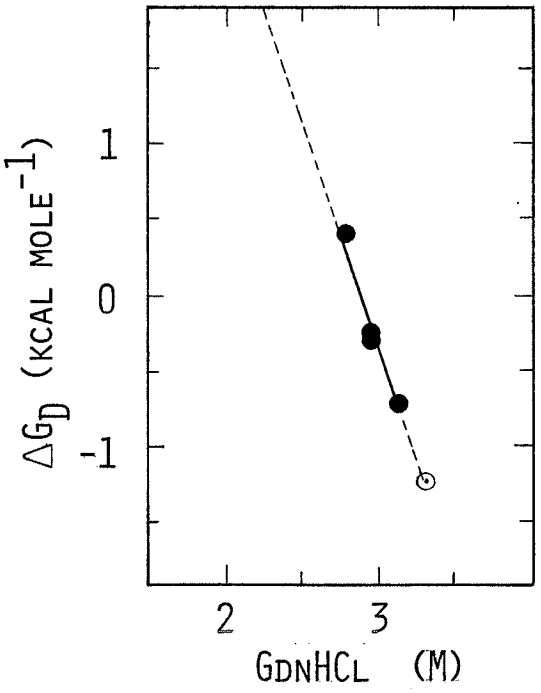


FIGURE 15

The variation of ΔG_D as a function of GdnHCl concentration in the presence of 0.45 M NH_4Cl.

FIGURE 16

The variation of ΔG_D as a function of GdnHCl concentration in the presence of 0.90 M NH_4Cl. Open faced points not used in regression analysis.

FIGURE 17

The variation of ΔG_D as a function of GdnHCl concentration in the presence of 0.50 (■) and 0.99 (●) M LiCl. Open faced points not used in regression analysis.

FIGURE 18

The variation of ΔG_D as a function of GdnHCl concentration in the presence of 0.10 (●) and 0.20 (■) M Phosphate.

FIGURE 15

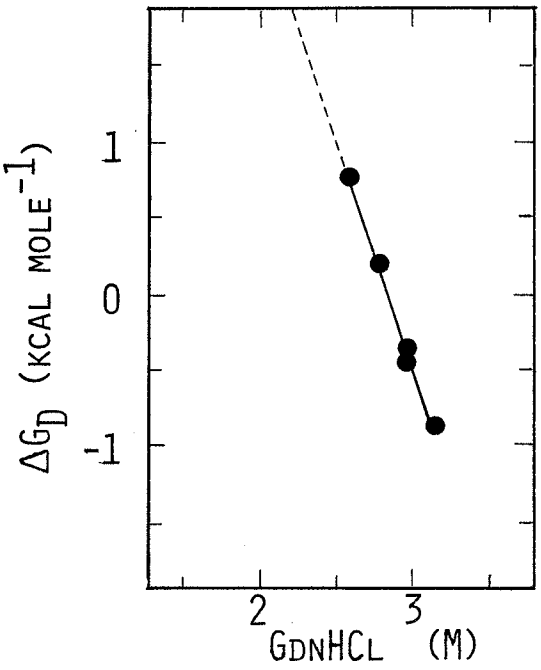


FIGURE 16

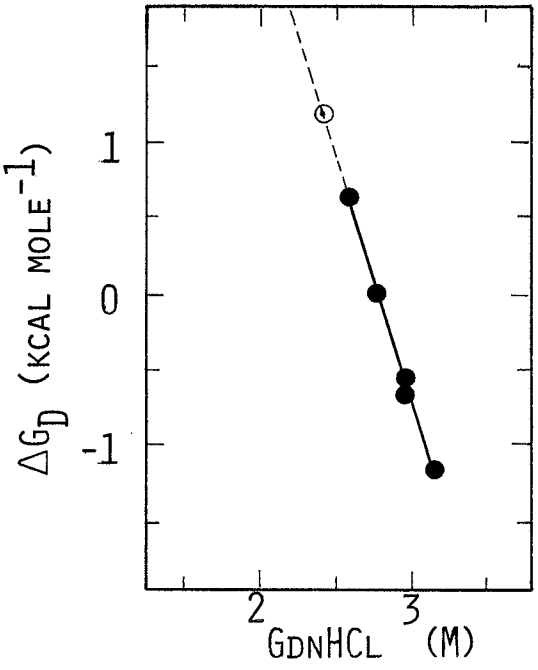


FIGURE 17

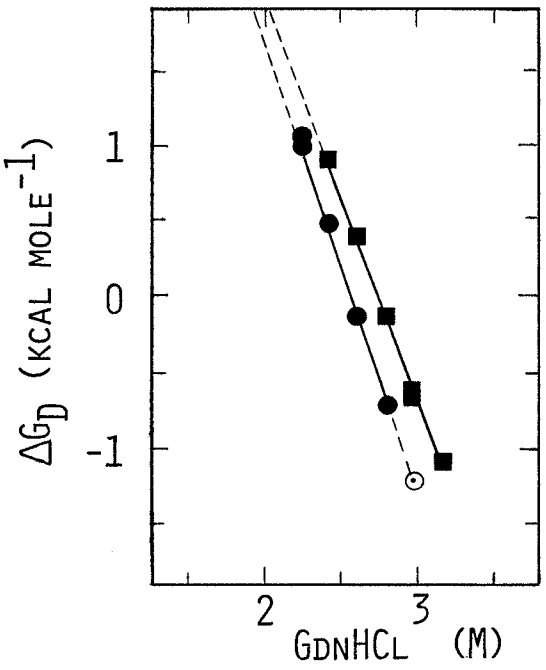


FIGURE 18

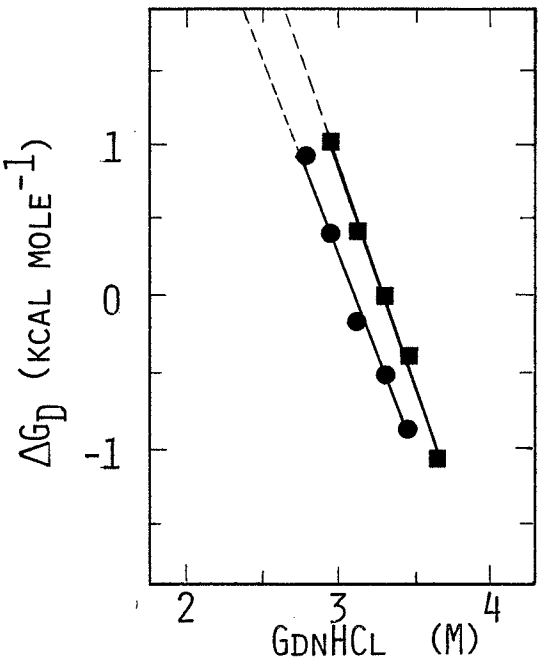


FIGURE 19

The variation of ΔG_D as a function of GdnHCl concentration in the presence of 0.15 (●) and 0.30 (■) M Na_2SO_4.

FIGURE 20

The variation of ΔG_D as a function of GdnHCl concentration in the presence of 0.15 (●) and 0.30 (■) M $(NH_4)_2SO_4$. Open faced points not used in regression analysis.

FIGURE 21

The variation of ΔG_D as a function of GdnHCl concentration in the presence of 0.20 (●) and 0.40 (■) M Li_2SO_4.

FIGURE 22

The variation of ΔG_D as a function of GdnHCl concentration in the presence of 0.25 (●) and 0.50 (■) M NaAc. Open faced points not used in regression analysis.

