A STUDY OF THE THERMAL DENATURATION OF DNA BY DIFFERENTIAL SCANNING MICROCALORIMETRY

by

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A dissertation submitted to the Graduate Faculty in Chemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1974

This manuscript has been read and accepted for the Graduate Faculty in Chemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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date — Chairman of the Examining Committee

5/14/74 — Leonard H. Schwartz
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ABSTRACT

A STUDY OF THE THERMAL DENATURATION OF DNA BY DIFFERENTIAL SCANNING MICROCALORIMETRY

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The applicability of techniques of differential scanning microcalorimetry to the study of biopolymers was evaluated. The enthalpy and activation energy of the thermal denaturation of calf thymus DNA were measured with the use of the Differential Scanning Calorimeter cell of the DuPont 900 Differential Thermal Analyzer. The enthalpy of the reaction, at pH 7.0, was found to be $9.4 \pm .2$ Kcal (mole base pair)$^{-1}$. Reaction enthalpy measured over the pH range 5.4 to 8.2, varied from 7.6 to 9.4 Kcal (mole base pair)$^{-1}$. The nature of the dependence of reaction enthalpy on hydrogen ion concentration is described by a bell-shaped curve. Using the approximation that $\Delta G=0$ at T_m, the entropy of the helix-coil transition was calculated to be from 20-25 e.u. (mole base pair)$^{-1}$ and was found to be dependent on pH. Free energy of stabilization at pH 7.0, ΔG_{298}^{o}, was estimated at -2,000 cal(mole base pair)$^{-1}$.

At physiological pH and temperature (7.0 and 37°C) the estimated value for stabilization energy is -1,700 cal (mole base pair). $^{-1}$ The results of the study of reaction kinetics show that the thermal denaturation of DNA is a two step reaction. Apparent second order effects on the kinetics of the second reaction step was attributed to the high polymer concentration of the DNA solutions used. The activation energy for this reaction step was found to be 69±5 Kcal. The energy barrier for the first stage, more difficult to measure precisely, was estimated to be 200 Kcal. An hypothesis for a mechanism of the thermal denaturation of DNA is presented. Removal of a layer of bound water is advanced as the central event of the initial stage of the reaction. Friction limited unwinding is believed to be responsible for the second stage.

ACKNOWLEDGEMENT

I wish to express my deep appreciation to Professor H.W. Hoyer for his patient guidance throughout the course of this project. I would like to thank Professors I. Blei and A. Santoro for their helpful suggestions concerning this project and manuscript. I also wish to express my appreciation to Professor H.M.J. Wijnen for inspiration to continue in graduate studies.

I should like to acknowledge the City University of New York for financial assistance; Professor D. Beveridge for making computer time available and A. Noguerola for assistance in writing the computer program.

I wish to express my sincere appreciation to my fellow graduate students for many helpful discussions and to the members of my family for their continual encouragement.

To my father and to my brother, Joel, I wish to express my deep gratitude for their spiritual and moral support. Last, but not least, I would like to thank my husband, David, for his understanding during my doctoral studies and for his help in the editing of this manuscript.

This work is dedicated to the cherished memory of my mother. Her love of learning and her dedication to teaching have been an inspiration to me all the years of my life.

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INTRODUCTORY COMMENTS

Deoxyribonucleic Acid, the nucleic acid component of chromosome nucleoprotein, is of enormous biological importance in that it is the principle repository of genetic information. In order to replicate itself, the key event in cell division, or to be a template for the manufacture of m-RNA, the first step in protein synthesis, the double helix must unwind. The mechanism of the unwinding, critical to an understanding of all fundamental biological processes, is not well understood. In the laboratory this material can be converted from its native, double helical, form to disordered single strands, generally referred to as random coils. This reaction, called the helix-random coil transition, occurs when DNA is placed in acidic or in alkaline solution or is heated above the transition temperature, usually about 80°C. This latter process, thermal denaturation, is the subject of the present investigation.

During the process of thermal denaturation the DNA molecule absorbs heat, this heat being the enthalpy of the helix-coil transition. Many, fairly similar values, for the enthalpy of the helix-coil transition of DNA molecules from different sources have been published. [1] In the past ten years there have been many reports on the measurement of the enthalpy of the helix-coil transition of the

synthetic analogs; poly(A + U),[2,3] poly(A + 2U),[2,3] poly A(double stranded),[4] poly(I + C),[5] as well as that of DNA under varying conditions.[6-8] In some of these studies, the reaction enthalpy was used to estimate two of the other state functions; free energy and entropy.[2,6,8]

There have been studies seeking to elucidate the nature of the helix stabilizing forces,[2,9,10] and of the mechanism by which the helix unwinds.[7,11,12] There is still a lack of complete agreement on the value of the enthalpy of the helix-coil transition. The nature of the forces stabilizing the helix, whether hydrogen bonding, hydrophobic interactions or other forces[10] remains an unsolved problem. Also, the mechanism of the helix-random coil transition is not at present clearly understood.

Differential thermal analysis offers the possibility of studying both the thermodynamics and, by the methods of Borchardt and Daniels[13] and of Piloyan,[14] the kinetics of the helix-random coil transition. It is hoped that a combined study of the thermodynamics and kinetics of this reaction will also yield information relevant to the question of the helix stabilizing forces.

SUMMARY

This work was undertaken with the aim of developing procedures for the application of microcalorimetric methods to the study of biopolymers. The research project addresses itself to two problems: the measurement of the enthalpy of the helix-coil transition of DNA and the measurement of the activation energy of the same reaction. A study of the effect of changes in pH and in polymer concentration upon the reaction enthalpy was made with the expectation of deriving information on the nature of the helix-stabilizing forces.

The data necessary for the determination of both enthalpy and activation energy of the helix-coil transition were obtained using the DuPont 900 Differential Thermal Analyzer. The Differential Scanning Calorimeter Cell of this instrument was found to have the sensitivity and reproducibility necessary for the quantitative measurement of the heat absorbed in the helix-coil transition of the biopolymer under study. Methods have previously been developed[13,14] for obtaining the activation energy of a reaction from a thermogram, the recorder output of the DTA. Of the two methods evaluated, the method developed by Borchardt and Daniels[13] was found to be the more satisfactory one for the study of the kinetics of the denaturation of DNA.

This study contributes to the field of nucleic acid research in the following ways:

(1) The advantages and limitations of differential thermal analysis as a tool for studying biopolymers have been evaluated.

(2) A value has been obtained for the enthalpy of the thermal denaturation of DNA at temperatures above 95°C.

(3) Measurement has been made of the variation in enthalpy of the helix-coil transition of DNA with change in pH.

(4) The activation energy of the helix-coil transition of DNA has been measured.

(5) Some additional evidence has been obtained which can be applied to the problem of elucidating the nature of the helix-stabilizing forces and the mechanism by which the DNA helix unwinds.

CHAPTER I

LITERATURE SURVEY

A. Introduction

The literature directly relevant to this research problem consists mainly of reports of calorimetric studies on DNA or synthetic polynucleotides and of kinetic studies on DNA. Studies of synthetic polynucleotides are significant as these biopolymers are analogs of DNA. The heat of interaction of poly A with poly U has been found to be temperature dependent [15] and this dependence has been attributed to a variation in the degree of ordering of poly A with change in temperature. This interpretation clearly implies that base stacking interactions are responsible, at least in part, for the stability of the poly(A + U) helix. The question of stacking interactions was examined in greater detail in calorimetric[2,3] and semi-empirical theoretical[2,9] studies of the doubly and triply stranded helices of poly A and poly U. It is generally believed[2,3,15] that these interactions account for approximately 50 percent of the stabilizing forces of the poly (A+U) helix and that the complete transition from double helix to random coil occurs only at 95°C or above, the temperature at which poly A exists completely in coil form. Enthalpy values corresponding to this complete transition have been estimated.[2,3]

The enthalpy of the thermal denaturation of DNA has been mea-

sured under alkaline conditions,[8,16] acidic conditions,[7] in 2.2 Molar urea[17] (in order to obtain complete disordering of the secondary structure) and at neutral pH with varying counterion concentrations.[6,8] The necessary corrections for heat of protonation and deprotonation of the bases add considerable uncertainty to the enthalpy values for the denaturation of DNA at extreme pH's making them less useful for comparison with the results of other studies. Linear dependence of transition enthalpy on transition temperature was reported in studies on the thermal denaturation of DNA at neutral pH.[6,8] The results were similar to those obtained in studies on poly(A + U).[2,3,15] The enthalpy values reported in these papers[6,8] differ by 1-2 Kcal (mole base pair).$^{-1}$ There has been uncertainty regarding the precise value of the enthalpy of the helix-coil transition.

Relaxation techniques, combined with U-V spectrophotometry, have been applied to the study of the thermal denaturation of DNA.[7,11,12] Considerable complexity has been observed in the kinetic responses of DNA to perturbations.[7,12] The magnitude of two energy barriers has been estimated.[12] The reaction rate has been found to be dependent on many factors, such as: pH,[7] solution viscosity,[11,12] and ionic strength,[11,12] Friction limited unwinding, as a rate determining step, is basic to the proposed mechanisms.[11,12] A well-constructed model proposed by Spatz and Crothers[12] is used as a basis of comparison for the model presented in a later part of this paper.