FIGURE 19

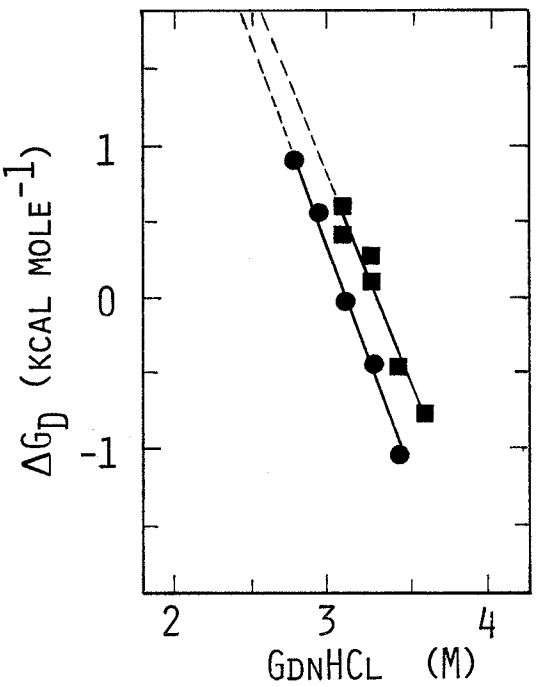


FIGURE 20

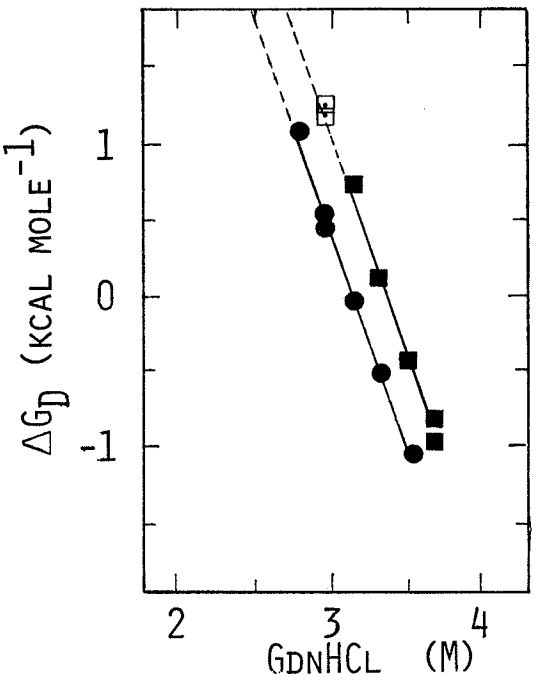


FIGURE 21

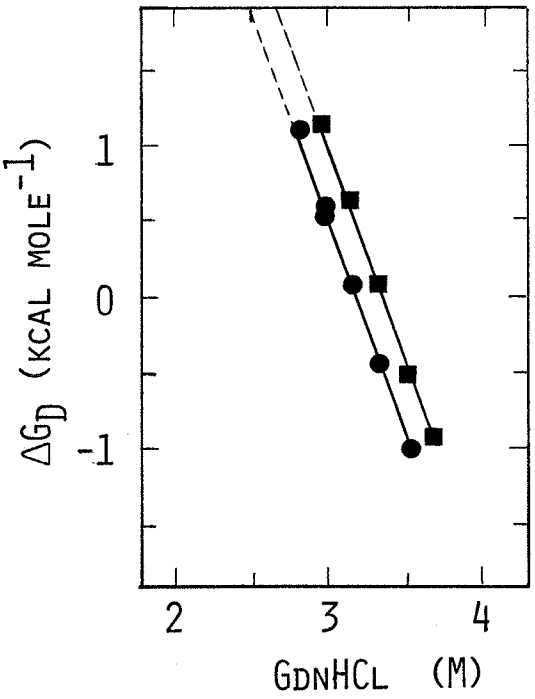


FIGURE 22

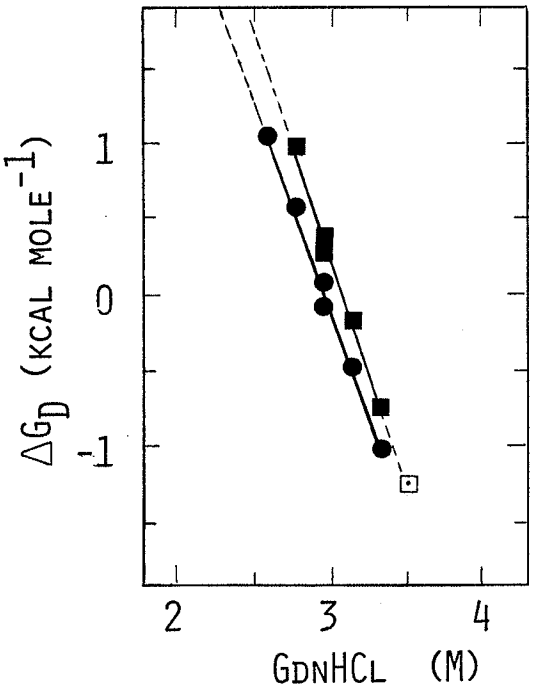


FIGURE 23

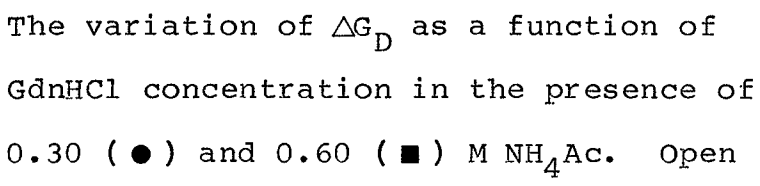


FIGURE 24

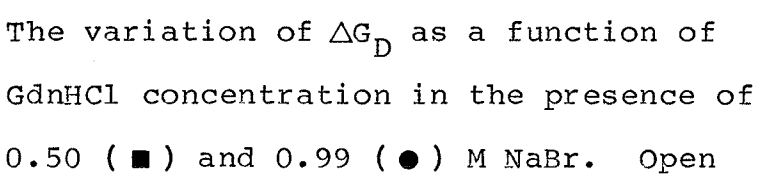


FIGURE 25

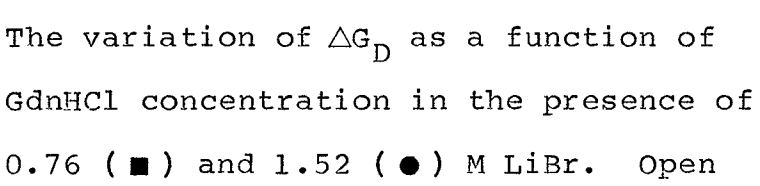


FIGURE 26

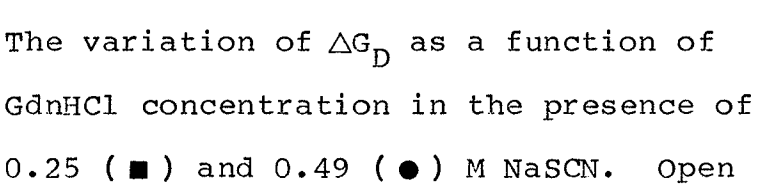


FIGURE 23

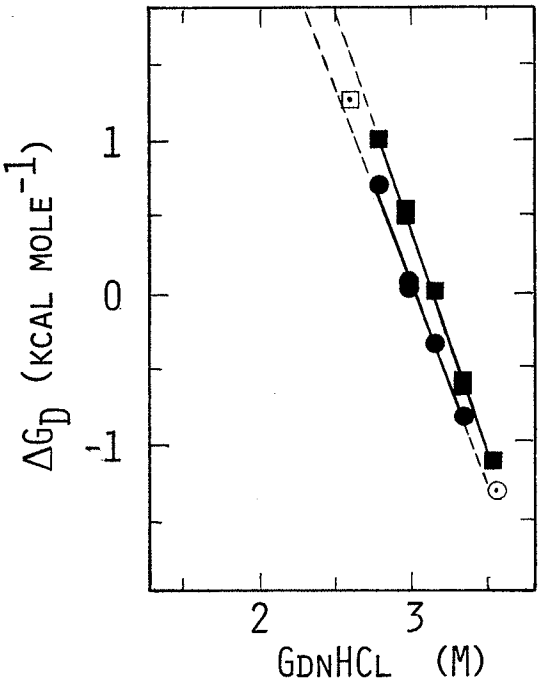


FIGURE 24

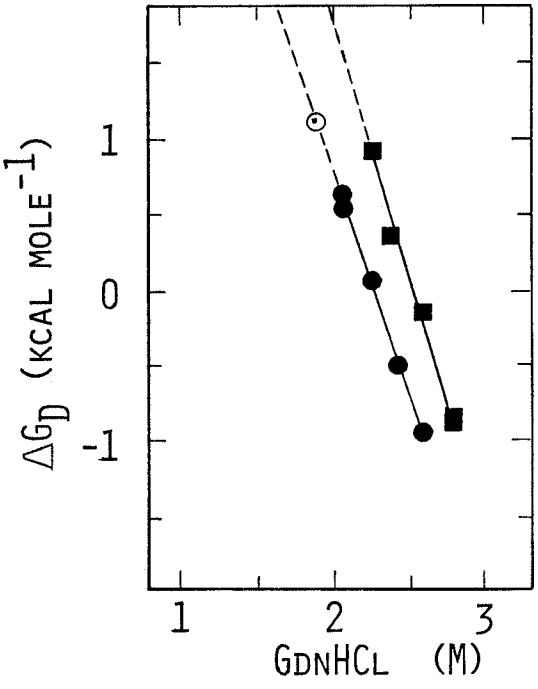


FIGURE 25

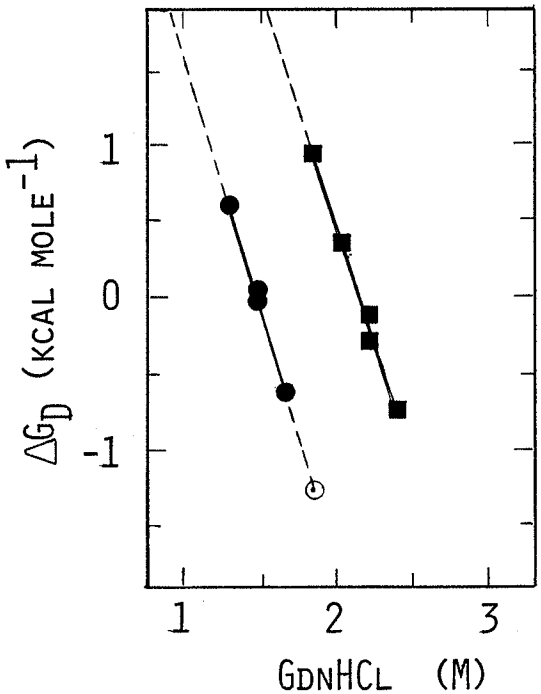


FIGURE 26

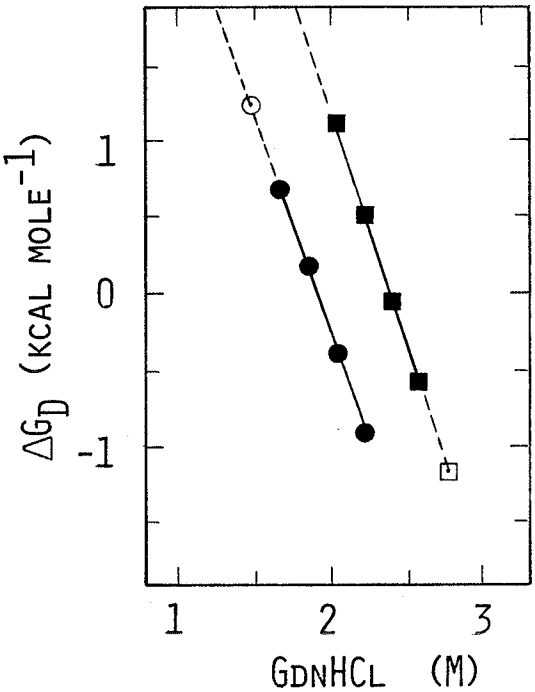


FIGURE 27

The variation of ΔG_D as a function of GdnHCl concentration in the presence of 0.09 (▲), 0.34 (■) and 0.69 (●) M LiSCN. Open faced points not used in regression analysis.

FIGURE 28

The variation of ΔG_D as a function of GdnHCl concentration in the presence of 0.35 (■) and 0.70 (●) M $NaClO_4^*$.

*Results obtained while faulty crystal in use.

FIGURE 29

The variation of ΔG_D as a function of GdnHCl concentration in the presence of 0.35 (■) and 0.70 (●) M $NaClO_4$.

FIGURE 30

The variation of ΔG_D as a function of GdnHCl concentration in the presence of 0.35 (■) and 0.70 (●) M $LiClO_4$.
Open faced points not used in regression analysis.

FIGURE 27

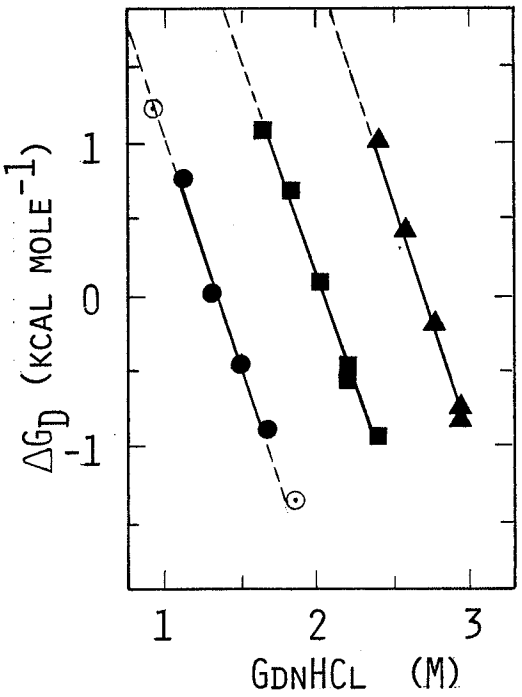


FIGURE 28

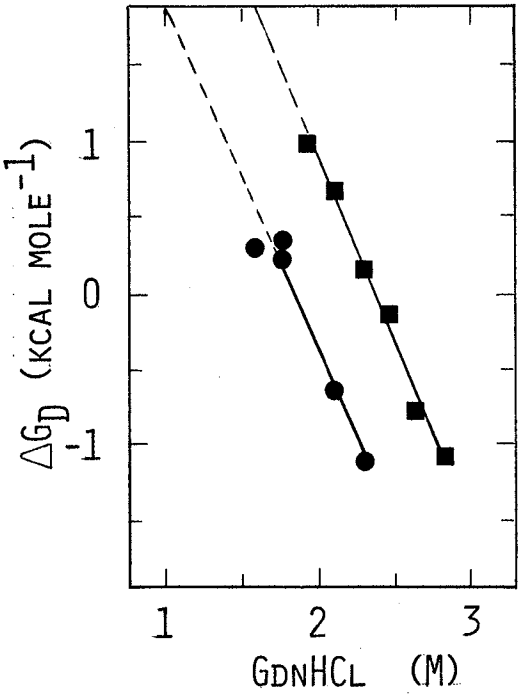


FIGURE 29

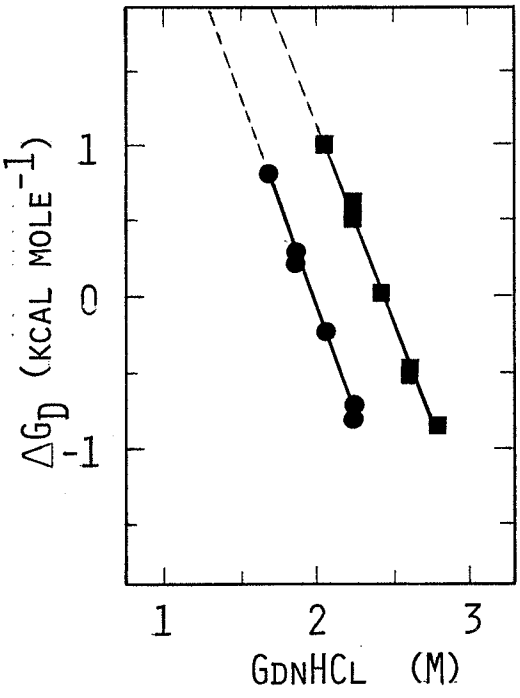
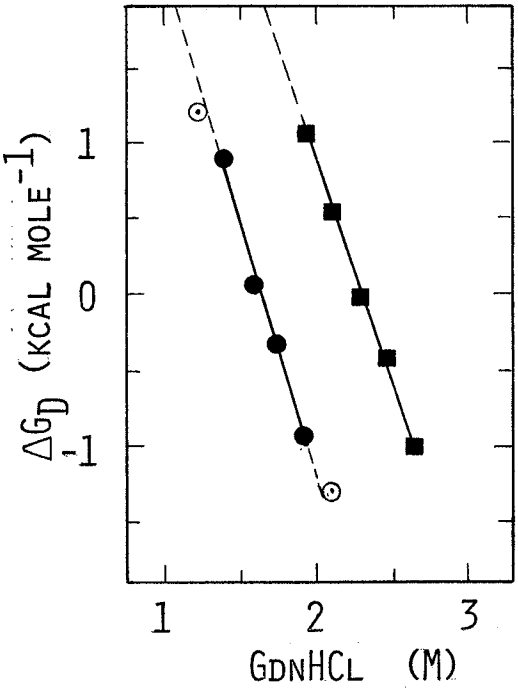


FIGURE 30



an estimate of the mdpt values and slopes. The ΔG_D^{salt} values were required to calculate the mdpt values. On the basis of the mdpt values alone each salt can be placed in one of three categories: Those salts that have a destabilizing effect on RNase A, those salts that have a stabilizing effect on RNase A and those salts that have no effect on the stability of RNase A.

B. REFITTED RESULTS (NON-BINDING SALTS)

1) Non-effectors

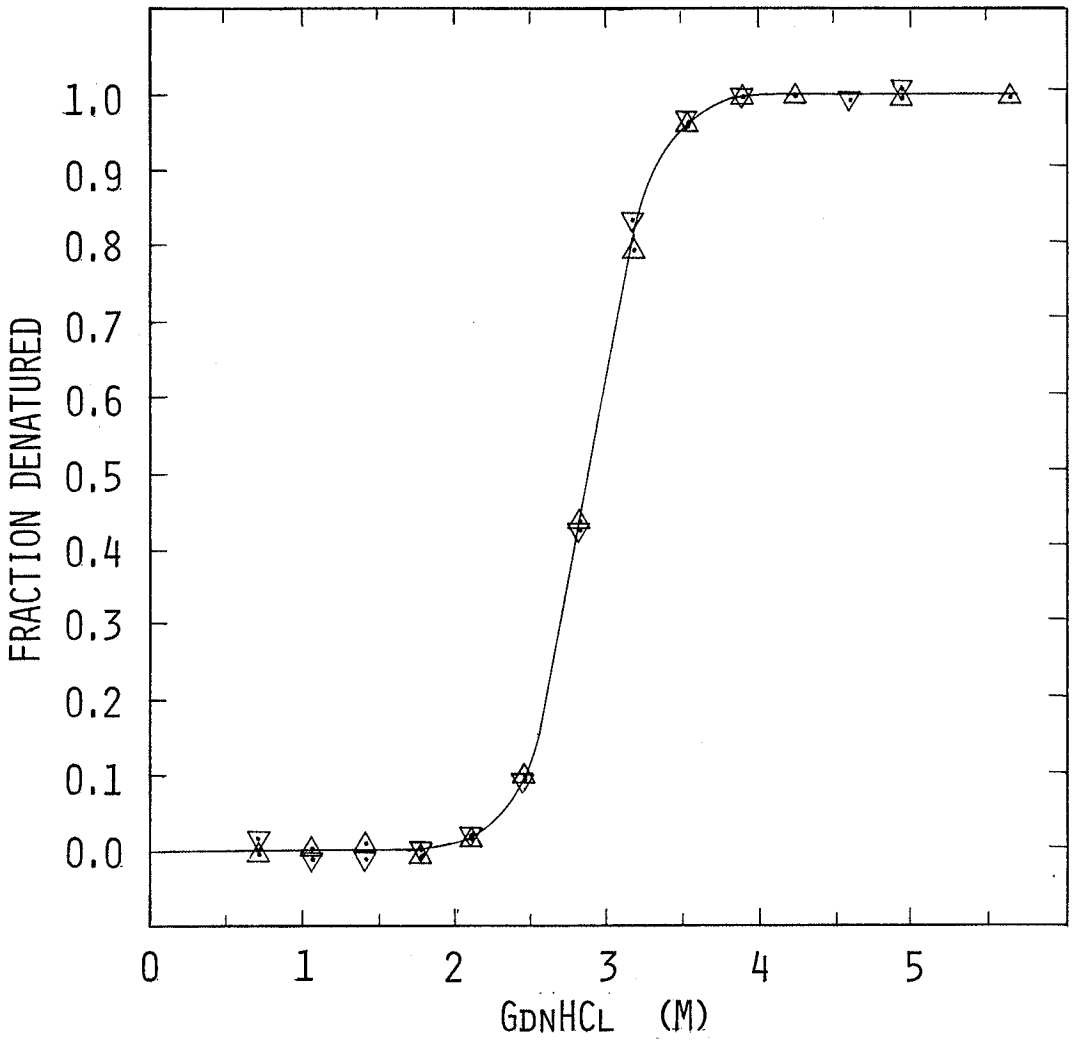
In this section the salts that have no effect on the stability of RNase A will be reanalysed. This analysis will yield a characterization of the effect of GdnHCl on the equilibrium between the two states, N and D. This first requires determining which salts have no effect on the stability of RNase A.