B. Enthalpy of the Helix-Coil Transition of the Synthetic Polynucleotides

In 1963 Rawitscher, Ross and Sturtevant[15] reported calorimetic measurements on the heat of interaction of poly A with poly U using twin adiabatic calorimeters.[19] According to the First Law of Thermodynamics, the heat of interaction should be the same as the heat of the reverse reaction. The heat of interaction was measured by Rawitscher and co-workers at 10^{o}, 25^{o} and $40^{o}C$. A temperature dependence was found for the measured heat of interaction. The experiments were reported to have been performed at pH 7 and in 0.1 Molar KCl. In order to estimate the value of ΔH at $58^{o}C$, the melting temperature of the poly(A + U) polymer in 1.0 Molar KCl, the computed values of $\Delta H/T$ were graphed as a function of $T_m - T$. Transition midpoint temperature here is $331^{o}K$ and T is the temperature in degrees Kelvin at which the value of ΔH is measured. The graph shown indicates a linear relationship. It was reported that extrapolation to $T_m - T = 0$ gives an estimate of $\Delta H_{331\,^{o}K}$ as -7.6 Kcal (mole base pair)$^{-1}$. From the assumption that this reaction is equivalent to a phase change, at the temperature of which the free energy is zero, the entropy of reaction was calculated to be -23 cal $\cdot$deg^{-1} (mole base pair)$^{-1}$. The same report contained a description and the results of a study of the conformational transition of poly A from the helical to the randomly coiled form in solutions of pH from 4 to 7. This was reportedly

done in order to determine if a value of enthalpy for the ordering of poly A from the random coil form would have to be included in the enthalpy of the transition.

$$\text{poly A}_{\text{coil}} + \text{poly U}_{\text{coil}} \longrightarrow \text{poly (A + U)}_{\text{helix}} \quad (1)$$

It has been reported that poly U is known to be in the randomly coiled form at neutral pH.[2,18] Rawitscher, Ross and Sturtevant[15] reported a value of 2.4 Kcal per mole of adenine residue as the heat of ionization of poly A. Using this heat of ionization to correct measurements of the heat liberated in the pH induced conformational change, -1550 cal (monomer mole)$^{-1}$ was calculated as the heat of the poly A transition at 25°C. Spectrophotometric studies were used to determine the fraction in the helical form at pH 7 and at the reaction temperatures. The correction for the heat of uncoiling of poly A gives a value for enthalpy of reaction (1) at 331°K of -8.7 Kcal (mole base pair)$^{-1}$.

The results reported by Rawitscher, Ross and Sturtevant are in general agreement with the subsequent and very thorough study of the reactions of poly (A + U) and poly(A + 2U) reported by Krakauer and Sturtevant.[3] The later work was done with a twin cell differential microcalorimeter. The instrument was described as having two cells of almost identical heat capacities, as long as no transition takes place. When a transition occurs, heat is directed to the lagging cell. The recorder output is heat absorbed as a function of

temperature. Enthalpy, ΔH, is taken as the value of the ordinate at the temperature midpoint of the transition. The transition temperature for the helix-coil transition of poly(A + U) was found to increase with increased salt concentration. Transition enthalpy also increased with salt concentration (or with increase in transition temperatures). Values of ΔH at several transition temperature were reported as follows: 7.38 $\pm$ .08 at 44.5°C, 7.95 $\pm$ .07 at 51°C, and 8.20 $\pm$ .24 at 58°C. These values were all reported in Kcal (mole base pair).$^{-1}$ The same transition, in the presence of potassium rather than sodium ions, had measured ΔH values (in Kcal) of 6.45, 6.85 and 7.6 at transition temperatures of 36°C, 49°C and 58°C respectively. The last value was taken from the earlier reported experimental work in that laboratory.[15] The comparison of ΔH of transition in the presence of sodium to that of the transition in the presence of potassium ion indicates a dependence of the enthalpy of the reaction on the nature of the counterion. Krakauer and Sturtevant attributed this as being due to an overall reaction written as follows:

$$A \longrightarrow B + i\ M^{+} \quad +n\ H_2O \qquad (2)$$

where A and B represent states of the polymer (helical and randomly coiled states in general) and i and n represent changes in the number

of moles of counterion M^+ and of water molecules bound to a nucleotide pair (or other reacting unit). The helix-coil transition of poly (A + 2U) was also studied. The enthalpy of the reaction

$$\text{poly (A + 2U)} \longrightarrow \text{poly A + 2 poly U} \qquad (3)$$

was reported as 11.3 and 12.7 Kcal (mole base pair)$^{-1}$ at transition temperature of 45°C and 68°C in the sodium salt solutions and 9.12 and 10.00 Kcal (mole base pair)$^{-1}$ in the presence of potassium counterion at those same temperatures. Using Hess's Law and the experimental values for the enthalpies of the complete helix-coil transition of the double and triple stranded polymers, reaction (3) and the reverse of reaction (1), the enthalpy of the following reaction was calculated.

$$\text{poly(A + 2U)} \longrightarrow \text{poly(A + U)} + \text{poly U} \qquad (4)$$

The reported values were 3.9 and 4.5 Kcal (mole base pair)$^{-1}$ at temperatures of 45°C and 68°C respectively. The significance of this result is, as reported, that a non-zero enthalpy for the reaction corresponding to reaction (4) is evidence that the transition

$$\text{poly A}_{\text{(helix)}} \longrightarrow \text{poly A}_{\text{(coil)}} \qquad (5)$$

does not account for the entire enthalpy of the helix-coil transition of poly(A + U). The results of Rawitscher et al.[15] demonstrated

that the heat absorbed by reaction (5) is part of the heat required for the uncoiling of the double stranded polymer while the results of Krakauer and Sturtevant[3] strongly suggest that there must be some other force maintaining the structure of double helical poly(A + U) and, by inference, the structure of DNA as well. This is a thermodynamic approach to resolving the basic stacking--hydrogen bonding controversy.

In the paper discussed previously, Krakauer and Sturtevant compared their results with the results published earlier by Neumann and Ackermann.[20] These earlier workers published findings on the study of the reactions of the poly A-poly U complexes in 1967[20] and a more complete, revised study in 1969.[2] In the earlier work, a twin cell adiabatic calorimeter was used to measure the enthalpy of the transitions of poly(A + U) and poly(A + 2U). Citing Felsenfeld and co-workers,[21, 22] it was assumed that poly A will be in completely random coil form only above 95°C. Incomplete disordering of the poly A helical structure would be expected to reduce the measured value of the heat absorbed in the helix-random coil transition of the poly A-poly U complexes. Therefore, the heat absorbed in the transitions of these complexes was measured at different transition temperatures (achieved by changing the ionic strength of the solution in which the complexes were dissolved). The curves of ΔH as a function of transition temperature were extrapolated to find the values of the transition enthalpies at 96°C. The values reported

were as follows:

$$\Delta H^{o}_{95^{o}} (A + 2U) = 12.5 \pm .5 \text{ Kcal (mole base pair)}^{-1}$$

$$\Delta H^{o}_{95^{o}} (A + U) = 8.5 \pm .5 \text{ Kcal (mole base pair)}^{-1}$$

$$\Delta H^{o} \text{ (Triple to double stranded)}^{*} = 4.5 \pm .5 \text{ Kcal (mole base pair)}^{-1}$$

and by the difference in values of the first two reactions, the enthalpy change for reaction (4) is 4.5 ± .1 Kcal per mole of (A + 2U) formed. Krakauer and Sturtevant (1968), in comparing their results at 70°C to those of Neumann and Ackermann (1967), found that their values were slightly higher than those of the earlier work. On the basis of private communications between the groups they considered the possibility that the lower enthalpy values were attributable to the higher polymer concentrations used by Neumann and Ackermann,[20] although it would logically be expected that enthalpy would increase with increasing polymer concentration. This possibility was tested by Ackermann[23] in a study in which the enthalpy of the poly(A + U) helix to random coil transition was measured at three different polymer concentrations from 0.948×10^{-3} to 3.76×10^{-3} moles (A+U) per kilogram of solvent. The measured enthalpies were the same for the polymer at all three

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* The equation given by Krakauer and Sturtevant (1968) is
poly(A + U) = ½ poly(A + 2U) + ½ poly A

concentrations used.

In the later study[2] Neumann and Ackermann reported on calorimetric measurements of the transition enthalpy of poly (A + U). Moreover, reaction enthalpy was measured at several different temperatures. As in previous studies [3, 15] the transition temperature was increased by increasing the salt concentrations of the solutions in which the double helical polymer dissolved. The graph of ΔH as a function of transition temperature was extrapolated to find the value of ΔH at 95°C. This extrapolated value was reported to be $9.3 \pm .5$ Kcal (mole base pair)$^{-1}$. The values listed by Krakauer and Sturtevant (1968) were shown on the same graph and were shown to lie along the curve best fitting those reported by Neumann and Ackermann.[2] The two sets of measurements thus appear to be in agreement. It should be recalled that the highest reaction temperature reported by Krakauer and Sturtevant is 68°C. The increase in enthalpy with temperature was attributed by Neumann and Ackermann to the increased degree of disordering of poly A after strand separation as the temperature is increased. By comparing the enthalpy value of the poly(A + U) disordering at 42°C, where

poly A is assumed to be 50 percent in ordered form, to the enthalpy value at 95°C, where poly A is assumed to be entirely in the random coil form, the enthalpy of the transition corresponding to equation 5 is estimated to be 4.5 ± .2 Kcal (mole adenine residue)$^{-1}$. Since the value calculated for ΔH^{o}_{95} for the poly(A + U) helix-coil transition is considerably higher, there is further evidence from thermodynamic data that there are forces other than the stacking interactions of poly A responsible for stabilizing the helical structure of poly(A + U).

Neumann and Ackermann also estimated entropy and free energy of the poly(A +U) disordering. Transition entropy was calculated to be 25.2 ± 1.5 cal(mole·deg)$^{-1}$ by assuming that

$$\Delta H = T\Delta S \qquad (6)$$

at the transition temperature, where free energy is zero. The assumption, of course, is that the helix-random coil transition can be treated thermodynamically as a phase change. Using the entropy value calculated from equation 6, the free energy ($\Delta G^{o}_{298^{o}K}$) of the transition was calculated to be 1.8 ± .2 Kcal (mole base pair)$^{-1}$ from the thermodynamic relationship

$$\Delta G^{o}_{298^{o}} = \Delta H^{o} - T\,\Delta S^{o} \qquad (7)$$

There is a further assumption here that both ΔH^o and ΔS^o are independent of temperature. The assumption regarding ΔH is not nullified by the finding of a dependence of ΔH on transition temperature if Neumann and Ackermann's explanation for this effect is valid. The enthalpy at 95°C can be considered to be ΔH^o, and may be independent of temperature if the complete disordering of the separate strands can be achieved at a lower temperature. There is some degree of support to this hypothesis in the results reported by Rialdi and Profumo[17] on the denaturation of DNA in concentrated urea solutions. This study will be discussed in some detail in a later section of the chapter.