The effect of NaCl was most extensively studied. RNase A was denatured in the presence of 0.1 M NaCl, 0.5 M NaCl and 1.0 M NaCl and the 0.1 M NaCl denaturation was followed both spectroscopically and by CD measurements. Figure 31 presents the combined 0.5 M and 1.0 M NaCl data. The overlapping of the results demonstrated that, as was expected, the stability of RNase A is completely independent of the NaCl concentration. The 0.1 M NaCl data presented earlier overlap these data as well. The 0.5 M and 1.0 M data have been combined and analysed before being presented in Table 1.

When a salt such as NaCl is shown to have no effect on

FIGURE 31

Normalized transition of the denaturation of RNase A by GdnHCl in the presence of 0.5 ($\triangle$) and 1.0 (∇) M NaCl.


FRACTION DENATURED
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
1
2
3
4
5
GDNHCL (M)

the stability of a protein then one of two conclusions can be drawn about the stabilizing/destabilizing effects of the individual ions. The most obvious and simplest is that neither the Na^+ ion nor the Cl^- ion has any effect upon the stability of the protein. The other possiblity is that either the Na^+ ion or the Cl^- ion has a stabilizing effect upon the protein while the counter ion has an equal and opposite destabilizing effect upon the protein. The net result in both cases is the same. For the purpose of this investigation it does not matter which conclusion is correct. If the former conclusion is correct then the relative effectiveness of the stabilizing/destabilizing capabilities of all the other ions studied can be expressed in absolute terms. If the latter conclusion is correct then the effectiveness of the stabilizing/destabilizing capabilities of each ion must be expressed in terms relative to that of the Na^+ and Cl^- ions. For no better reason than to make the reporting of the results easier the former conclusion was adopted as the "correct" one.

By studying the stabilizing/destabilizing effects of salts containing either Na^+ or Cl^- ions the effect of various other ions was established. When RNase A was denatured with 0.1 M KCl present instead of 0.1 M NaCl the same results as above were obtained. This indicated the K^+ ion was as ineffective as the Na^+ ion in altering the stability of RNase A.

Although a slightly lower midpoint value was obtained

when the effect of 0.49 M CsCl on the stability of RNase A was determined further investigation involving two other concentrations of CsCl failed to reveal a change in the stability of the protein. Similarly, Rb^+ was found to have no effect on the stability of RNase A.

The effect of NH_4Cl on the stability of RNase A was investigated at two different concentrations. With the first NH_4Cl concentration no stabilizing or destabilizing of the RNase A could be detected. When a higher concentration was used a slight shift in the midpoint of transition was noted. These results indicated that the NH_4^+ ion possessed a slight destabilizing ability. As this result was unexpected the transition curves were given closer scrutiny.

Figures 32 to 34 present the normalized curves of the NH_4Cl, NaAc and NH_4Ac data respectively. Examination of the two NaAc normalized curves revealed that they were identical in all respects except that they were each displaced from the normalized NaCl curve to a different extent. That is, these two curves would have been coincident if they had possessed the same midpoint of transition value. Comparison of the two NH_4Ac normalized curves, and the previously presented NaBr and $NaClO_4$ normalized curves revealed the same findings.

Unlike the above described curves the NH_4Cl normalized curves were initially coincidental diverging in the transition region to yield two different midpoints of

FIGURE 32

Comparison of the normalized transition curves of the denaturation of RNase A by GdnHCl in the presence of (1) 0.45 and (2) 0.90 M NH_4Cl.

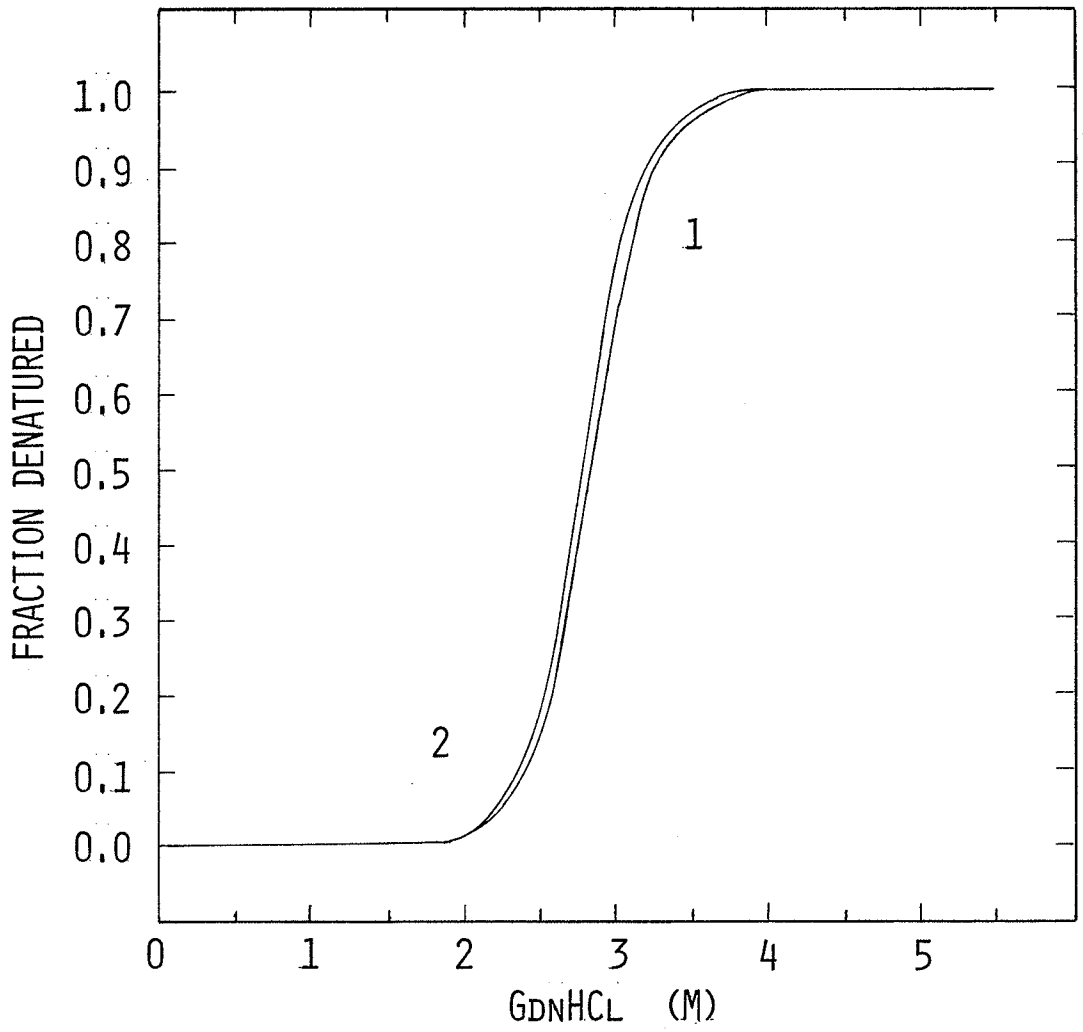

FRACTION DENATURED
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
1
2
3
4
5
GDNHCL (M)
1
2

FIGURE 33

Comparison of the normalized transition curves of the denaturation of RNase A by GdnHCl in the presence of (1) 0.25 and (2) 0.50 M NaAc.

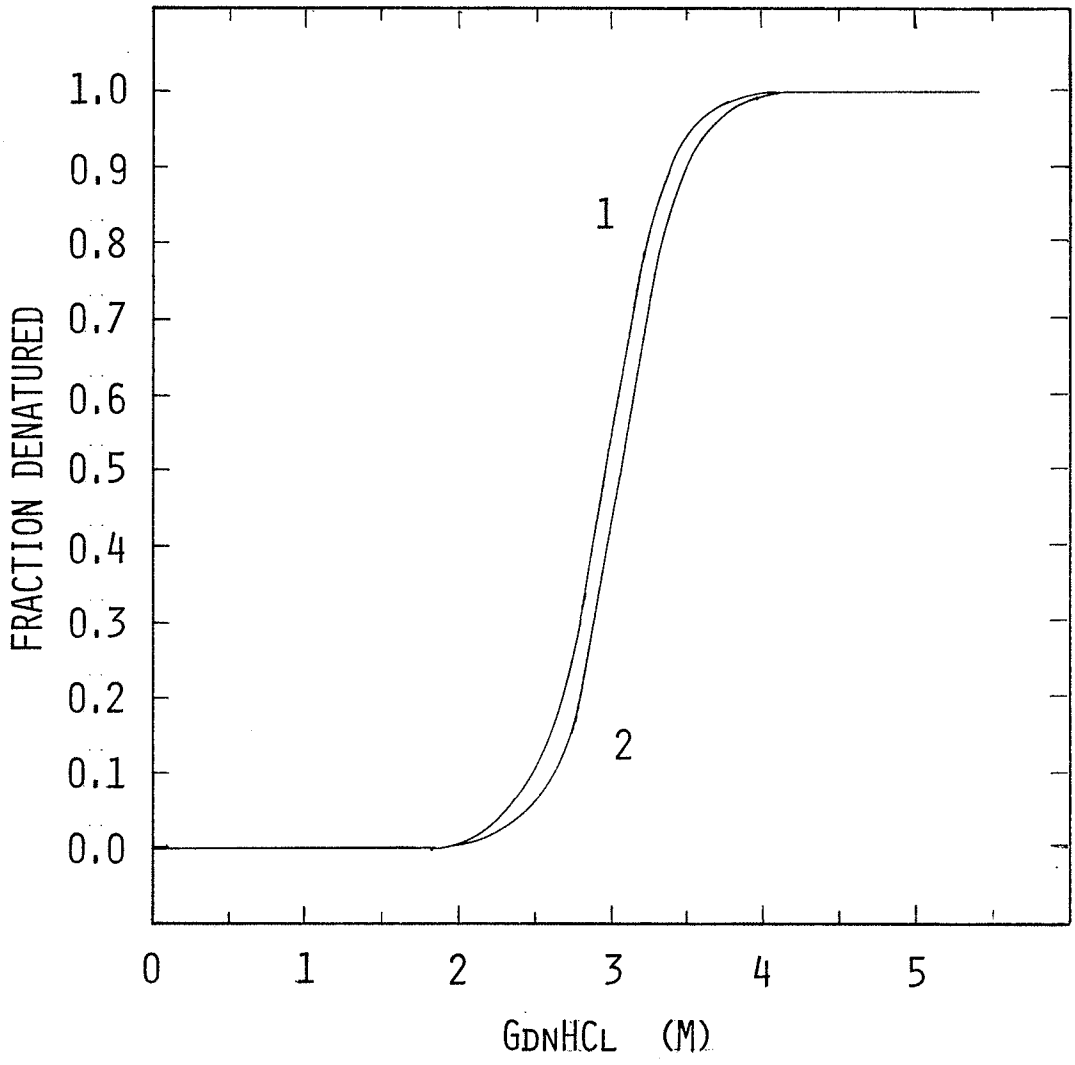
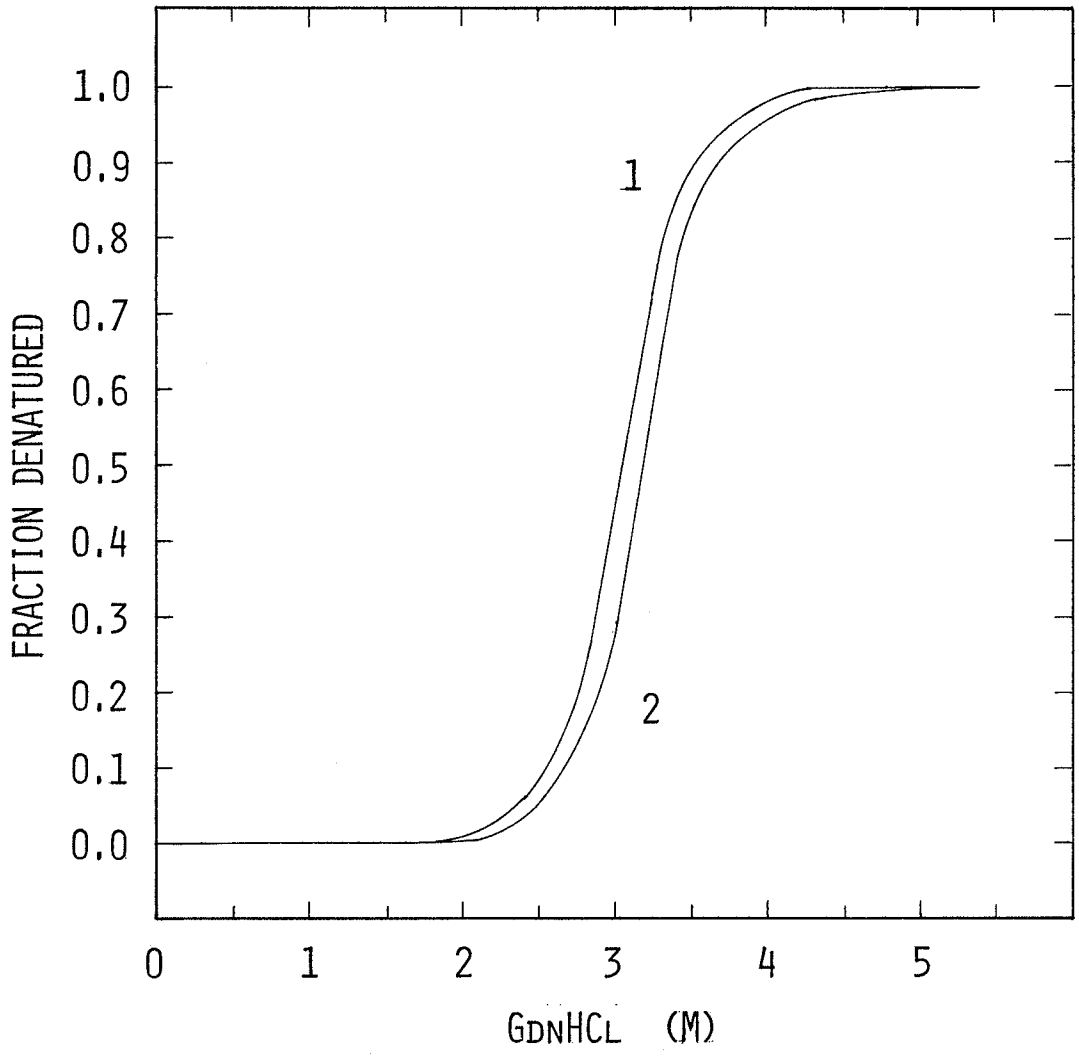