Neumann and Ackermann (1969) further interpreted their results with relation to estimation of the stacking energy of the molecule. A molar stacking energy, ϵ, was calculated where

$$\epsilon = - RT \ln \gamma, \qquad (8)$$

γ being a measure of the cooperativity and is the reciprocal of a "nucleation parameter" used in the statistical treatment of the formation of the helix from the randomly coiled separate chains. In Neumann and Ackermann's statistical treatment a "cooperative equilibrium" of stacked and unstacked bases is assumed. Trial values of γ are used in an equation relating

degree of conversion θ to τ and to a stability constant, s, taken from an optical density curve at 260 mμ. Graphs of θ as a function of 1n s are compared to the experimental optical density curve, and the best fit was found at $\tau = 200$. The molar stacking energy associated with this value is 3.2 Kcal/mole (A + U) at 298°C.

A different semi-empirical method of calculating the extent of base stacking in poly(A + U) was used by Pörschke[9] who studied short chain nucleotide complexes. Using the thermodynamic equations for the different possible transitions of the poly A-poly U complexes as a set of simultaneous equations he solved for the thermodynamic values that gave the best fit of a calculated to an experimental "phase diagram" of T_m of transition as a function of sodium ion concentration. Values for transition enthalpy obtained by Krakauer and Sturtevant[3] and Neumann and Ackermann[2] are used in the equations to reduce the number of unknowns. The particular source of a given value is somewhat unclear in the paper. It appears that the experimental values for transition enthalpy reported by these workers were used by Pörschke as stacking enthalpy. A stacking factor, x, expressing the fraction of the enthalpy

due to stacking interactions and related to temperature dependence of transition enthalpy, was found to be 0.7* by fitting the equations to experimental hyperchromicity curves for the short length nucleotide complexes. Pörschke reports the stacking enthalpy for poly A in the (A + U) complexes is -7.9 Kcal $(\text{mole})^{-1}$. This value corresponds to the enthalpy of the reaction that is the reverse of reaction (5). In addition, the formation enthalpy of poly(A + U) from the randomly coiled single strands was estimated to be -10.9 Kcal/mole based on experimental values best fitting the curves. From this method a calculated stacking enthalpy of approximately 8 Kcal/mole is also obtained using the value of 0.7 for the stacking factor. This is quite high compared to the Neumann and Ackermann value of 3.2 Kcal per mole.[2] If a stacking factor 0.7 suggests that 70 percent of the transition enthalpy is due to base stacking interactions, this value is also high compared to the ratio of 3.2 to 9.3 Kcal.

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* This author interprets Pörschke's value of a stacking factor of 0.7 as intending to suggest that 70 percent of the transition enthalpy is due to base stacking.

In summary, these studies on the poly A-poly U complexes suggest that some, but certainly not all, of the transition enthalpy of the uncoiling of the double stranded polymer is due to the stacking of the bases in poly A. Transition temperature is found experimentally to be dependent on the concentration of the counterion in solution. One group[3] found the transition enthalpy to be dependent on the nature of the counterion, supporting the idea of binding of counterions, and, by inference, water to the polymer. The transition enthalpy was measured to be between 7.6 and 9.3 Kcal/mole. When the temperature dependence of the transition enthalpy is taken into account, the higher value, corresponding to a transition temperature of 95°C, would appear to be in agreement with the other results. Based on Ackermann's interpretation this can be considered to be ΔH^{o}. This explanation also considers the change in enthalpy as being due to change in transition temperature, rather than being due to increase in counterion concentration directly. A dependence of enthalpy on counterion concentration cannot be ruled out as being at least a contributory factor to the observed enthalpy changes.

Poly(A + U) is an analog of DNA. Calorimetric investigations on the naturally occuring polymer would be expected to yield similar results.

C. Enthalpy of the Helix-Coil Transition of Deoxyribonucleic Acid

In this section a number of published papers[6-8,16,17] on the measurement of the enthalpy of the helix-random coil transition of DNA at different conditions of pH or in the presence of other materials will be reviewed.

Sturtevant and co-workers measured the heat absorbed by the denaturation of DNA from four different organisms, G-C content varying from 37-64 percent.[7] The measurements were done in acid solution and most were done at 25°C. The transition enthalpy of salmon spern DNA was measured at 5°, 25° and 40°C. Since the experiments were performed in acid solution, correction had to be made for the heat of dilution of the acid, HCl, and more importantly, for the heat of ionization of the bases. When these corrections were made on the calorimetric data (correcting to a pH of 6), the values for the transition enthalpies (ΔH_{25}°, pH_6) ranged from 7.8 to 8.3 Kcal per mole of base pair. No dependence of ΔH on G-C content could be found. Sturtevant's paper (Sturtevant et. al, 1965) also included a report of a kinetic study of the helix-random coil transition of salmon sperm DNA. The results of the kinetic study will be discussed in the last section of this chapter.

Barber[16] studied the helix-coil transition of four types of DNA in alkaline solution. In this study, the pH of the solution, rather than transition temperature was varied. The transition pH showed

a linear dependence on sodium ion concentration. Barber interpreted this as showing that alkaline denaturation of DNA can be described by an equation comparable to equation 5

$$M_x^{a+} \text{ DNA}_{\text{helix}}^{n-} + y\, M^{a+} = M^{a+}_{(x+y)} \text{ DNA}_{\text{coil}}^{(n+z)-} + zH^+ \quad (9)$$

where n represents the number of phosphate residues, x the number of bound counterions and z the number of lost protons. A linear dependence of transition pH(and, therefore, stability) on the G-C content of the DNA was also shown. Total enthalpy for the transition from helix to deprotonated coil was reported as varying between 12.0 and 13.4 Kcal(mole base pair).$^{-1}$ The high value corresponded to the transition enthalpy for calf thymus DNA. After correction for heat absorbed in deprotonation, the values of ΔH were reported to be from 6.2 to 7.6 Kcal(mole base pair).$^{-1}$ The calculations of the fraction of bases ionized at each pH (and in 0.1M NaCl) was made from the known pKa values for the bases in very dilute solutions and those in 0.2 M NaCl. It was also reported, without data, that the pK_a values vary with salt concentration.

Rialdi and Profumo[17] studied the helix-coil transition of N4 coliphage DNA in urea solution using a Calvert Calorimeter. This calorimeter has an operating temperature of 27°C. Urea concentrations lower than 0.8 moles per liter caused no changes, at 27°C, in the secondary structure of DNA and urea concentrations 2.2 Molar

or higher caused complete disruption of the secondary structure. Comparison of the heat absorbed by DNA - urea solutions containing less than 0.8 moles per liter urea to the measured heat of dilution of urea was reported to have shown little or no urea - DNA interaction. Calculations from calorimetric measurements on DNA - urea solutions* in which the urea concentration was 2.2 Molar gave a result which was assumed to correspond to the enthalpy of the helix-random coil transition of DNA. The reported enthalpy value was 9.5 $\pm$ 1.5 Kcal(mole base pair).$^{-1}$ The pH of a 2.2 Molar urea solution was stated to be 3.25. No statement was made about the possibility of protonation of the bases at this pH.

The Rialdi and Profumo study is interesting because it reports a direct measurement of what is believed to be the heat of the complete transition of DNA from the ordered double helix to the single stranded random coil. This value is very close to the value, 9.3 Kcal, reported by Neumann and Ackermann[2] for the transition enthalpy of the poly(A + U) helix. It is

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* Reported to include a correction for the heat of dilution of urea.

certainly not in conflict with the DNA enthalpy values discussed previously,[7, 16] if we accept the reason proposed for enthalpy dependence on temperature.[2, 3, 15] Barber's[16], as well as Sturtevant's[7] DNA studies were done at low temperatures, without special provision to obtain complete disordering of the secondary structure of the separated strands. Rialdi and Profumo's work[17] is also very interesting from the point of view of the H-bonding versus base-stacking controversy since urea is known to be a hydrogen bond disruptor, and the measured enthalpy is certainly no lower than that measured by other workers under what would be assumed to be more optimum conditions. This author does not wish to place too heavy emphasis on the results of Rialdi and Profumo's study and believes that interpretations of these results must be made with caution as the presence of a high concentration of urea could cause many changes.

A calorimetric study of T_2 phage DNA is of special interest with reference to the present study. In this study by Privalov the transition enthalpy is studied as a

functi on of pH and of ionic strength with the aim of deriving from the enthalpy values, and their temperature dependence, the free energy of stabilization of DNA.[6] Based on the partial derivitive of free energy with respect to 1/kT being the reaction enthalpy, written in equation form as

$$\frac{\partial \Delta G}{\partial (1/kT)} = \Delta H(T), \qquad (10)$$

the following equation can be derived to calculate free energy at a given temperature T_o from enthalpy and transition temperature, assuming that ΔH is independent of temperature:

$$\Delta G(T_o) = \Delta H(T_t) \left[\frac{T_o - T_t}{T_t}\right] * \qquad (11)$$

The calorimetric measurements were reported to have been made using an automatic model of a precision scanning microcalorimeter. The calorimeter has 0.5 milliliter gold cells and can be used to study reasonably dilute solutions (approximately 0.05 percent) of this size with good precision. Two of the

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*Equations 10 and 11 are as written in Privalov's paper - see equations 7 and 9 in that paper.

graphs in the report show the dependence first of transition temperature on pH and pNa and then of the transition enthalpy on these two variables. The graph shows for transition temperature and transition enthalpy a linear dependence on sodium ion concentration, the slope of the line being small in the latter case. The pH dependence of both transition temperature and transition enthalpy is shown as a bell-shaped curve, fairly flat in the pH region 5-8. The values of ΔH were also listed with the corresponding pH value in table form. The value of ΔH at pH 7.0 was reported to be 9.65 Kcal (mole base pair)$^{-1}$. This corresponds to a transition temperature of 84.8^{o}C. The salt concentration was 0.2 moles per liter NaCl. Free energy was calculated according to equation 11 , Privalov's equation 9 . The dependence of free energy on pH and on pNa was shown on a graph. The calculated free energy showed a linear dependence on pNa. The curve drawn through the points on the ΔG-pH graph was bell shaped, symetrical about pH 7, and flat in the region pH 5-8. The separately listed values show an approximately 10 percent decrease in the magnitude of the free energy in going from pH 7 to pH 8.5 but an extremely small decrease in going from pH 7 to pH 5. There were no enthalpy values measured at intermediate pH's. Special attention was called

to the value of ΔG_{37^o} at pH 7 and NaCl concentration 0.2 moles $\cdot$ liter^{-1} calculated to be 1.2 Kcal(mole base pair)$^{-1}$ since this value represents the estimated value of the free energy of stabilization of DNA under physiological conditions.

A very recent study by Shiao and Sturtevant[8] is the investigation of the dependence of the enthalpy of the helix-coil transition of calf thymus DNA in 0.15M phosphate buffer on pH in the alkaline region, and of the dependence of the enthalpy at pH 7, on sodium ion concentration. The reported values* of enthalpy in alkaline solution show no trend with change in pH. Corresponding entropy values, calculated by assuming ΔG is zero at the transition temperature, were all listed as being between 27 and 30 entropy units per mole. The enthalpies found at pH 7 with varying sodium ion concentration were shown graphed as a function of T_m. The enthalpy value shown for the thermally induced transition of calf thymus DNA at pH 7 is 7.2 Kcal (mole base pair)$^{-1}$. The graph of enthalpy as a function of T_m is shown to be linear. Extrapolation of the graph to a transition midpoint temperature of 95^{o}C gives an enthalpy value of approximately 8 to 8.5 Kcal(mole base pair)$^{-1}$. This is lower by approximately 2 Kcal than the value, 9.65 Kcal, reported by Privalov.[6] The enthalpy value

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* corrected for phosphoric acid ionization and shown before correction as ΔH_{app}.

is also low when compared to the results previously reported by Sturtevant[7] for the heat absorbed in the acid induced transition of four different types of DNA. In the earlier paper the enthalpy of transition at 25°C was reported to be approximately 8 Kcal and there has been substantial evidence cited here demonstrating that transition enthalpy varies with transition temperature. Comparison of results of these studies is made more difficult by the fact that the final enthalpy values reported in the earlier study were obtained after substantial corrections were made on the calorimetric data for the heats of ionization of the bases. A similar problem occurs when attempting to use the transition enthalpy reported by Barber[16] for the alkaline induced transition. Shiao and Sturtevant obtained a value of 800 cal(mole base pair)$^{-1}$ for the free energy of stabilization of the DNA double helix at 37°C and in the presence of 0.1 moles per liter of sodium counterion.[8] Their calculations included the use of a correction factor for change in enthalpy with temperature; when applying the Gibbs-Helmholtz equation to the measured reaction enthalpy. The equation used, including the correction terms, is

$$\Delta G = \Delta H_{T_m} \left(1 - \frac{T}{T_m}\right) + \Delta Cp \left(T - T_m + T \ln \frac{T_m}{T}\right) \quad (12)$$

where T is the temperature at which ΔG is being calculated, T_m is the experimental transition midpoint temperature and Δ Cp is the change in heat capacity with increasing temperature, measured from the slope of the enthalpy-T_m graph. Interestingly, a calculation of ΔG from Privalov's value for ΔH using equation 12 gives a result no different from the one published.

To summarize, the work of Privalov[6] and of Shiao and Sturtevant[8] show a definite dependence of enthalpy of the helix-random coil transition on T_m and/or counterion concentration as was found for the synthetic polynucleotides[2,3,15]. Otherwise, the studies do not show agreement in detail. All of the DNA studies mentioned report values for enthalpy of the helix-coil transition measured at counterion concentration of 0.1-0.2 moles per liter but otherwise very different experimental conditions, with T_m values ranging from 25°C to 85°C. The reported enthalpy values ranged from 7.2 to as high as 10 Kcal per mole. In comparing values it is difficult to find any trend with T_m, or

other variable. The two studies measuring the heat absorbed in the transition induced by alkalinity of the solution report the highest and the lowest value, the low value[16] reported at 25°C and the high[8] at both 57°C and at 69°C. The study of the acid induced transition[7] gave an enthalpy value more in the middle of the range of reported values but at a T_m of 25°C.

Shiao and Sturtevant[8] reported substantially different values for an alkali induced transition than for a heat induced transition (at pH 7 and at a slightly higher T_m but lower salt concentration). It must be noted that the measured heat absorbed in the acid and alkali induced transitions must be corrected to a very significant degree for heats of ionization of the bases. The two studies of thermally induced transitions,[6,8] however, report very different values under comparable conditions. Clearly, additional data on the helix-coil transition of DNA would not be superfluous.

D. Kinetics of the Helix-Coil Transition of Deoxyribonucleic Acid

Another important aspect of the study of the helix-coil transition is the study of the kinetics of the reaction. Earlier

studies generally attempted to follow the optical densitity changes brought about by heating DNA. A number of these studies have been reviewed by Rice and Doty[24] and by Peacock and Walker.[25] Assuming first order kinetics, the reported activation energy values were from 35 to 145 Kcal·mole^{-1}. Later studies relied on other techniques such as relaxation kinetics.

The temperature-jump method of studying reaction kinetics was applied to the DNA helix-coil transition by Spatz and Crothers[12]. Temperature perturbations from 0.25° to 18° were applied to the sample by passing different electric currents through it. The effect of the perturbations on the DNA molecule was followed by monitoring the optical density in the 270 mμ region. The studies were done on DNA in alkaline solution, buffered with phosphates. Temperature perturbations of different magnitudes produced different kinetic reponses. A "slow" effect was observed on graphs of optical density as a function of time when small temperature perturbations were introduced but disappeared under the influence of large temperature perturbations. The "slow" effect

was found to be temperature sensitive. It was found to have little dependence on molecular weight and appeared not to be "friction limited". The slow effect was attributed to a nucleation event producing locally uncoiled regions, not to an untwisting of large sections of the molecule. The activation energy for this process was estimated to be a minimum of 100 Kcal/mole and possibly as much as an order of magnitude higher. An effect referred to as the "fast" effect was observed on samples exposed to large temperature perturbations. The same sort of temperature perturbations produced another effect, labeled the "instantaneous" effect. The fast effect was found to be sensitive to viscosity changes (using dextran as an inert filler) although there were some unexplained anomalies in the reported results. This effect is said to be an actual unwinding of the molecule. The instantaneous effect was reported to have been too rapid for kinetic study by the methods available. It is attributed to "melting of a few base pairs at the end of helical sections, with the twist taken up in adjacent coil region" but reported to possibly be due to unstacking of bases in regions of the molecule that are single stranded at the beginning of the reaction. The proposed over-all mechanism was shown schematically.

It indicates an initial disordering in A-T rich regions with the size of the disordered regions increasing, possibly by the mechanism described for the instantenous effect, until the disordering is over a large enough region to permit the unwinding of the molecule.

Another kinetic study by relaxation methods was reported by Massie and Zimm.[11] They used "pH jumps" and "salt jumps" as well as temperature jumps for the perturbations of the system. The change in absorbance with time for the sample of DNA after any of these perturbations was followed on a spectrophotometer. The slope of the initial straight line portion of the resulting curve gives the rate constant; the reciprocal of the rate constant in this case being the relaxation time. Perturbations introduced by changes in pH and the temperature perturbations at pH 9 (where single stranded breaks were expected to be minimal) at varying ionic strength showed a dependence of relaxation time on ionic strength. The combination of experiments comprising the study showed dependence of relaxation time on bulk viscosity, concentration, molecular weight, extent of transition and the nature of the solvent. All of this evidence led Massie and Zimm to believe that the rate of

transition was "hydrodynamically limited".

Sturtevant and co-workers studied the kinetics of the helix-coil transition of DNA in acid solution using the stopped flow technique.[7] The reciprocal of unreacted fraction (as measured by absorption) is plotted against the product of the derivative of that parameter, at t=0, time of reaction, t_r. The curves of three of these dimensionless plots at differing acidities were shown along with the theoretical curves for first, second and third order reactions. The curves for this data at all three pH values failed to follow well any of the theoretical curves. Use of reaction half-times was considered to be the best way of treating the data for a complex reaction such as the helix-coil transition of DNA. Half times as a function of the reciprocal of absolute temperature gave a linear plot with an apparent activation energy of 26 Kcal(mole)$^{-1}$. It was noted that half-times are valid representations of rate constants only for first order reactions. Therefore, this is an apparent first-order activation energy. It was also noted that the term "mole" has no precise meaning in the case of this reaction. Reaction half-times were found to be, within experimental error, linear functions of pH. From the similarities of pH and temperature dependence of transition enthalpy and reaction

half-times it was concluded that reaction enthalpy and activation enthalpy were related by a constant. The results of the experiments were believed to be consistent with a co-operative reaction mechanism, having an initial phase essentially first order and the rate determining step being the disordering of a segment of the helical chain.

These studies indicate a complex reaction mechanism, likely a co-operative mechanism, having two or more phases. Neither reaction order of the rate determining step nor activation energy has been unequivocally evaluated. A great deal of study is still required on the kinetics of the helix-random coil transition of deoxyribonucleic acid.

CHAPTER II

DIFFERENTIAL THERMAL ANALYSIS

A. Introduction

The Differential Thermal Analyzer and some of the more specialized calorimetric instruments* are adaptable to a wide range of scientific investigations in analytic, organic, polymer and biochemical areas and have a sizeable number of commercial applications. DTA has previously been used in this laboratory to study the cis-trans isomerization of stilbene[26] and of oleic to eliadic acids,[27] the Diels-Alder reaction of cyclopentadiene,[28] the polymerization of styrene,[29] the decomposition of dimethyl hydrazine,[30] as well as the behavior of liquid crystals and of biopolymers on heating[32,33] and the determination of critical temperatures.[34]

The DTA has the advantages of sensitivity, enabling the use of small samples; versatility, making possible the study of many kinds of thermal transitions at slow or rapid heating rates; ease of sample handling, making possible a series of analyses in rapid succession; and a fair degree of precision, making possible its use in quantitative work. In addition, techniques have been developed to determine activation energy of reaction,[13,14]

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* The Beckman thermogravimetric analyzer and the Perkin-Elmer differential scanning calorimeter.

whose thermal behavior has been recorded on the DTA.