FRACTION DENATURED
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
1
2
3
4
5
GDNHCL (M)
1
2

FIGURE 34

Comparison of the normalized transition curves of the denaturation of RNase A by GdnHCl in the presence of (1) 0.30 and (2) 0.60 M NH_4Ac.


FRACTION DENATURED
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
1
2
3
4
5
GDNHCL (M)
1
2

transition. Of all the salts investigated this was the only occurrence of dissimilar normalized transition curves.

Divergence of this same sort will result when the same denaturation process is observed at two slightly different temperatures. A small temperature difference would explain why a slight destabilization was noted for one concentration of NH_4Cl but not the other. Based upon the 0.45 M NH_4Cl data the NH_4^+ ion was classified as as ion that had no effect on the stability of RNase A and the destabilization noted at the higher concentration should be attributed to a minor difference in temperature.

All five of these chloride salts were satisfactorily shown to have no effect upon the ability of GdnHCl to denature RNase A. The slope and midpoint of transition obtained from the ΔG_D plot should have been the same in each case. Any differences should be attributed to the small number of data points that were available for the least mean squares analysis. Collectively there were sufficient data to obtain a reliable characterization of the effect of GdnHCl on the N to D equilibrium. The average slope value, excluding the 0.90 M NH_4Cl data, was $-3.04(\pm 0.04)$ kcal $mole^{-1}$ M^{-1} and the average midpoint value was $2.88(\pm 0.02)$ M GdnHCl.

With this new average slope value new estimates of ΔG_D^{salt} were obtained. The data presented in Figures 10 to 30 were refitted to a line with the new fixed slope. The data in each of these figures were averaged to obtain, for

each set of data, an average point, (X,Y), through which the line was placed. This function was extrapolated to zero concentration to obtain a new ΔG_D^{salt} value. The uncertainty in this new value is equal to the uncertainty in the Y value. The new ΔG_D^{salt} values and midpoint values are presented in Table 2. The uncertainty in the new midpoint value is simply equal to the uncertainty in the ΔG_D^{salt} value divided by the fixed slope. Comparison with the results presented in Table 1 revealed that the midpoint values remain unaffected and only minor changes occurred in the ΔG_D^{salt} values.

2) Effectors

All ions other than Na^+, K^+, Cs^+, Rb^+, NH_4^+, and Cl^- were identified as possessing either stabilizing or destabilizing cababilities. Li^+, Br^-, ClO_4^- and SCN^- all displayed significant concentration dependent destabilizing capabilities. The SO_4^{-2}, phosphate and acetate ions all displayed concentration dependent stabilizing capabilities.

Four lithium salts and two ammonium salts were utilized to study the additivity of the stabilizing/destabilizing effects of the ions. Three of these salts, $(NH_4)_2SO_4$, Li_2SO_4 and NH_4Ac, displayed concentration dependent stabilizing capabilities while the other three salts, LiBr, LiSCN and $LiClO_4$, displayed concentration dependent destabilizing capabilities.

TABLE 2

"FITTED" RESULTS CHARACTERIZING RNase A DENATURATION

Salt	[Salt]	ΔG_D^{salt}	Slope	midpoint
	M	kcal/mole	kcal/mole/M	M
NaCl*	0.1	8.69(±0.04)	-3.04	2.86(±0.01)
NaCl (UV)	0.1	8.89(±0.03)	-3.04	2.92(±0.02)
NaCl*	0.5 + 1.0	8.78(±0.03)	-3.04	2.89(±0.01)
KCl*	0.1	8.83(±0.01)	-3.04	2.90(±0.01)
CsCl*	0.49	8.53(±0.04)	-3.04	2.81(±0.01)
RbCl (UV)	0.50	8.79(±0.04)	-3.04	2.89(±0.01)
NH_4Cl	0.45	8.62(±0.03)	-3.04	2.83(±0.01)
LiCl	0.50	8.36(±0.04)	-3.04	2.75(±0.01)
LiCl	0.99	7.80(±0.01)	-3.04	2.57(±0.01)
Phosphate*	0.10	9.45(±0.05)	-3.04	3.11(±0.02)
Phosphate*	0.20	10.00(±0.04)	-3.04	3.29(±0.01)
Na_2SO_4*	0.15	9.49(±0.03)	-3.04	3.12(±0.01)
Na_2SO_4*	0.30	10.15(±0.05)	-3.04	3.34(±0.02)
$(NH_4)_2SO_4$	0.15	9.60(±0.02)	-3.04	3.16(±0.01)
$(NH_4)_2SO_4$	0.30	10.28(±0.03)	-3.04	3.37(±0.01)
Li_2SO_4	0.20	9.60(±0.01)	-3.04	3.16(±0.01)
Li_2SO_4	0.40	10.18(±0.01)	-3.04	3.35(±0.01)
NaAc	0.25	9.00(±0.03)	-3.04	2.96(±0.01)
NaAc	0.50	9.35(±0.02)	-3.04	3.07(±0.01)
NH_4Ac	0.30	9.20(±0.06)	-3.04	3.02(±0.02)
NH_4Ac	0.60	9.58(±0.02)	-3.04	3.15(±0.01)

TABLE 2 (CONTINUED)

Salt	[Salt]	ΔG_D^{salt}	Slope	midpoint
	M	kcal/mole	kcal/mole/M	M
NaBr (UV)	0.50	7.70(±0.04)	-3.04	2.53(±0.01)
NaBr (UV)	0.99	6.91(±0.02)	-3.04	2.27(±0.01)
LiBr (UV)	0.76	6.55(±0.03)	-3.04	2.15(±0.01)
LiBr (UV)	1.52	4.51(±0.03)	-3.04	1.48(±0.01)
NaSCN (UV)	0.25	7.27(±0.01)	-3.04	2.39(±0.01)
NaSCN (UV)	0.49	5.80(±0.02)	-3.04	1.91(±0.01)
LiSCN (UV)	0.09	8.27(±0.03)	-3.04	2.72(±0.01)
LiSCN (UV)	0.34	6.25(±0.04)	-3.04	2.06(±0.01)
LiSCN (UV)	0.69	4.08(±0.04)	-3.04	1.34(±0.01)
$NaClO_4^*$	0.35	7.25(±0.09)	-3.04	2.38(±0.03)
$NaClO_4^*$	0.70	5.65(±0.13)	-3.04	1.86(±0.04)
$NaClO_4$	0.35	7.38(±0.05)	-3.04	2.43(±0.02)
$NaClO_4$	0.70	5.96(±0.03)	-3.04	1.96(±0.01)
$LiClO_4^*$	0.35	7.01(±0.02)	-3.04	2.31(±0.01)
$LiClO_4^*$	0.70	5.01(±0.07)	-3.04	1.65(±0.02)

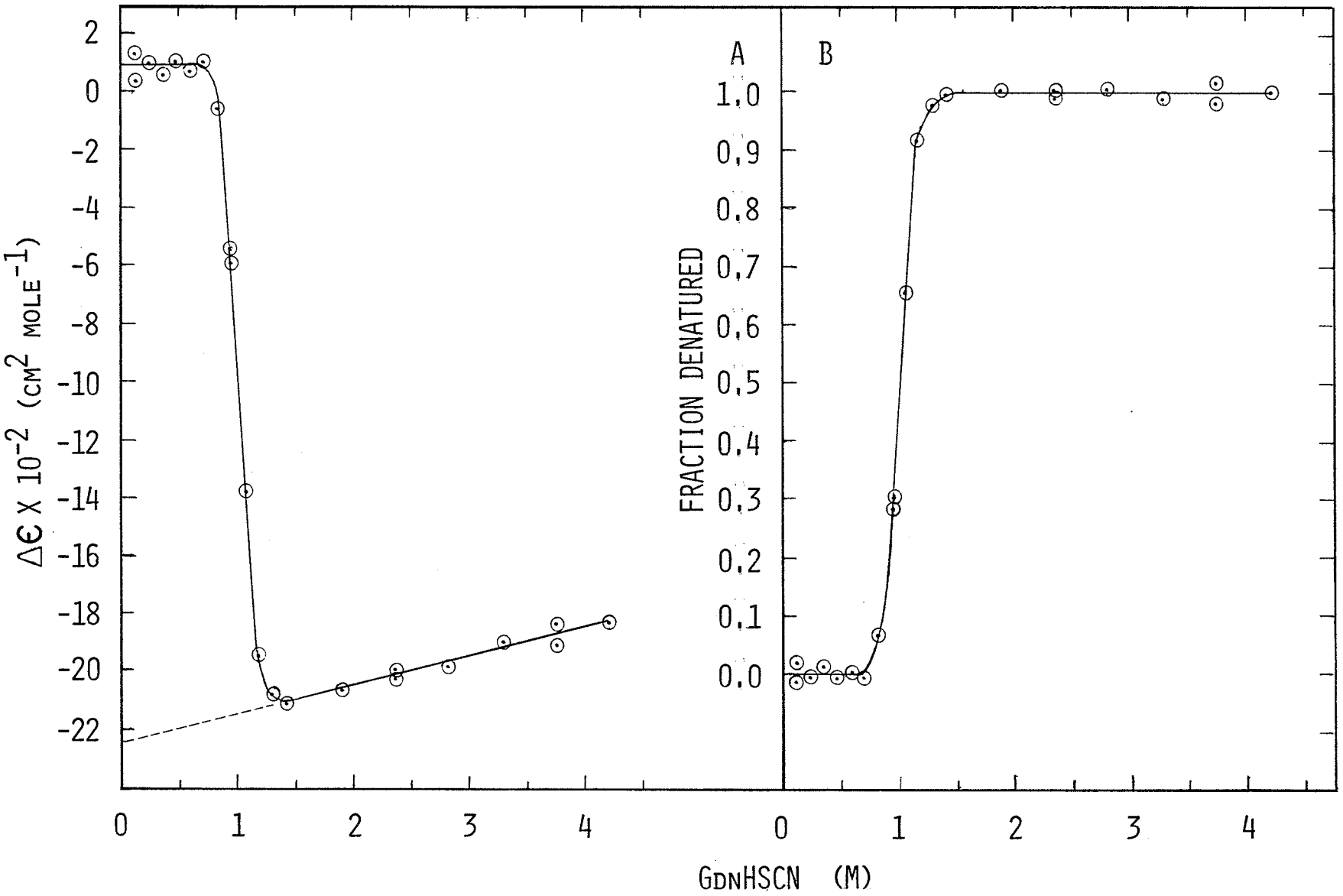
C. VERIFICATION

In this section an attempt will be made to verify, using experimentally obtained data, that the slope used to refit the results should not be influenced by the presence of salt unless that salt directly interacts with either the native state or denatured state of the RNase A or with the GdnHCl. There are two different ways by which this verification can be obtained. The first method involves denaturing the RNase A with a GdnHCl-salt mixture. In this method the salt concentration is varied keeping the ratio of salt concentration to the GdnHCl concentration constant instead of maintaining a fixed salt concentration. In this manner when the GdnHCl concentration approaches zero so does the salt concentration. The ΔG_D plot obtained from this denaturation procedure and the ΔG_D plot obtained using a fixed salt concentration should intersect at the point where the varied salt concentration and the fixed salt concentration are equal.