In this chapter the operating principles of the DuPont 900 DTA will be discussed with special emphasis on the Differential Scanning Calorimeter Cell. A comparison of the two techniques for activation energy determination by DTA is the subject of the last section of the chapter.

B. Principles of Operation of the 900 DTA

In the differential thermal analyzer a sample and a thermally inert reference (each in contact with one of a pair of thermocouples) are heated simultaneously in an insulated block. A third thermocouple is in contact with a zero degree reference such as an ice-water slurry and is part of the temperature programming system which controls the heating of the block at a preset linear rate. The reference material selected must be thermally inert in the temperature region being studied, must increase in temperature at a rate which is constant and is dependent on the rate of heat input (solid line, figure 1) and must be of nearly identical heat capacity to the sample under study. Where the sample and reference materials have the same heat capacity the sample will increase in temperature at the same rate as the reference until a transition temperature is reached. Upon reaching a transition temperature the sample will remain at that temperature until the transition is complete. It will then resume its temperature rise, gradually reaching the temperature of the reference

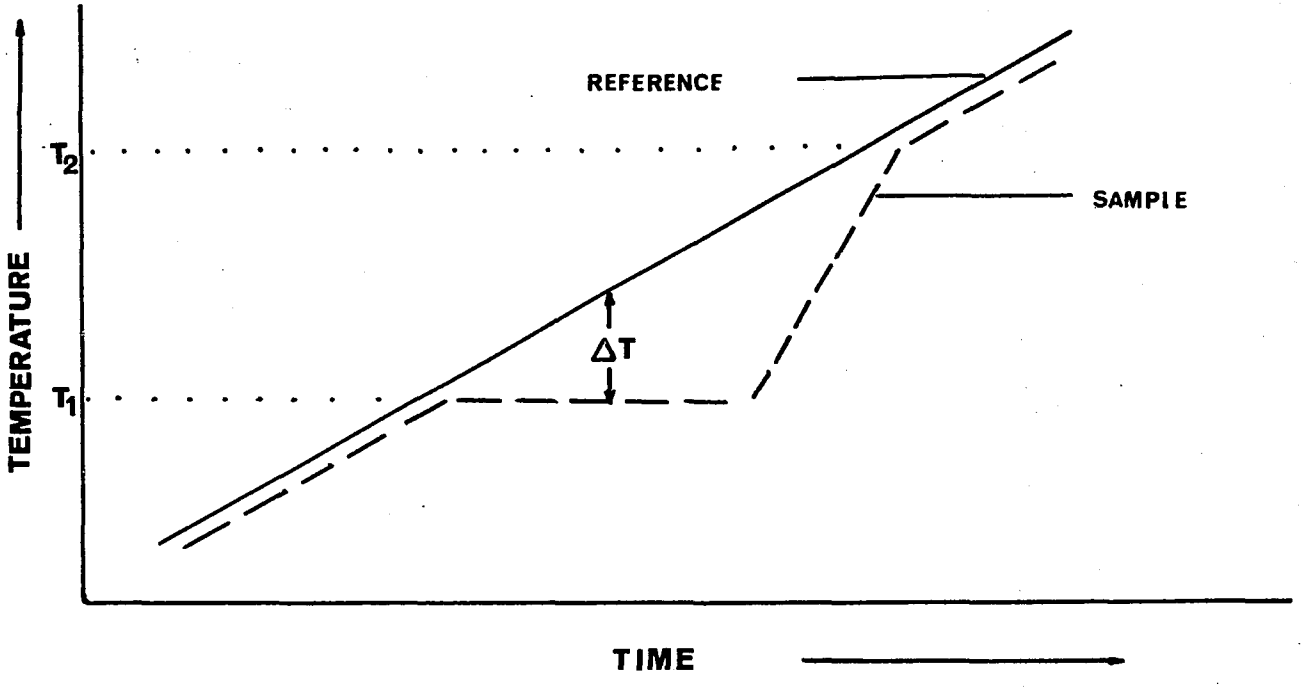

Figure 1. Thermal behavior of sample and of inert reference.

(dotted line, figure 1).

The recorder monitors the voltage difference between the reference and sample thermocouples at each temperature during the heating process. As shown by the thermocouple diagram (figure 3) the net voltage output (signal) from the thermocouples is zero when the reference and sample are at the same temperature since the individual thermocouples would then have identical voltage outputs. When there is a temperature difference between reference and sample, as when the sample undergoes a thermal transition, there is a net signal from the thermocouples to the recorder. The signal is proportional to the temperature difference between the reference and the sample. In addition, the signal can be negative or positive showing whether the sample is at a lower temperature (due to an endothermic transition) or at a higher temperature (due to an exothermic transition) in comparison to the reference. The recorder pen is deflected along the y-axis by the thermocouple signal previously discussed while also moving along the x-axis in response to a signal from the thermocouple monitoring sample temperature. Therefore the pen records the temperature difference between sample and reference (Δt) on the y-axis and sample temperature on the x-axis. The trace along

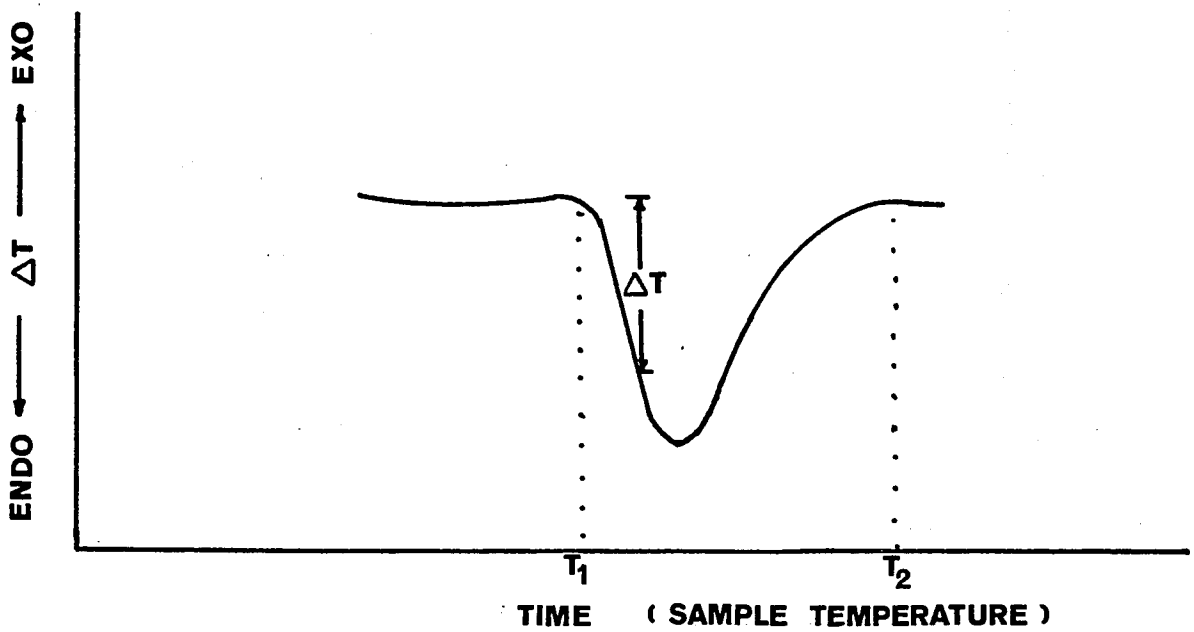

Figure 2. Thermogram of DNA in phosphate buffer, illustrating thermal behavior of samples as recorded by DTA. Temperatures, T_1 and T_2 refer to initial and final temperatures of the endothermic peak (see figure 1).

the x-axis can be taken as a recording of time since the rate of heating is linear. The recorder output, called a thermogram, is a recording of Δt as a function of time (or temperature).

An idealized thermogram is shown in figure 2. The area of the peak can be found by drawing in a baseline and tracing the peak with a planimeter. Assuming the heat capacity of the sample is constant in the region of the transition, the area under the curve is proportional to the heat absorbed or released in the transition (or reaction). Enthalpy of reaction can be calculated from the sample weight, the peak area and the calibration coefficient using equation 13.[35]

$$\Delta H \text{ (mcal/mg)} = \frac{E \cdot A \, \Delta T_s \, T_s}{M \, a} \qquad (13)$$

E is a calibration coefficient, A is the peak area, ΔT_s is the y-axis sensitivity, T_s is the x-axis sensitivity, M is the sample mass and $\underline{a}$ is the heating rate. In practice, calibration is done at the particular heating rate and the sensitivity settings which the transition is being studied . Equation 13 reduces to

$$\Delta H \text{ (mcal/mg)} = \frac{E \cdot A}{M} \qquad (14)$$

The calibration coefficient is found from the measurement

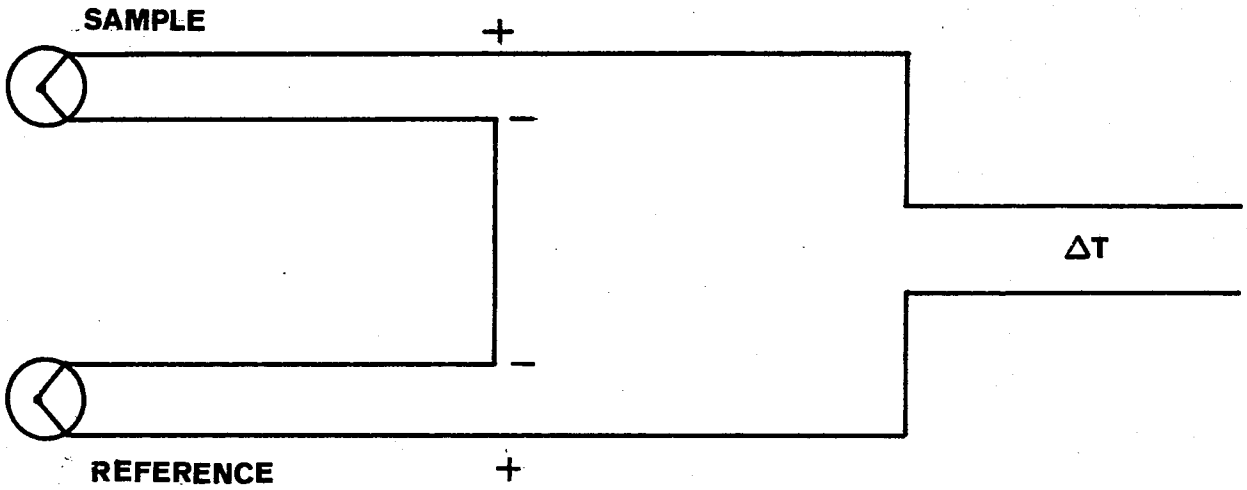

Figure 3. Diagram of differential thermocouple. Redrawn from Instruction Manual, 900 DTA (reference 35).