An example of this method is presented whereby RNase A was denatured using guanidinium thiocyanate, GdnHSCN, in the presence of buffer and 0.1 M NaCl. The denaturation was followed spectroscopically and the data obtained were analysed in the same manner as were the GdnHCl denaturation data. Figures 35A and B present the transition curve and the normalized transition curve respectively. The transition is much steeper than those presented in the previous figures and the midpoint of transition occurs at a

FIGURE 35

Transition curve (A) and normalized transition curve (B) of the denaturation of RNase A by GdnHSCN in the presence of 0.10 M NaCl.


A
B
2
0
-2
-4
-6
-8
-10
-12
-14
-16
-18
-20
-22
$\Delta\epsilon \times 10^{-2}$ (CM^2 MOLE^{-1})
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
FRACTION DENATURED
0
1
2
3
4
GDNHSCN (M)

much lower denaturant concentration. Because of the steepness of the transition ΔG_D values could be obtained at only two different concentrations of GdnHSCN.

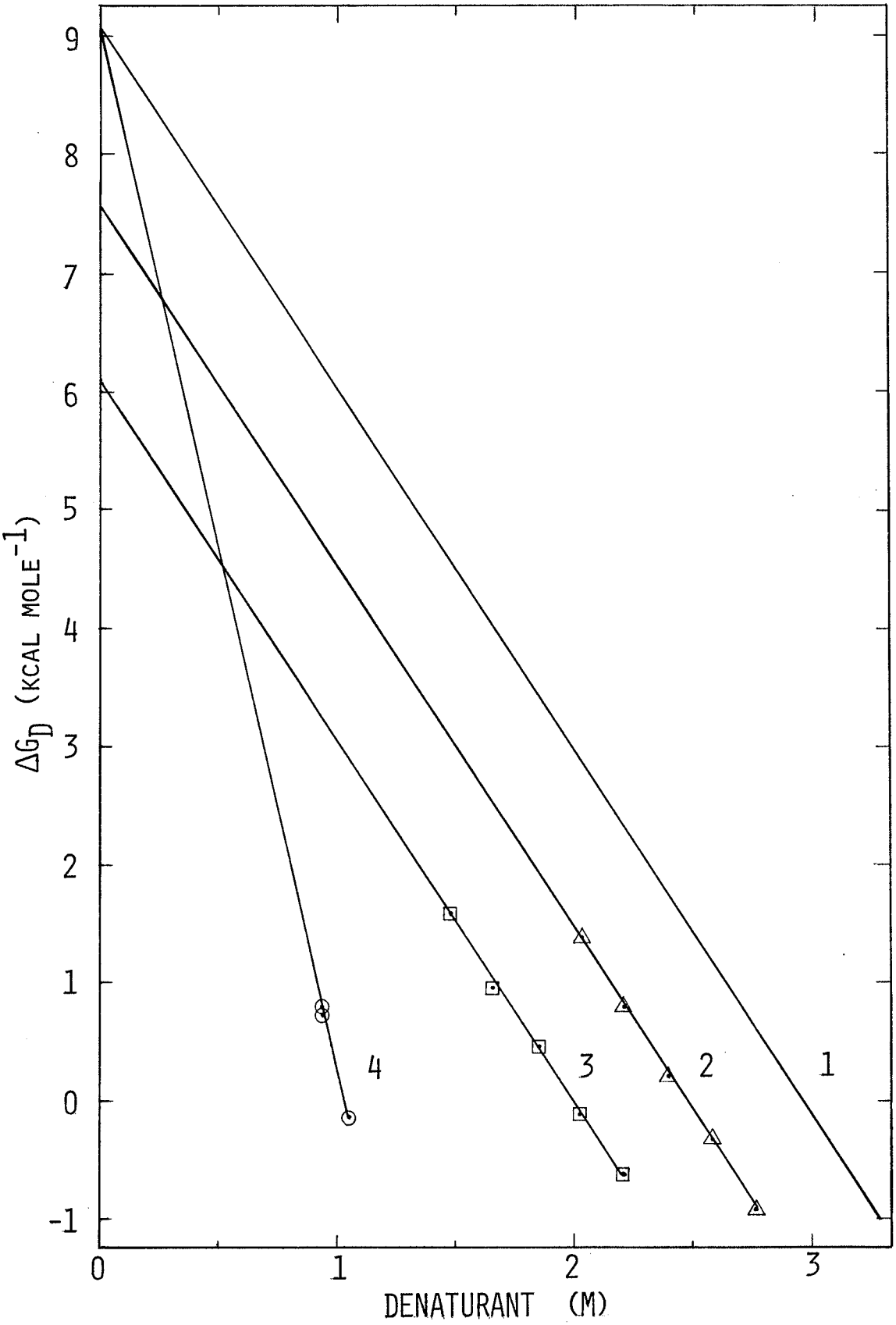
These values are presented in Figure 36. Line 1 of this figure presents the average NaCl data and is used to represent the GdnHCl denaturation of RNase A in the presence of 0 M NaSCN. Lines 2 and 3, respectively, present the 0.25 M and 0.49 M NaSCN data as treated in the described manner. In addition to the three GdnHSCN data points a value of ΔG_D equal to 8.8 kcal $mole^{-1}$ at zero GdnHSCN concentration was included in obtaining Line 4. This value represents $\Delta G_D^{H_2O}$ and should be the same whether obtained from the linear extrapolation of GdnHCl data, GdnHSCN data or even urea data when available (7,14).

If the methods of treatment of the data have been valid up to this point then Lines 2 and 3 should intersect Line 4 at 0.25 M and 0.49 M denaturant respectively. The actual points of intersection are 0.27 M and 0.52 M denaturant respectively. This is surprisingly good agreement considering the small number of points available to define the GdnHSCN ΔG_D plot. This agreement proves that at least in the case of SCN^- destabilization the use of the fixed slope value of -3.04 kcal $mole^{-1}$ M^{-1} to characterize the ΔG_D plot is justified.

The method just described leaves no doubt as to whether use of the fixed slope is or is not valid. The next method is equally good at verifying the use of a fixed slope and

FIGURE 36

The variation of ΔG_D as a function of denaturant concentration. Line 1 represents the denaturation of RNase A by GdnHCl in the absence of SCN^- ions (average NaCl data). Lines 2 and 3 represent the denaturation of RNase A by GdnHCl in the presence of 0.25 and 0.49 M SCN^- ion respectively (0.25 and 0.49 M NaSCN data respectively). Line 4 represents the denaturation of RNase A by GdnHCl in the presence of an equal concentration of SCN^- ions (GdnHSCN data).


9
8
7
6
5
4
3
2
1
0
-1
0
1
2
3
ΔG_D (KCAL MOLE^{-1})
DENATURANT (M)
1
2
3
4

has the added advantage of not requiring additional denaturation data. The only way the lines in Figure 36 can remain parallel and still intersect the GdnHSCN ΔG_D plot at the appropriate places is if their midpoint of transition values are linearly dependent upon the salt concentration. In addition to the linear dependence the value obtained by plotting the midpoint of transition values against the salt concentration and extrapolating to zero salt concentration should yield the midpoint of transition value obtained in the absence of stabilizing or destabilizing salts. This relationship between the midpoint of transition and slope will be expanded upon in the Discussion.

The midpoint values were plotted as a function of the salt concentration. In all but one case, LiSCN, these plots consisted of two points each. A line was drawn through these two points and extrapolated back to zero concentration. These plots are presented in Figures 37 and 38. In each case this extrapolated value should be the previously observed value of 2.88(±0.02) M GdnHCl. These extrapolated values, $mdpt_0$, are presented in Table 3. Due to the method by which these values were obtained the associated uncertainty cannot be predicted.

Figure 37 clearly reveals that for all the salts presented, with the exception of the additional set of $NaClO_4$ data and the $LiClO_4$ data, the extrapolated midpoint value is as expected. The $mdpt_0$ value predicted from the $NaClO_4$ data obtained while the properly

TABLE 3

PREDICTED MIDPOINT OF TRANSITION

AT ZERO MOLAR ION/SALT CONCENTRATION

ION	$mdpt_0$	SALT	$mdpt_0$
	M		M
Na^+	2.88	$(NH_4)_2SO_4$	2.95
Cl^-	2.88	Li_2SO_4	2.97
K^+	2.88	NH_4Ac	2.89
Cs^+	2.88	LiBr	2.82
Rb^+	2.88	LiSCN	2.89
NH_4^+	2.89	$LiClO_4^*$	2.97
Li^+	2.93		
Phosphate	2.93		
SO_4^{-2}	2.90		
Ac^-	2.85		
Br^-	2.80		
SCN^-	2.87		
ClO_4^{-*}	2.94		
ClO_4^-	2.90		

FIGURE 37

The variation of the midpoint of transition as a function of non-binding salt concentration. NH_4Ac (▼), NaAc (△), NH_4Cl (■), LiCl (○), NaBr (◇), LiBr (▽), $NaClO_4$ (▲), $LiClO_4$ (□) and NaSCN (●).

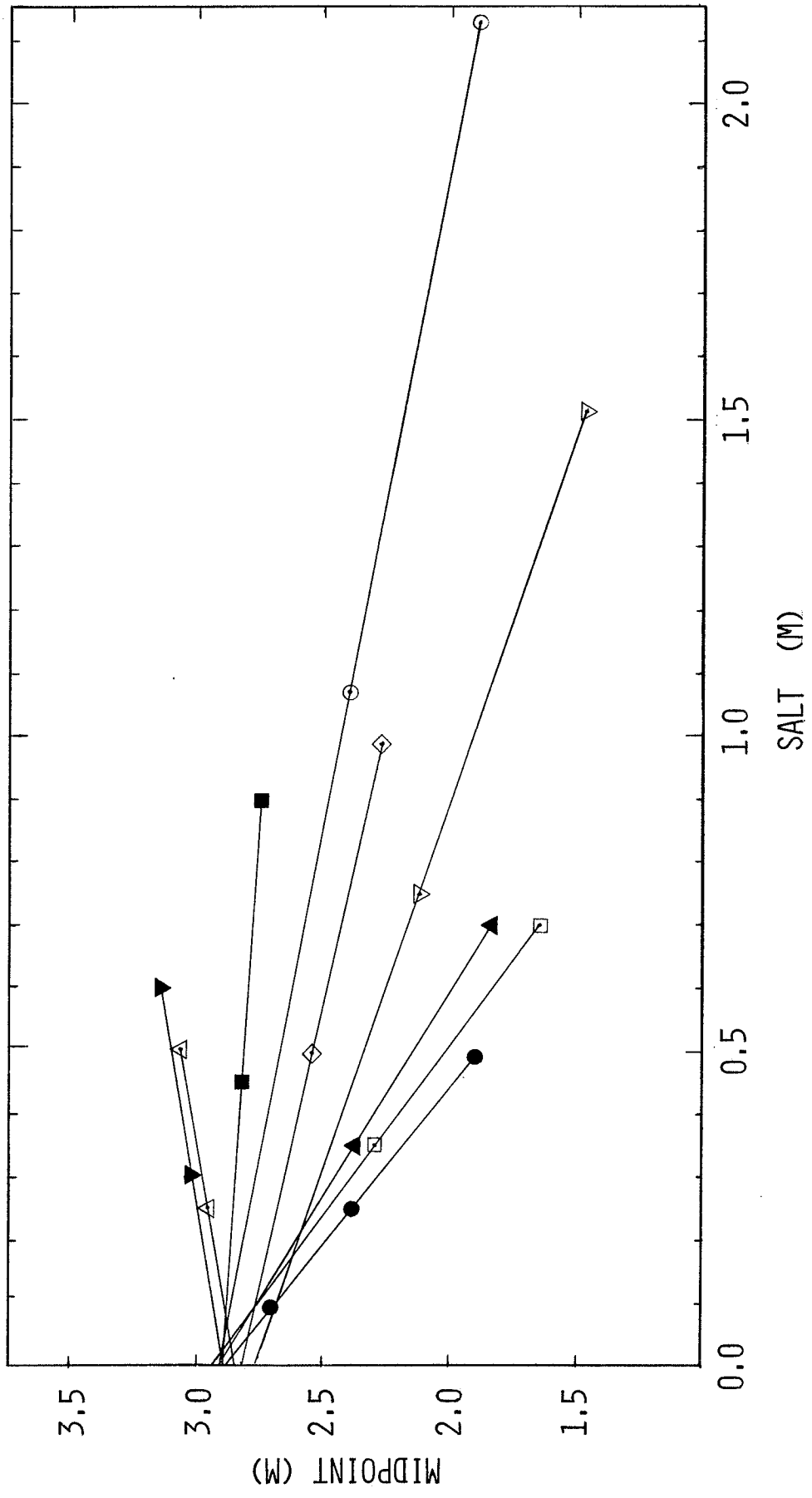
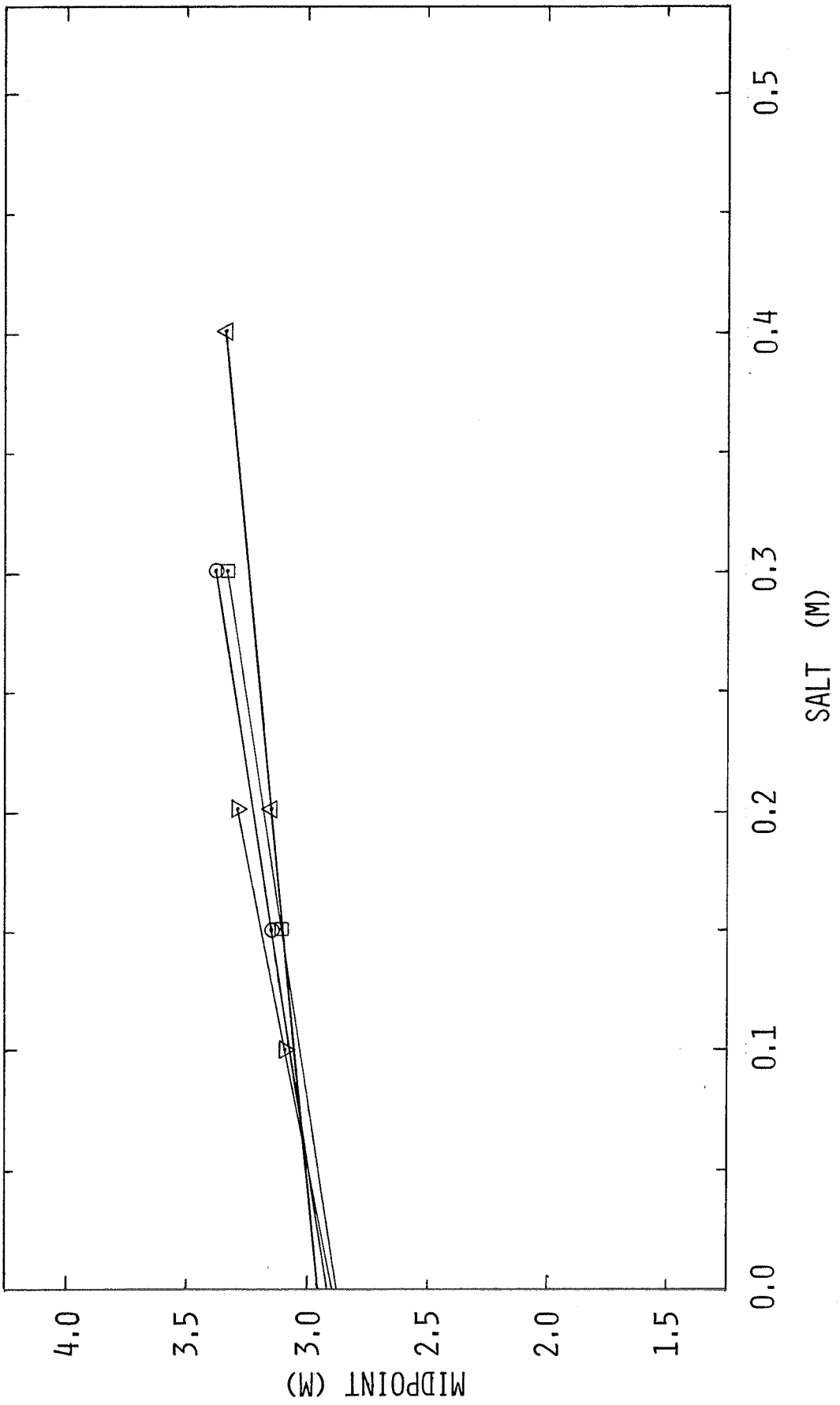