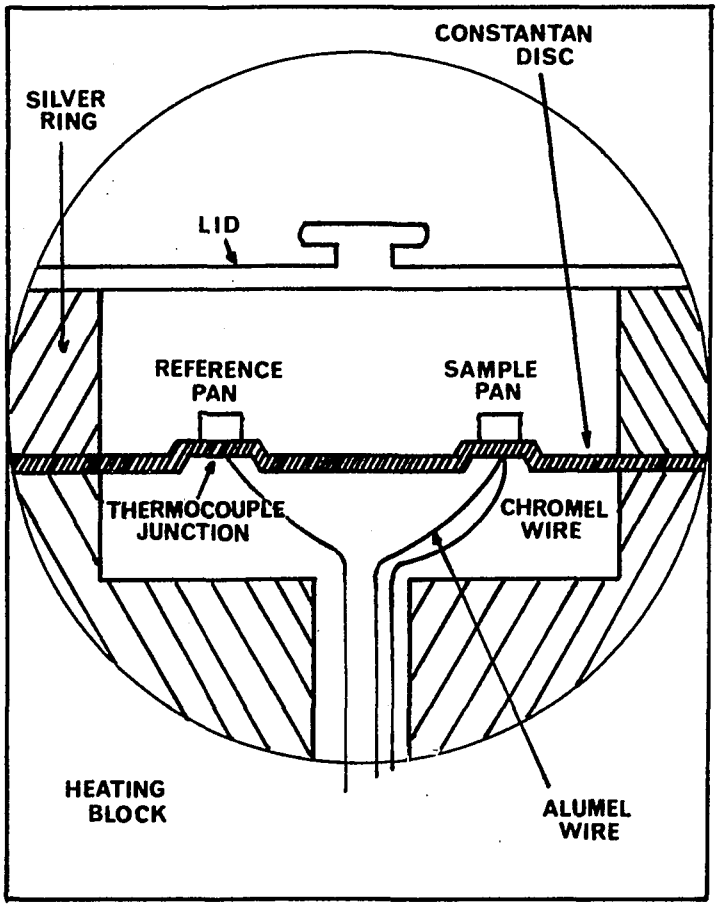

Figure 4. Cross-sectional diagram of DSC cell, redrawn from Instruction Manual, 900 DTA (reference 35).

of the peak area of the melting transition of a material with a precisely known heat of fusion. The material used for calibration of the instrument is chosen also on the basis of having a melting point approximately the same as the temperature of the transition or reaction under study. Alternately, calibration coefficients for the DTA can be determined at a series of temperatures, using different materials for calibration, and the calibration coefficient at any given temperature can be read from a calibration coefficient - temperature curve.

C. The Differential Scanning Calorimeter Cell

The experimental work in this study was done with the Differential Scanning Calorimeter cell of the DTA. It is similar in operating principle to the calorimeter cell on which the generalized previous description was based but it is more sensitive and has a y-axis deflection more directly proportional to the differential of enthalpy with time (dH/dt).[36,37]

In the DSC cell the sample and reference thermocouples are embedded in platforms on a constantan disc (figure 4). The disc serves as a path of heat transfer between sample and reference. The disc is also an element of the chromel-constantan

thermocouples used to measure temperature differences between sample and reference. The alumel wire under the sample platform forms, with the chromel disc, a chromel-alumel thermocouple. This thermocouple is part of a circuit connecting the sample platform with the cold junction thermocouples (0^{o} reference), the recorder, and the temperature programmer (see figure 5).[35] Based on the signal from the chromel-alumel thermocouple, the sample temperature is recorded on the x-axis, of the thermogram. The difference in output of the two chromel-constantan thermocouples goes to an amplifier and then to the recorder, with the signal determining the y-axis deflection of the pen. The y-axis deflection is almost directly proportional to dH/dt, as shown by the following analysis.

If we assume that temperature change of the sample (dT_s) is due to the difference between the rate of heat input to the sample and the rate at which heat is absorbed by the reaction, the mathematical expression is

$$dTs = \frac{dHs - dH/dt}{C_s} \qquad (15)$$

where dH_s is the total rate of heat input to the sample, dH/dt is the

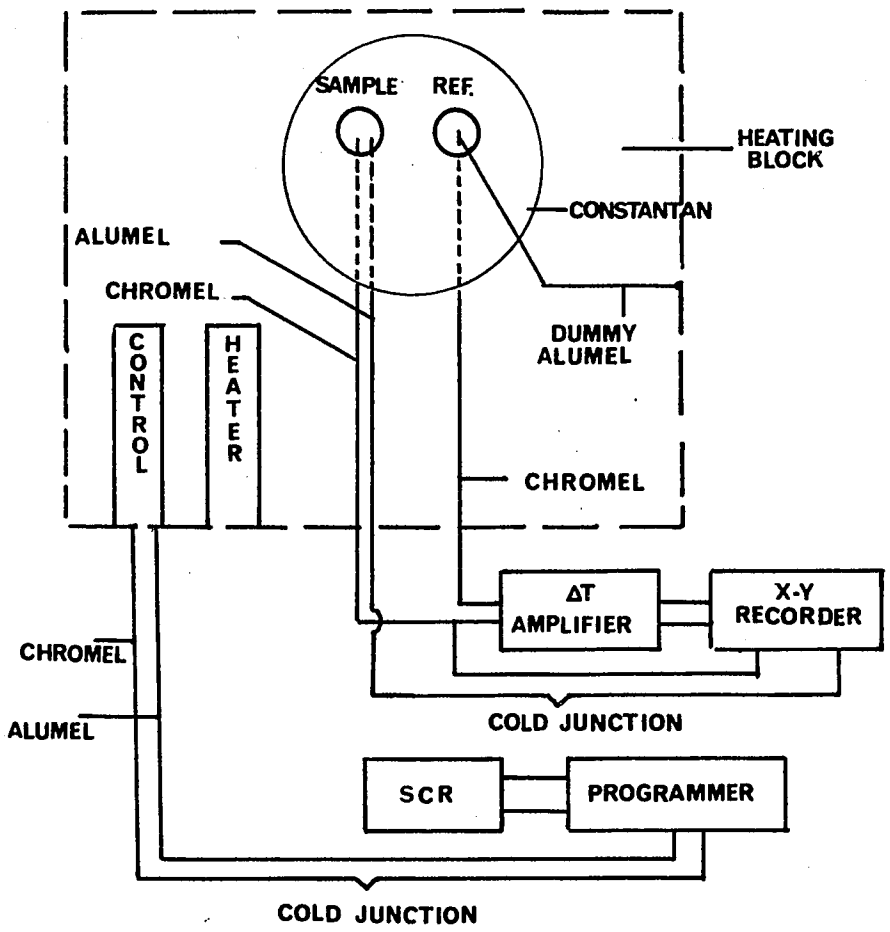

Figure 5. Schematic diagram of DSC cell. Redrawn from Instruction Manual, 900 DTA. (reference 35).

differential with respect to time of the reaction enthalpy, and C_s is the heat capacity of the sample. Heat to the sample comes from two sources; the heat transferred uniformly from the heater to all parts of the disc, and the heat transferred along the constantan disc from warmer to cooler parts of the disc. When the sample undergoes an endothermic transition or reaction the absorption of heat will give rise to small temperature differences between the sample platform and other regions of the disc. Therefore:

$$dH_s = dH_{sd} + dH_{sh} \qquad (16)$$

where dH_{sd} is the rate at which heat transferred to the sample along the disc and dH_{sh} is the rate at which heat is supplied to the sample from the heater. Equation 15 then becomes

$$dT_s = \frac{dH_{sd}}{C_s} + \frac{dH_{sh}}{C_s} - dH/dt \qquad (17)$$

While the sample is undergoing a transition the reference is linearly increasing in temperature due to absorption of heat and

$$dT_r = \frac{dH_{rh}}{C_r} \qquad (18)$$

where dH_{rh} is the heat rate at which absorbed by the reference from the heater and C_r is the heat capacity of the reference. Since both

the sample and the reference are situated in geometrically equivalent places on the disc for purposes of heat absorption and the reference is chosen to have roughly the same heat capacity as the sample, the first term on the right side of equation 17 and the term on the right hand side of equation 18 should be equal. Equations 17 and 18 can be combined to express the difference in rates of temperature increase between the sample and the reference.

$$dT_s - dT_r = d(\Delta T_{sr}) = dH_{sd}/C_s - dH/dt \qquad (19)$$

Furthermore, the reference can be considered to be at the same temperature as the rest of the disc since it is not undergoing a transition, and the rate of heat transfer to the sample from the other parts of the disc arises from Δt between sample and disc (or reference). Equation 19 then becomes

$$d(\Delta t)_{sr} = \Delta t_{sr} - dH/dt \qquad (20)$$

where Δt_{sr} is the difference in temperature between the sample and the reference and $d(\Delta t)_{sr}$ the instantaneous rate of change of the former quantity. Since there is good heat conductance along the disc, Δt_{sr} will be kept small and $d(\Delta t)_{sr}$ will be kept extremely

small as heat flow will tend to compensate for the tendency for Δt_{sr} to increase. If $d(\Delta t_{sr})$ is significantly smaller than Δt_{sr}, equation 20 can be rearranged to

$$\Delta t_{sr} = dH/dt \qquad (21)$$

A derivation for equation 21, using quantities involving heat absorption by reference and sample holders as well as the quantities discussed here and analyzing exothermic rather than endothermic reaction can be found in Baxter's article on the DSC cell.[37]