MIDPOINT (M)
3.5
3.0
2.5
2.0
1.5
0.0
0.5
1.0
1.5
2.0
SALT (M)

FIGURE 38

The variation of the midpoint of transition as a function of binding salt concentration. $(NH_4)_2SO_4$ (○), Na_2SO_4 (□), Li_2SO_4 (△), and phosphate (▽).


MIDPOINT (M)
4.0
3.5
3.0
2.5
2.0
1.5
SALT (M)
0.0
0.1
0.2
0.3
0.4
0.5

functioning crystal was in place compares very well with the other $mdpt_0$ values. The $mdpt_0$ value obtained from the additional $NaClO_4$ data is somewhat higher. In keeping with this finding the $mdpt_0$ value predicted from the $LiClO_4$ data is also higher than the other values. This is to be expected as both sets of data were obtained while the faulty crystal was in place.

The average $mdpt_0$ value, excluding the additional $NaClO_4$ result and the $LiClO_4$ result, is 2.87(±0.01) M GdnHCl. This is the value expected if the observed shifts in the midpoints of transition are linearly dependent upon the concentration of added salt. This result can be considered sufficient proof of the validity of using the fixed slope value of -3.04 kcal $mole^{-1}$ M^{-1} to characterize the ΔG_D plot for all cases presented in Figure 37.

Figure 38 presents a similar treatment of the data obtained from the SO_4^{-2} salts and the phosphate salt. As can be seen from this figure all the extrapolated midpoint values are higher than those presented in the previous figure. The average $mdpt_0$ value based on these salts is 2.94(±0.01) M GdnHCl. These higher values cannot be attributed to instrumentational difficulties as were the previous two anomalous results. This means that there is no justification in fixing the slope of the ΔG_D plots of these salts with the previously determined value.

D. REFITTED RESULTS (BINDING SALTS)

This finding indicates one of two things. Either the salt is binding to the native state thereby increasing the stability of the protein or the salt is binding to the guanidinium ion thereby decreasing the effective concentration of the denaturant. Although both situations will have the same effect on the midpoints of transition they are readily distinguishable by their effects on the slope of the ΔG_D plots. With the latter situation the slope of the ΔG_D plots will remain unaffected because neither the protein nor the denaturing capability of the free GdnHCl is affected. The plots need only be shifted to lower GdnHCl concentrations to correct for the binding.

If the salt binds to the protein the product will most likely be less susceptible to denaturation by GdnHCl. Although there is the possiblity that the product will be more susceptible to denaturation by GdnHCl this situation is very unlikely and will not be considered. Examination of the original data presented in Table 1 reveals that the average slope value of the phosphate and SO_4^{-2} salts is $2.81(\pm 0.04)$ kcal $mole^{-1}$ M^{-1}. This reduction in the slope reflects the difference in the ability of GdnHCl to denature RNase A when either SO_4^{-2} or phosphate is bound to this protein.

This binding must be accounted for in the calculation of ΔG_D^{salt}. This is accomplished by rewriting the equilibrium equation, $N \rightleftharpoons D$, as

$$N' \rightleftharpoons D + ion(s) \qquad (18)$$

where N and D still represent the (unbound) native and denatured states respectively and N' represents the bound native protein-ion complex. The new equilibrium constant, K', used to calculate ΔG_D must now take in to account the salt concentration. The change in salt concentration that arises from the release of the bound salt upon denaturation is neglible and the salt concentration, [salt], can therefore be considered to be constant. K' can be calculated using the equation

$$K' = (f_D/f_N)\,[salt] = K\,[salt] \qquad (19)$$

and ΔG_D can be calculated using the equation

$$\Delta G_D = -RT\ \ln(K') = -RT(\ln(K) + \ln[salt]) \qquad (20)$$

The ΔG_D values obtained should still be linearly dependent upon the concentration of the denaturant. Therefore, a plot of -RT ln(K) against denaturant concentration should still be expected to yield a straight line with a slope indicative of the effectiveness of the GdnHCl's ability to denature the native protein-ion complex. However, the intercept will now be equal to the ΔG_D^{salt} value plus RT ln[salt] instead of simply being equal to

ΔG_D^{salt}.

Table 4 presents the ΔG_D^{salt} values obtained for the SO_4^{-2} and phosphate salts when the binding of the ions is taken in to consideration. Note that the slope used to refit the data was the average slope obtained from the SO_4^{-2} and phosphate data only and not the average slope obtained from the non-binding salts. Another point that should be noted is that the midpoints of transition listed in Table 4, still defined as the concentration of GdnHCl at which the equilibrium constant, K, is equal to a value of one, no longer occur when ΔG_D is equal to zero.

E. ADDITIVITY

The data presented so far can confidently be used to compare qualitatively the stabilizing and destabilizing effects of the various salts and/or ions. However, the purpose of this experiment is to compare these effects quantitatively. To achieve this goal the effects must be compared directly. This can be accomplished by directly comparing the shift in the midpoint of transition or change in the free energy of stability of the protein arising from the addition of a fixed concentration of each salt. The shift in the midpoint of transition arising from the addition of 0.5 M salt was arbitrarily chosen for this purpose.

In many cases the effect of the addition of 0.5 M salt was not measured. However, a prediction of the shift in the midpoint of transition, $mdpt_{0.5}$, arising from the addition

TABLE 4

"FITTED" RESULTS CHARACTERIZING RNase A DENATURATION IN THE PRESENCE OF BINDING SALTS

Salt	[Salt]	ΔG_D^{salt}	Slope	mdpt
	M	kcal/mole	kcal/mole/M	M
Phosphate*	0.10	10.09(±0.05)	-2.81	3.11(±0.02)
Phosphate*	0.20	10.19(±0.04)	-2.81	3.29(±0.01)
Na_2SO_4*	0.15	9.89(±0.03)	-2.81	3.12(±0.01)
Na_2SO_4*	0.30	10.09(±0.05)	-2.81	3.34(±0.02)
$(NH_4)_2SO_4$	0.15	10.00(±0.02)	-2.81	3.16(±0.01)
$(NH_4)_2SO_4$	0.30	10.19(±0.03)	-2.81	3.37(±0.01)
Li_2SO_4	0.20	9.82(±0.01)	-2.81	3.16(±0.01)
Li_2SO_4	0.40	9.96(±0.01)	-2.81	3.35(±0.01)

of 0.5 M salt can now be achieved without question for all salts using the data in Figures 37 and 38. In most cases the $mdpt_{0.5}$ values were obtained by interpolation rather than extrapolation, adding to their credibility.

These values are presented in Table 5 along with the algebraic difference between the $mdpt_0$ values and the $mdpt_{0.5}$ values. Guanidinium is included as addition of 0.5 M $GdnH^+$ prior to denaturation is virtually equivalent to shifting the axes of the denaturation profile or ΔG_D plot 0.5 M GdnHCl units to the right.

With the results presented in this table the effects of various salts and even individual ions on the stability of RNase A can be compared quantitatively. In addition these results can be used to predict the effects of the salts containing more than one ion with stabilizing/destabilizing capabilities.

Table 6 presents the "observed" shifts in the midpoint arising from the addition of six salts containing more than one such ion. These "observed" shifts are the same as previously presented in Table 5. The "predicted" shifts are the algebraic sums of the "observed" shifts obtained for the individual ions involved. The "observed" value presented for $LiClO_4$ is based on data obtained while the faulty crystal was in place. Two "predicted" values are reported for the $LiClO_4$ salt. The first value is based on the "observed" shifts obtained for both the ions while the spectropolarimeter was in proper working order. The

TABLE 5

PREDICTED MIDPOINT OF TRANSITION

AT 0.5 MOLAR ION/SALT CONCENTRATION

ION/SALT	$mdpt_{0.5}$ M	difference M
Na^+	2.88	0
Cl^-	2.88	0
K^+	2.88	0
Cs^+	2.88	0
Rb^+	2.88	0
NH_4^+	2.88	0
Li^+	2.75	-0.18
$GdnH^+$	--	-0.50
Phosphate	3.83	+0.90
SO_4^{-2}	3.63	+0.73
Ac^-	3.07	+0.22
Br^-	2.53	-0.27
SCN^-	1.91	-0.96
ClO_4^-*	2.16	-0.74
ClO_4^-	2.23	-0.67
$(NH_4)_2SO_4$	3.65	+0.70
Li_2SO_4	3.45	+0.48
NH_4Ac	3.11	+0.22
LiBr	2.48	-0.44
LiSCN	1.75	-1.14
$LiClO_4$*	2.03	-0.94

TABLE 6

OBSERVED AND PREDICTED MIDPOINT OF TRANSITION VALUES

Salt	"Observed difference" M	"Predicted difference" M
$(NH_4)_2SO_4$	+0.70	+0.73
Li_2SO_4	+0.48	+0.37
NH_4Ac	+0.22	+0.22
LiBr	-0.44	-0.45
LiSCN	-1.14	-1.14
$LiClO_4$	-0.94	-0.85 (-0.92)

bracketed value is based on the "observed" shifts obtained while the faulty crystal was still in place. The results in Table 6 clearly indicates that it is not always possible to predict the stabilizing/destabilizing capability of a salt solely from the observed effects of the constituent ions. For four of the six salts, $(NH_4)_2SO_4$, NH_4Ac, LiBr and LiSCN, the "observed" and "predicted" shifts are in perfect agreement. The "predicted" shift in the midpoint of transition arising from the addition of 0.5 M $LiClO_4$ as obtained from the data collected while the CD spectropolarimeter was not functioning properly agrees remarkedly well with the "observed" value obtained from the data collected at the same time. This should be considered evidence enough that the destabilizing effect of $LiClO_4$ can be predicted from the destabilizing effects of its constitutive ions.

The lack of agreement between the "observed" and "predicted" values for Li_2SO_4 cannot be attributed to experimental error. The difference is real and should be expected. The reasons for this will be presented in the Discussion.

IV. DISCUSSION

The method of comparing the effects of neutral salts on the stability of proteins presented in this dissertation offers several advantages over those methods currently in use. However, before discussing these advantages or comparing the results obtained by this method to those obtained by other methods the validity of the assumptions made during the analysis should be reviewed.

A. ASSUMPTIONS

Several basic assumptions were made before the denaturation process could be thermodynamically analysed. These assumptions can be considered basic as they are required for the method proposed here as well as for the methods this procedure was designed to replace. The main assumption upon which the analysis was based was that the denaturation process proceeded via a reversible two-state mechanism. Also, to normalize the transition curves the solvent effects had to be assumed to be linearly dependent upon the denaturant concentration. And, to obtain the ΔG_D^{salt} values the Gibbs free energy changes were assumed to be linearly dependent upon the denaturant concentration.