Where the length of the deflection of the y-axis is directly proportional to dH/dt the total area of the endotherm can be expressed as

$$A = \int_{x=x_1}^{x=x_2} y\,dx = \int_{t=t_1}^{t=t_2} dH/dt \cdot dt = \Delta H \qquad (22)$$

D. Activation Energy Measurements by DTA

1) Piloyan Method

Piloyan and his co-workers[14] developed a method of calculating activation energy, based on an Arrhenius-type plot, using values obtained by direct measurements on a thermogram. In the derivation of the equations used, the initial assumption is

$$\Delta t = S \frac{d\alpha}{d\tau} \qquad (23)$$

where τ is a reaction coordinate, α is the extent of the reaction,

$d\alpha/d\tau$ is the reaction rate, S is the total area under the curve and Δt is the temperature difference between the reference and the sample as measured by the length of the line from the baseline to curve at any given value of temperature, or time. (see figure 6) It can be shown that this assumption is justified.* Furthermore,

$$\text{Rate of reaction} = \frac{d\alpha}{d\tau} = A_o f(\alpha) \exp(-E/RT) \quad (24)$$

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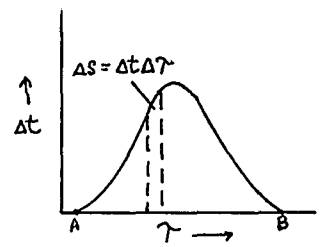
* If we draw an inverted endotherm (or an exotherm) as at right and divide it into small area segments as for a numerical integration, one area segment Δs is equal to the product of Δt and $\Delta\tau$ and,



$$\lim_{\Delta\tau \to 0} \Delta s = \Delta t \, d\tau \quad (a)$$

If we let a_n represent the partial area under the curve to point n

$$a_n = \int_A^n \Delta t \, d\tau \quad (b)$$

and the total area under the curve S is mathematically expressed as

$$S = \int_A^B \Delta t \, d\tau \quad (c)$$

The extent of the reaction to a given point can be expressed as the ratio of the partial area at that point to athe total area of the endotherm. If we let represent the extent of the reaction at any point

$$\alpha = a/S \quad (d)$$

and the rate of reaction $\frac{d\alpha}{d\tau}$ can be expressed as

$$\frac{d\alpha}{d\tau} = \frac{1}{S} \frac{d}{dt} \left\{ \int_A^{n \, d\tau} \Delta t \, d\tau \right\} = \frac{\Delta t}{S} . \quad (e)$$

Since $\frac{d\alpha}{d\tau} = \frac{\Delta t}{S}$, by rearrangement,

$$S \frac{d\alpha}{d\tau} = \Delta t \quad (f)$$

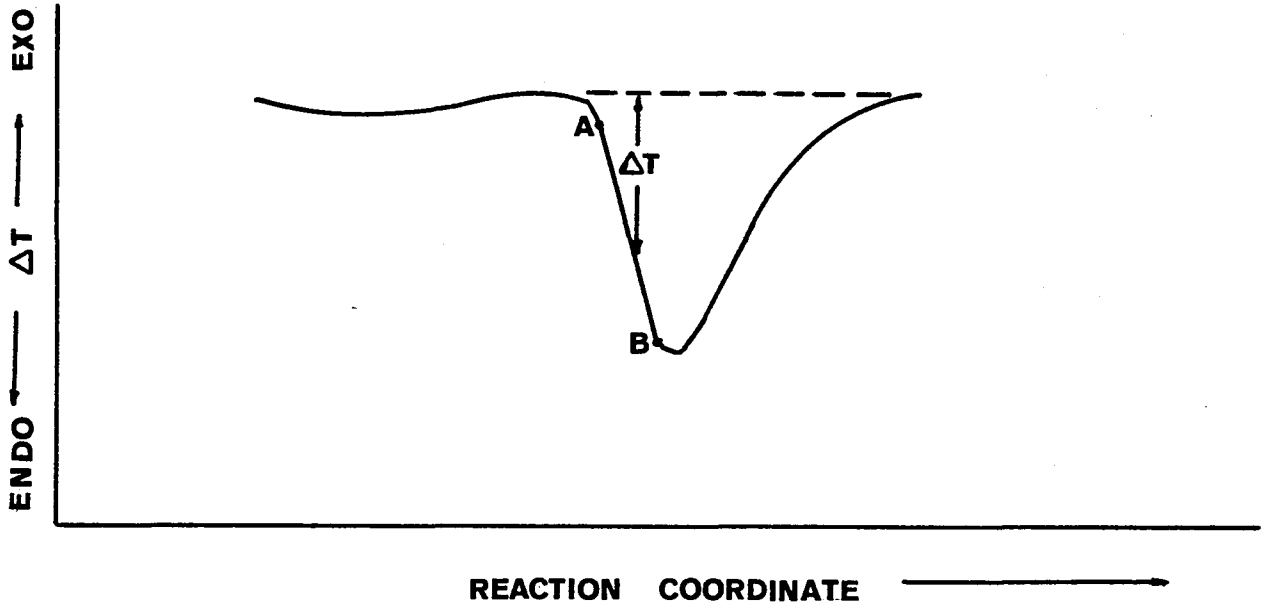


Figure 6. Thermogram of DNA in phosphate buffer illustrating method of Piloyan for calculation of activation energy.

By substitution of equation 23 into equation 24 and taking the logarithm of both sides the following equation is obtained

$$\ln \Delta t = C - f(\alpha) - E/RT \qquad (25)$$

where C is a constant. $F(\alpha)$ is, in the general case,* $(1-\alpha)^n$. Piloyan neglects $f(\alpha)$, an approximation said to be good for values of α between 0.05 and 0.8, corresponding to the region between points A and B on figure 6, simplifying equation 25 to

$$\ln \Delta t = C - E/RT \qquad (26)$$

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* If we let α, extent of reaction, be represented by $1-c/c_o$, where c is the concentration of reactant in solution at time t, then

$$c - c_o(1-\alpha) = c_o - c_o\alpha \qquad (a)$$

and

$$\frac{dc}{dt} = -c_o \frac{d\alpha}{dt} \qquad (b)$$

From the general expression for reaction rate

$$\frac{-dc}{dt} = k c^n \qquad (c)$$

and it follows therefore, that

$$-\frac{dc}{dt} = k c_o (1-\alpha)^n \qquad (d)$$

and that

$$c_o \frac{d\alpha}{dt} = k c_o^n (1-\alpha)^n . \qquad (e)$$

By rearrangement

$$\frac{d\alpha}{dt} = k c_o^n (1-\alpha)^n \qquad (f)$$

By substituting A exp (-E/RT) for k, and the general term τ for t,

$$\frac{d\alpha}{d\tau} = A_o (1-\alpha)^n \exp(-E/RT) \qquad (g)$$

The graph of the logarithm of Δt as a function of the reciprocal of absolute temperature has a slope equal to $-E/R$. For a zero order reaction $(1-\alpha)^n$ is equal to one and certainly can be neglected. To test the validity of the approximation the difference between $\log (1-\alpha)^n$ for $\alpha = .05$ and $\alpha = 0.8$ was compared to the difference between $\log \Delta t$ for the corresponding Δt values from a sample thermogram for both the n=1 and n=2. The Δt values for the thermograms in this study are very similar for a given temperature and set of experimental conditions. The difference between the corresponding $\log (1-\alpha)$ values and for the n=1 case and approximately 1.2 times the difference between the $\log (1-\alpha)$ values for the n=2 case*. Equation 26 is then an exact equation for a zero order reaction, a good approximation for a first order reaction and a fair approximation for a second order reaction.

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*For values of $\alpha = 0.05$ and 0.8, the values of $1-\alpha$ are 0.95 and 0.2, the values of $\log (1-\alpha)$ are -0.02 and .7; and the values of $(1-\alpha)^2$ are -0.04 and -1.4. The difference between the two values of $(1-\alpha)$ is 0.68 and the differences between the two values of $(1-\alpha)^2$ is 1.36. Corresponding values, on this representative thermogram, for Δt are .08 and 1.4 and $\log \Delta t$ are -1.1 and 0.6. The difference between the $\log \Delta t$ values is 1.7.

The Piloyan method has the advantage of simplicity. The derivation is straight forward. Of greater importance is the fact that the calculations are uncomplicated and can be done without the aid of a computer. The values for Δt are measured from the thermogram in units of length, and, if semilogarithmic paper is used, the only necessary calculation is the determination of the slope of the graph. The method also has a number of disadvantages. The number of points from which slope is determined is small unless the peak is spread out over a large temperature region. More than half of the thermogram cannot be analyzed, a problem that can be serious if the reaction is expected to give a complex kinetic picture. As previously shown, neglecting $f(\alpha)$ can introduce a significant amount of error.

2) Borchardt and Daniels Method

The limitations of the Piloyan method of determining activation energies are not found in the method developed by Borchardt and Daniels.[13] For the latter method equations have been derived making it possible to use the entire curve of the endotherm for data for an Arrhenius plot from which activation energy can be calculated. Slope of the plot, and therefore, activation energy, is determined from many points on the graph.

Since the entire peak can be analyzed, it is possible to recognize complexities in the kinetic picture. Borchardt and Daniels make assumptions involving instrumental accuracy, which will be discussed, but the equations used are derived directly from the Arrhenius equation and from the general expression for the rate of a reaction. Two additional advantages of this method are that there are separate equations derived for DTA and DSC and there is an expression for reaction order in the equations, enabling the experimentor to obtain additional information. The difficulty with this method is that the calculations are long and tedious and it is necessary to use a computer if more than a few thermograms are to be analyzed.