Prior to the advent of high resolution scanning microcalorimeters, verifying that a denaturation process proceeded via a two-state mechanism could sometimes be a

very difficult task. Non-coincidence of normalized transition curves could be used to disprove the assumption whereas coincidence was not considered sufficient proof of the reverse situation. Kinetic studies usually proved useful in determining if a particular denaturation process proceeded via a two-state mechanism. However, kinetic studies were not applicable to this investigation because of the large heats of dilution that would have been involved.

Since the advent of high resolution scanning microcalorimeters the validity of the two-state assumption as applied to thermal denaturation studies can now be verified using true thermodynamic criteria. Privalov has reported that the ratio between the calorimetrically determined enthalpy change and the van't Hoff enthalpy change is very close to unity, as is required for a two-state denaturation process (34). Biltonen and Freire have determined that at no temperature does the fraction of intermediates exceed five percent of the total population (35-37). Pfeil has stated that "cooperativity of protein folding seems to be independent on the nature of the denaturing action, and more likely a latent feature of the protein structure" (8). He based his conclusion on a comparison of thermal denaturation results obtained by scanning calorimetry and the chemical denaturation results Creighton obtained by using urea gradient electrophoresis (38). The evidence presented in the above cited studies supports the assumption that the denaturation process

involved in this investigation can, to a very good approximation, be considered an all-or-none process.

In the present study the process was assumed to be reversible. This assumption could easily be verified by standard renaturation studies. This would involve denaturing the RNase A in excess GdnHCl, allowing an equilibrium to be reached and then reducing the GdnHCl concentration by dilution with a buffer-salt solution. Ahmad has performed such an experiment using LiBr, LiCl and NaBr and found that the process is reversible (26). Although this same experiment should have been performed on every one of the salts used in this study and repeated for several different concentrations the results reported by Ahmad were considered sufficient proof to justify making the assumption. In the future when this technique is applied to the study of other denaturants verification of the reversibility of the denaturation process should be established concomitantly.

Of the three methods currently used to estimate G_D at zero concentration of GdnHCl only one was readily applicable to this investigation. However, this was not the reason for assuming that the linear extrapolation method was the best method to use in this experiment. Several investigators have concluded that when either GdnHCl or urea is used as the denaturant the linear extrapolation method should be used to estimate ΔG_D at zero denaturant concentration. Pace and Vandenburg arrived at this

conclusion based on the results of experiments whereby they extended the denaturant concentration range by monitoring the same transition at several different pH values. After correcting for the differences attributable to the pH the data were plotted together and found to lie on the same straight line (16). Schellman proposed that the linear dependence of ΔG_D on denaturant concentration should be expected on the basis of theoretical considerations (39). Agreement between $\Delta G_D^{H_2O}$ values obtained by thermal denaturation studies and solvent denaturation studies has been used as "presumptive evidence for the linear model" (15). Also, the values of $\Delta G_D^{H_2O}$ obtained from the analysis of urea, GdnHCl and GdnHSCN denaturation data for the same protein should be the same. Ahmad and Bigelow have reported such a comparison (7). They found that for RNase A and several other proteins the $\Delta G_D^{H_2O}$ values obtained by linear extrapolation of the three sets of data were in very good agreement.

The linearity of the solvent effects, is an assumption that cannot be proven. As explained previously, for a quantitative analysis of the denaturation the solvent effect functions must be removed. Actual measurements cannot be made on denatured protein in the absence of denaturant nor on native protein in the presence of high concentrations of denaturant. Definition of the solvent effects is accomplished by extrapolating the observed low GdnHCl concentration function and observed high GdnHCl concentration function into the transition region. The less

pronounced solvent effects are more easily and more reliably extrapolated into the transition region. This was the case with the pre-transition region solvent effects. The more pronounced solvent effects, such as the post-transition region solvent effects, require more precise definition as they have a greater effect on the shape of the transition curve. Assuming that the solvent effect functions are linear is common practice with most investigators in this field of study. This assumption is as applicable to this analysis as it is to the normal solvent denaturation analysis this procedure was designed to replace.

The last assumption, common to this analysis and all other methods used to directly compare the stabilizing and destabilizing effects of various ions, was that neither the Na^+ nor the Cl^- ions have any effect on the stability of RNase A other than the non-specific stabilization that results from monopole-monopole interactions. This charge shielding stabilization or electrostatic salting-in effect depends only on the ionic strength of the solvent and is therefore independent of the salt type. As in all experiments dealing with the effects of individual ions the effect of one ion must be used as a reference. In this experiment the Na^+ ion was chosen for this purpose. By arbitrarily setting the Na^+ ion's effect on the stability of RNase A (in an aqueous NaCl and cacodylate buffer solution) at zero then the effects of all other ions could be expressed in absolute terms. It should therefore be

noted that the absolute stabilizing and destabilizing effects reported here are all relative to the effect of the Na^+ ion.

The treatment of the denaturation data presented in this dissertation differs from all previously reported treatments. The difference arises from the assumption made regarding the slopes of the ΔG_D plots. After obtaining and comparing the midpoints of transition of the GdnHCl denaturation of RNase A in the presence of several different concentrations of NaCl, KCl, CsCl, RbCl and NH_4Cl these salts were determined to have no specific effect on either the native or denatured states of the protein or on the effectiveness of the denaturant. Given that the midpoints of transition are the same for all these salts and the same value of ΔG_D^{salt} ($= \Delta G_D^{H_2O}$) must be obtained for all these salts then the slope of the ΔG_D plots must be the same in each case. The average slope obtained from a least mean squares treatment of data obtained using these five salts was then assumed to be the value expected for all salts. This excludes only those salts possessing an ion that directly interacts with either the native state of the protein, the denatured state of the protein or the denaturant. To verify this assumption the average slope value obtained for all other salts used (excluding those containing SO_4^{-2} or phosphate) was found and compared to the average slope value found for the five salts mentioned above. As expected the two values were found to be equivalent.

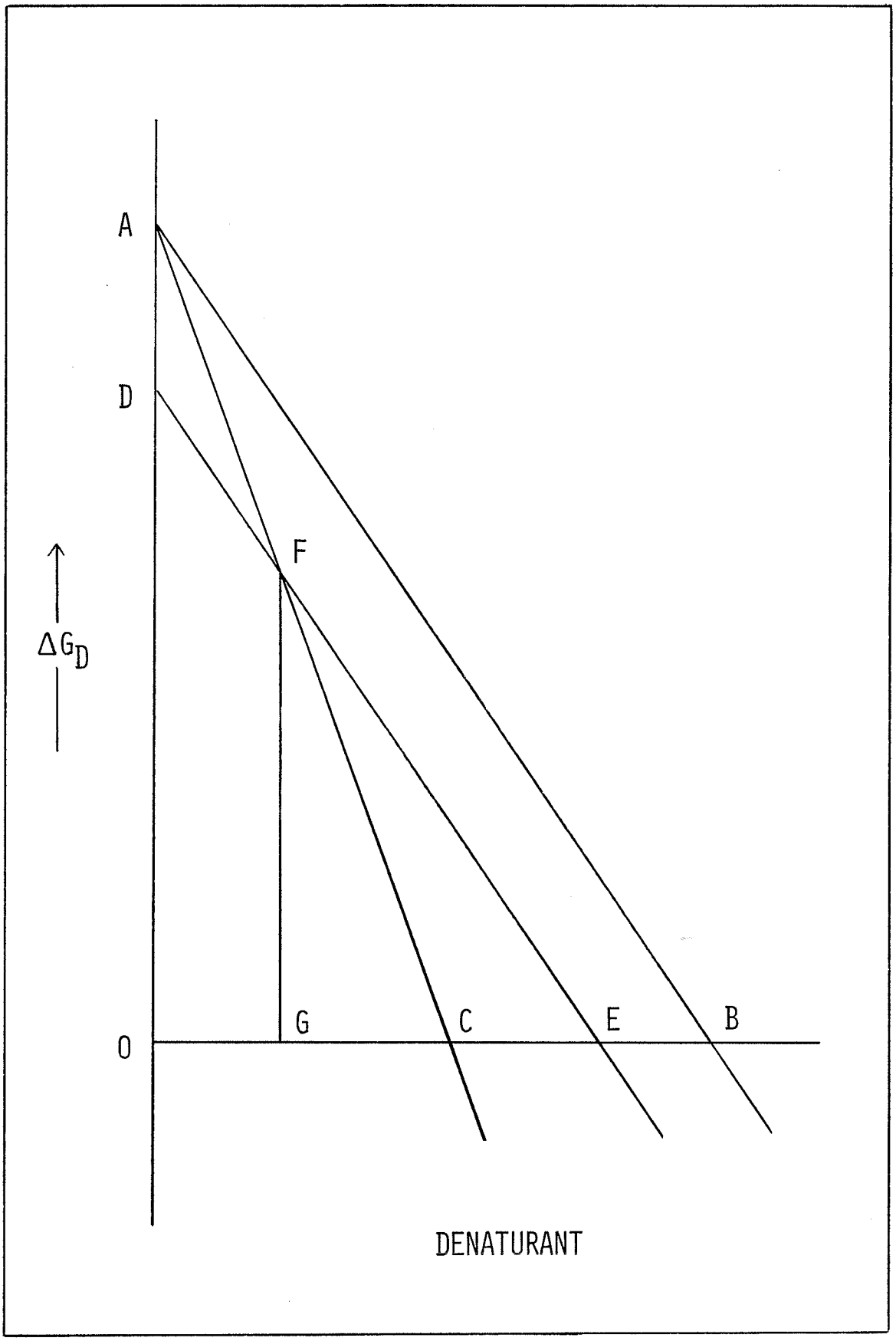
Two different methods to further verify this assumption were presented. The first method involved a comparison of the ΔG_D values obtained from GdnHSCN denaturation of RNase A with some values obtained from refitted NaSCN data. The results indicated that the proposed treatment and therefore the assumption upon which the treatment was developed was valid.

This method of verification was only included as a means of introducing the second method of verification. Using this method to individually verify that the assumption was valid for all the salts studied would have required a great deal of additional denaturation data involving some salts that are not readily available. However, the data already presented contained sufficient information to verify the assumption.

Observing a linear relationship between the midpoints of transition and the concentration of salt is sufficient verification of the assumption. This can be shown using the following hypothetical situation, presented in Figure 39. This Figure presents three hypothetical ΔG_D plots. Line AB represents the extrapolated ΔG_D values obtained for a denaturant, MCl, consisting of two ions, M^+ and Cl^-. The M^+ ion is solely responsible for the destabilizing capability of the salt. Line AC represents the extrapolated ΔG_D values obtained for a denaturant, MX, consisting of two ions, M^+ and X^-, both of which possess destabilizing capabilities. The same estimate of $\Delta G_D^{H_2O}$ is obtained when

FIGURE 39

Variation of ΔG_D as a function of the concentration of several hypothetical denaturants.


A
D
F
ΔG_D
G
C
E
B
O
DENATURANT

these two plots are extrapolated back to zero concentration of denaturant. Line DE represents the extrapolated ΔG_D values obtained for the denaturant MCl in the presence of a fixed concentration of the salt, NaX, which consists of two ions, Na^+ and X^-, with only the X^- ion possessing a destabilizing capability. The concentration of NaX added is less than the concentration of MX required to reach the midpoint of transition.

The only restriction placed on this hypothetical situation, other than that the extrapolation of the G_D values to zero salt concentration for both the MCl salt and MX salt results in the same ΔG_D value, is that the same relationship exists between the midpoint of transition and NaX salt concentration as was observed for all the salts other than those possessing either SO_4^{-2} or phosphate. That is, a plot of the midpoints of transition as a function of the NaX concentration is linear and the intercept of the function is equal to the midpoint of transition obtained when the protein is denatured with MCl salt in the absence of NaX. Because of this latter restriction the shift in the midpoint of transition observed for Line DE will occur somewhere between the midpoint of transition observed for the two pure denaturants. The actual value will dependent on the ratio of the X^- concentration, $[X^-]$, and the MX salt midpoint of transition value, $[MX]_{1/2}$. The relationship

$$[X^-] / [MX]_{1/2} = EB / BC \qquad (21)$$

must be observed. Line AC and DE must intersect at a point F such that

$$GC / OC = EC / BC \qquad (22)$$

Given this information the triangles ABO and FEG can be shown to be congruent therefore proving Lines AB and DE to be parallel. This proof holds true for all denaturants as long as the same N to D transition is induced by all denaturants involved. Excellent agreement between the hypothetical situation presented here and actual results using GdnHCl, GdnHSCN and NaSCN was obtained.

If the native state, denatured state or denaturant directly interacts with one or more of the ions in solution then deviations from the proposed trends will occur. When a strong salt-denaturant interaction occurs low concentrations of the salt will appear to stabilize the protein against the action of the denaturant. This apparent stabilization is the direct result of a decrease in the effective concentration of the denaturant. This particular situation was not observed during this investigation. However, Ahmad and Bigelow have presented some data initially suggesting that low concentrations of some salt denaturants could actually stabilize RNase A against denaturation by urea (5,6). These results were later explained as denaturant-

denaturant interactions between the carbonyl oxygen of the urea and the ions, Li^{+} and Ca^{+2}, of the salts used in their studies (26). Using their data the results that would be obtained if analysed using this proposed method is easily summarized. Plotting the midpoints of transition against the salt concentration would yield a curve, concave downward, instead of a straight line. The initial increase in the midpoint of transition occurs as a result of the reduced effective concentration of the denaturant and the later decrease in the midpoint of transition starts once an equilibrium between the two denaturants has been reached.

The deviations from the proposed trends that resulted when either a SO_4^{-2} or phosphate salt was used can be explained as the result of a direct interaction between these two ions and the native state of the protein. This binding, which would account for all the anomalous results observed while using these salts, has been reported for RNase S and RNase A (40,41). The specific binding of both the SO_4^{-2} and phosphate ion was found to take place at the active site.

The bound protein can virtually be considered a different protein from unbound RNase A. As the denaturing capability of GdnHCl varies from one protein to another it is not surprising to find that the slope of the ΔG_D plot obtained in the presence of these ions differs from that found in the absence of these ions. Equally

predictable is the fact that the slope of these plots, although dependent upon the presence of the ion, is independent of the concentration of the ion.

Only one other assumption was made during this investigation. To calculate the ΔG_D^{salt} values based on the SO_4^{-2} and phosphate data the assumption was made that only one ion binds to the active site of the native state of the protein. Although this assumption was made based on prior knowledge it was not essential and had little bearing on the outcome of the experiment.

B. ADVANTAGES

Besides being based on known facts and well founded or well precedented assumptions, the method of comparing the effects of neutral salts on the stability of proteins presented in this dissertation offers several advantages over the presently used methods. A few of these advantages are briefly presented.

A greater number of salts can be investigated. Normally the study is limited to salts that are quite soluble in aqueous solution. The effects of sparingly soluble salts which otherwise could not be investigated using the standard chemical denaturation procedure may now be studied.

The mechanism of chemical denaturation more closely approaches a two-state mechanism with guanidinium or urea than it does for almost any other type of denaturant (13). This technique is readily applicable not only to RNase A but

to any protein for which the GdnHCl denaturation process can be shown to proceed via a two-state mechanism. RNase A was chosen for this particular study because of the wealth of information already available on its denaturation. Also, this protein is commercially available in phosphate free crystalline form.

The changes in the midpoint of transition that are used to measure the salts' ability to stabilize or destabilize RNase A are easily obtained and the precision with which they can be measured allows for the detection of even the slightest effect on the stability of the protein. By varying the salt concentration and monitoring the changes in the midpoints of transition that result any specific binding involving the salt and the protein or the salt and the denaturant can be detected without the need of any additional information.

Both the native state of RNase A and the GdnHCl denatured state are well characterized and any deviation from the N to D denaturation process should be easily detected. This is unlike the thermal denaturation process where any other partially denatured state that might be produced can easily be mistaken for the thermally denatured state.

The products of ordinary chemical denaturation studies depend on the denaturant used (2-4). Urea and guanidinium salts yield the most extensively unfolded state. This state is devoid of all elements of the native structure (9,10).

The denatured states obtained in other denaturants are "intermediate" states containing residual structure. This residual structure, which is not necessarily the same for each denaturant, can be removed by addition of urea (6,7,42-44). As a result the denaturing action of different denaturants should not be directly compared.

By far the most important advantage is the improvement in the estimates of the changes in the Gibbs free energy that can be obtained using this method. The Gibbs free energy changes obtained from solvent denaturation studies are a measure of the protein's ability to withstand that particular kind of denaturing action. As such, only the Gibbs free energy change obtained from a reversible complete unfolding of RNase A should be used as a measure of the protein's stability. The approach offered here satisfies both these requirements. Any observed changes in the Gibbs free energy obtained in this study can be taken to represent the true effect of the salt on the stability of the protein. This is instead of the normally obtained measure of the protein's ability to withstand being forced into some partially unfolded state that might not exist under any other possible set of circumstances.

C. COMPARISON WITH OTHER METHODS

Because of the uniqueness of this experiment and the limited amount of published data that are currently available on this particular aspect of protein denaturation a detailed comparison of the results presented in this

dissertation with those obtained by other methods is not possible. Instead, the currently accepted beliefs and the results upon which these beliefs were formulated will be compared to the results and conclusions presented here. The bulk of the comparisons that can be made with previous studies involve the work of von Hippel and Wong (17-19). They were one of the few groups to attempt a quantitative study of the effects of salts on the stability of RNase A. The approach used by von Hippel and Wong was very similar to that proposed here. The main difference between this study and theirs is that they studied the effects of the presence of salts on the thermal denaturation of RNase A. It is from their work that the majority of the commonly accepted beliefs were derived.

Qualitatively, the rank ordering of the effective abilities of the salts to stabilize or destabilize RNase A against complete unfolding follows the classical Hofmeister series. Although some of the salts used by von Hippel and Wong differ from those used here, they too noted this same similarity between the Hofmeister series and their rank order of the relative molar effectiveness of the various ions (18,19).

When von Hippel and Wong initially published their findings they concluded that the "neutral salts lower (or raise) T_m [the thermal midpoint of transition] approximately linearly with increasing concentration" (18). They arrived at this conclusion despite the obvious

curvature contained in their plots. In a later review article von Hippel described these same plots as "not all linear, most of them showing some curvature (concave downward) at low salt concentration" (19).

The results presented here verify von Hippel and Wong's insightful conclusion. The curvature that was apparent in their results is not surprising considering the system they chose to study. At very low salt concentrations a transition between the native state and thermally denatured state would have been taking place as desired. However, as the concentration of salt was increased the chances that the end product of denaturation was the thermally denatured state decreased. This change in end product would have easily gone undetected. As mentioned earlier, it is difficult to distinguish one partially denatured state from another. When the higher concentrations of salt were used the product of denaturation was most likely the salt denatured form and therefore instead of observing the effect of the presence of salt on the thermal denaturation of RNase A they were observing the effect of temperature on the salt denaturation of RNase A.

Another insightful conclusion reached by von Hippel and Wong, equally as valid as the first yet based on the same questionable data, stated that "the total effect of a given salt on T_m is approximately the algebraic sum of the effects of its constituent ions" (18). They only attempted to predict the T_m value of one salt, LiBr. Their

predicted and observed values differed by slightly less than three degrees. Considering the magnitude of the T_m values that were involved this is a noteworthy difference. To give this value some perspective this difference is equivalent to the change in T_m that they noted upon addition of approximately 2.8 M LiCl. And, even though they concluded that NaCl has stabilizing capabilities at no concentration of this salt did they note a three degree change in T_m. In his previously mentioned review, von Hippel admits that the principle of ion additivity "is not clearly brought out in Fig. 6 (the figure from which this data was taken)" (19). However, they offered no further evidence to support their conclusion.

Again, the results presented here support their conclusion, at least when non-binding ions such as Na^+, Li^+, Cl^- and Br^- are the only ones involved. The lack of agreement between their results and the appropriate conclusion is not surprising. This, as in the case previously discussed, was the result of the unsatisfactory experimental design. This design flaw was also directly responsible for several other conclusions that have since been proven to be incorrect.

More recent work by Ahmad, in which mixed denaturants were employed in the same manner as in this experiment, conclusively established the additivity of the effects of Li^+ and Br^- (26). However contrary to what was found in this study Ahmad proposed that the slopes of the ΔG_D plots

are affected by the presence of low concentrations of salt denaturants and the extrapolated values of ΔG_D found at zero salt concentration are unaffected. The work presented here shows that this proposal is impossible when dealing with non-binding ions such as those used in his investigation. Similarly pre-transition region concentrations of partial denaturants were shown to affect the stability of RNase A contrary to the results presented by Ahmad.

D. SUMMARY

The results from this study confirmed that the effects of the constitutive ions of salts on the overall stability of proteins are additive, provided that the ions involved do not specifically bind the protein or the denaturant. Also, the results from this study confirmed the proposal that the magnitude of the effect of a salt on the stability of a protein is linearly dependent upon the concentration of the salt. For the first time ever absolute values regarding the effects of salts on the Gibbs free energy change for the complete unfolding of a protein were presented. This study also revealed a means by which the specific binding of a salt to a protein could be detected without any additional information other than that provided in denaturation profiles. The specific binding of SO_4^{-2} and phosphate to RNase A was successfully predicted.

The method proposed in this dissertation has been shown to be superior to that of any other method currently used to

determine the effects of partial denaturants on the stability of proteins. This method allows for a direct quantitative evaluation of the effectiveness of various salts to shift the equilibrium between the native and fully unfolded states of a protein. The quantity used to represent this shift can either be the directly observed change in the midpoint of transition or the change in the Gibbs free energy associated with the protein under physiological conditions. Both values are very useful as one, the change in the midpoint of transition, is an extremely sensitive indicator of changes to either the protein or its environment and the other, the change in the Gibbs free energy of stability, offers the first true thermodynamic measure of the effect of partial denaturants on the stability of proteins.

REFERENCES

1. Bigelow, C.C. (1964) J. Mol. Biol. 8:696-701
2. Sarfare, P.S. and Bigelow, C.C. (1967) Can. J. Biochem. 45:651-658
3. Kugimiya, M. and Bigelow, C.C. (1973) Can. J. Biochem. 51:581-585
4. Sharma, R.N. and Bigelow, C.C. (1974) J. Mol. Biol. 88:247-257
5. Ahmad, F. and Bigelow, C.C. (1978) Can. J. Biochem. 56:1003-1005
6. Ahmad, F. and Bigelow, C.C. (1979) J. Mol. Biol. 131:607-617
7. Ahmad, F. and Bigelow, C.C. (1982) J. Biol. Chem. 257:12935-12938
8. Pfeil, W. (1981) Mol. Cell. Biochem. 40:3-28
9. Tanford, C. (1968) Adv. Prot. Chem. 23:121-282
10. Lapanje, S. (1978) Physicochemical Aspects of Protein Denaturation, John Wiley & Sons, New York
11. Tanford, C. (1970) Adv. Prot. Chem. 24:1-95
12. Ellerton, H.D. and Dunlop, P.J. (1966) J. Phys. Chem.70:1831-1836
13. Pace, C.N. (1975) CRC Crit. Rev. Biochem. 3:1-43
14. Greene, R.F. and Pace, C.N. (1974) J. Biol. Chem. 249:5388-5393
15. Schellman, J.A. and Hawkes, R.B. (1980) in Protein Folding (Jaenike, R. ed.) 331-341,

Elsevier/North-Holland Biomedical Press, Amsterdam

16. Pace, C.N. and Vandenburg, K.E. (1979) Biochemistry 18:288-292
17. von Hippel, P.H. and Wong, K.-Y. (1964) Science 145:577-580
18. von Hippel, P.H. and Wong, K.-Y. (1965) J. Biol. Chem. 240:3909-3923
19. von Hippel, P.H. and Schleich, T. (1969) in Structure and Stability of Biological Macromolecules (Timasheff, S.N. and Fasman, G.D. eds.) Vol. 2 417-574, Marcel Dekker, Inc. New York
20. Herskovits, T.T., Behrens, C.F., Siata, P.B. and Pandolfelli, E.R. (1977) Biochim. Biophys. Acta 490:192-199
21. Castellino, F.J. and Barker, R. (1968) Biochemistry 7:4135-4138
22. Gordon, J.A. (1972) Biochemistry 11:1862-1870
23. Warren, J.R. and Gordon, J.A. (1976) Biochim. Biophys. Acta 420:397-405
24. Schrier, E.E., Ingwall, R.T. and Scheraga, H.A. (1965) J. Phys. Chem. 69:298-303
25. Pace, C.N. and Marshall, H.F. (1980) Arch. Biochem. Biophys. 199:270-276
26. Ahmad, F. (1984) J. Biol. Chem. 259:4183-4186
27. Lapanje, S. (1971) Biochim. Biophys. Acta 243:349-356
28. Nozaki, Y. (1972) in Methods in Enzymology (Hirs, C.H.W. and Timasheff, S.N. eds.) Vol. 26 43-58

29. Takase, K., Nitta, K. and Sugai, S. (1974) Biochim. Biophys. Acta 371:352-359

30. International Critical Tables (1930) Washburn, E.W. (ed.) Vol. VII McGraw-Hill Book Co., Inc. New York

31. CRC Handbook of Chemistry and Physics 57th Ed. (1976) Weast, R.C. (ed.) CRC Press, Inc. Cleveland

32. Chen, Y.H. and Yang, J.T. (1977) Anal. Lett. 10:1195-1207

33. Chen, Y.H., Yang, J.T. and Martinez, H.M. (1972) Biochemistry 11:4120-4131

34. Privalov, P.L. and Krechinashvili, N.N. (1974) J. Mol. Biol. 86:665-684

35. Biltonen, R.L. and Freire, E. (1978) CRC Crit. Rev. Biochem. 5:85-124

36. Freire, E. and Biltonen, R.L. (1978) Biopolymers 17:463-479

37. Freire, E. and Biltonen, R.L. (1978) Biopolymers 17:481-496

38. Creighton, T.E. (1979) J. Mol. Biol. 129:235-264

39. Schellman, J. (1978) Biopolymers 17:1305-1322

40. Kartha, G., Bello, J. and Harker, D. (1968) in Structural Chemistry and Molecular Biology (Rich, A. and Davidson, N. eds.) 29-37 Freeman San Francisco

41. Wyckoff, H.W., Tsernoglou, D., Hanson, A.W. Knox, J.R., Lee, B. and Richards, F.M. (1970) J. Biol. Chem. 245:305-328

42. Aune, K.C., Salahuddin, A., Zarlengo, M.H., and

Tanford, C. (1967) J. Biol. Chem. 242:4486-4489

43. Contaxis, C.C. and Bigelow, C.C. (1981) Biochemisrty 20:1618-1622

44. Ahmad, F., Contaxis, C.C. and Bigelow, C.C. (1983) J. Biol. Chem. 258:7960-7963