In their derivation of equations for the Arrhenius plots, Borchardt and Daniels assumed that the heat capacities and heat transfer coefficients for both reference and sample are equal. They also assumed that there is uniformity of temperature within the cells. Since small, identical metal cups are used as sample and reference holders and the sample solvent is used as the reference material, the above conditions are met. An additional assumption is that heat transfer is by conduction alone. This condition is not always met in DSC operation[35] but is met in

the temperature range in which solutions are usually studied.[13] A basic assumption is that the area of the endothermic peak is equal to the enthalphy of the reaction,

$$\Delta H = A \qquad (27)$$

and that the heat evolved or absorbed by the sample is in proportion to the number of moles that have reacted

$$dH \propto dn \qquad (28)$$

An equivalent expression for equation 28 is

$$-dn/dt = -n_o/A \times dH/dt \qquad (29)$$

where n_o is the initial number of moles and A is the total area of the peak. Equation 27 has been demonstrated to be correct for DSC operation by the discussion in this chapter on heat transfer in the DSC cell (see pages 43 to 47). Equation 28 and 29 follow directly from equation 27.

The number of moles reacted at any given time during the reaction is given by the equation

$$n = n_o - n_o \, a / A \qquad (30)$$

where a is the partial area under the curve at any given time (or

temperature) as shown in figure 7. Substitution of equations 29 and 30 into the general expression for rate of reaction gives the following expression* for rate constant, k

$$k = \frac{\left(\frac{AV}{n_o}\right)^{x-1} \frac{dH}{dt}}{(A-a)^x} \qquad (31)$$

where x is the order of the reaction.

A plot of ln k as a function of the reciprocal of absolute temperature should give a straight line whose slope is -E/R. In practice, a computer is used to find all values of a by numerical integration from the values of dH/dt measured along the ordinate of the thermogram. The computer is also used to evaluate k for all values of x at each value of T. The data set (values of k for a given value of x) that best fits a straight line determines the reaction order. Activation

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*Using as the general expression for reaction rate

$$\frac{-d(Y)}{dt} = k(Y)^x \qquad (a)$$

where (Y) is concentration and x is reaction order, by substituting n/V for (Y)

$$\frac{-d(n/V)}{dt} = \frac{1}{V}\frac{dn}{dt} = k\left(\frac{n}{V}\right)^x \qquad (b)$$

the rate constant k is given in the following expression

$$k = \frac{1}{V}\frac{dn}{dt}\left(\frac{V}{n}\right)^x = V^{x-1}\frac{dn}{dt}\cdot\frac{1}{n^x} \qquad (c)$$

Since

$$\frac{-n_o}{A}\cdot\frac{dH}{dt} = \frac{-dn}{dt} \qquad (d)$$

and $$n = n_o - \frac{n_o a}{A} \qquad (e)$$

the expression for k becomes

$$k = \frac{\left(\frac{AV}{n_o}\right)^{x-1} \frac{dH}{dt}}{(A-a)^x} \qquad (f)$$

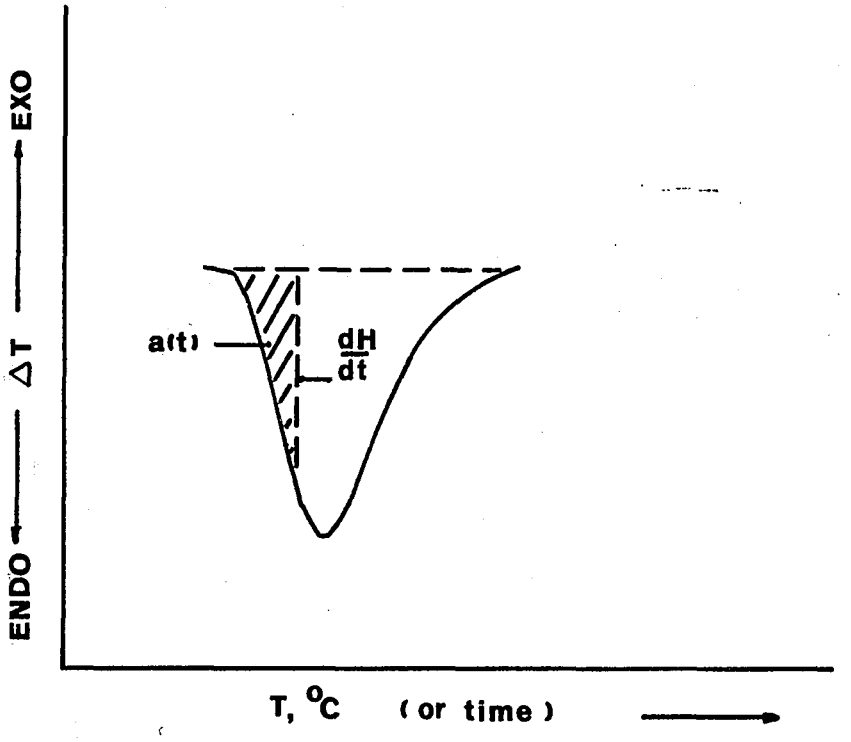

Figure 7. Thermogram of DNA in phosphate buffer, illustrating method of Borchardt and Daniels for calculation of activation energy.

energy is calculated from the slope of that line. In this study non-integer values of x were tried, as well as the more obvious small integers.

The Borchardt and Daniels method was developed for the kinetic study of simple reactions, reactions which can be described by a single rate constant. Also, the activation energy should not vary with temperature.[13] In this study, the Borchardt and Daniels methods used for the study of a more complex reaction, the helix-coil transition of DNA.

CHAPTER III

EXPERIMENTAL

The details concerning materials used in this work will be presented in this brief chapter.

DNA Polymer: All DNA solutions used in this work were prepared from the sodium salt of calf thymus DNA. The material was purchased lyophilized and used to prepare DNA solutions without further treatment. All of the polymer used for the enthalpy and activation energy studies was purchased from Sigma Chemical Company. The control number was 101C-9520. Earlier work, including the studies on salt concentration effects, was done with solutions prepared from DNA control number 41C-9560 from Sigma Chemical Company, and prior to that from control number 4776 from Nutritional Biochemical Company.

Reference Materials: The inert references used for thermograms of the DNA - buffer solutions were samples of the same buffer solutions in which the DNA was dissolved. These solutions were inert as long as no leaks occurred in the sealed pans. The buffer was used as the reference material in order to cancel out any spurious effects in the sample due to the heating of the solution and in order to have the heat capacities of the reference and sample

as nearly identical as possible.

Sample Holders: The sample and reference solutions were placed in small coated aluminum pans, or cups, DuPont part number 900757. The part number for the matching covers is 900687. The covered pans were sealed with a special sealing die and die press, DuPont part number 900733. When properly sealed these pans were found to withstand the pressures generated by the heating of the aqueous solutions to temperatures somewhat above 120°C.

Buffers; The DNA polymer was dissolved in phosphate buffers prepared by mixing ACS grade mono and disodium phosphate solutions of the appropriate concentration to the desired pH. Measurement of pH was done with a small diameter, combination Silver-silver chloride electrode* purchased from the A.H. Thomas Company. The electrode was used with a Beckmann Zeromatic II pH meter. Spot checks were done on the final DNA-buffer solutions using narrow range pH paper.

Preparation of Samples: The dry polymer was weighed out in the sample pan using a Mettler micro balance. The buffer solution was added and the sample reweighed in order to be able to determine

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*single electrode, no reference electrode required.

weight percent of DNA in solution. After reweighing, the sample cover was placed on the pan and the pan sealed with the die press. All of the prepared solutions were number coded and refrigerated approximately 20 hours. The actual time varied from 18 to 26 hours. This was done in order to be certain that the DNA had ample time to dissolve in the buffer. The precautions were taken because the resulting gels were very viscous.

Calibration: For the thermograms, special recorder paper, purchased from DuPont (part number 900325) was used. This paper has an x-axis temperature scale corrected for the specific heating rate and for errors in temperature recording arising from the use of the Chromel-Alumel thermocouples in the DTA. A voltage transformer was used with the DTA to provide voltage stabilization. The heating rate of the instrument was frequently checked during the recording of the thermograms as the calibration coefficient is dependent on heating rate. Calibration of peak areas was done with ACS grade benzoic acid and with indium samples provided with the instrument by DuPont. The calibration coefficient (see appendix 1`) used for conversion of peak areas to heat

of reaction is the average of nine trials with a standard deviation of 3 percent of the value.

CHAPTER IV

PRESENTATION OF RESULTS

A. Introduction

In this chapter the nature and shape of the DNA thermograms are described and a substantial number of kinetic plots are shown, in order to faciliate understanding of the results presented. Several sets of data relating to various aspects of the thermal denaturation of DNA are presented. Full discussion of the results is undertaken in chapter V.

B. Description of Thermograms

Thermograms of the thermal denaturation of DNA were obtained on two of the DTA cells; the Calorimeter cell and the DSC cell.

1) DNA Thermograms - Calorimeter Cell

Some preliminary work was attempted with the Calorimeter cell of the DTA. For this cell, the sample and reference thermocouples are contained in a pair of silver sample holder assemblies. The holder assemblies are obtained in pairs from the manufacturer of the DTA and are understood to be matched for electrical response to temperature variation. The sample and

reference materials were sealed in small glass sample tubes by rotating the tops of the tubes in a pinpoint flame. The tubes were then placed upright in the cuplike upper portion of the sample holder assemblies.

These preliminary trials indicated that quantititive results would be difficult to obtain with this experimental arrangement. In addition to the expected difficulty of finding a substantial fraction of the tubes improperly sealed there were major problems due to the tubes tilting in the holder assemblies and therefore making poor contact with the thermocouples. There were also significiant difficulties due to the relatively large, and unmatched, heat capacities of the glass tubes. The thermograms often showed badly sloping baselines or regions containing anomalous sets of endothermic and exothermic peaks, as shown by figure 8. In general, the thermograms lacked both reproducibility and clear definition of the endothermic peak attributable to the helix-coil transition of the DNA polymer. Figure 9 shows an example of the most reproducible pattern. The lack of sharp definition of the endothermic peak can be seen clearly by contrasting this thermogram to one obtained using the DSC cell, shown in figure 12.

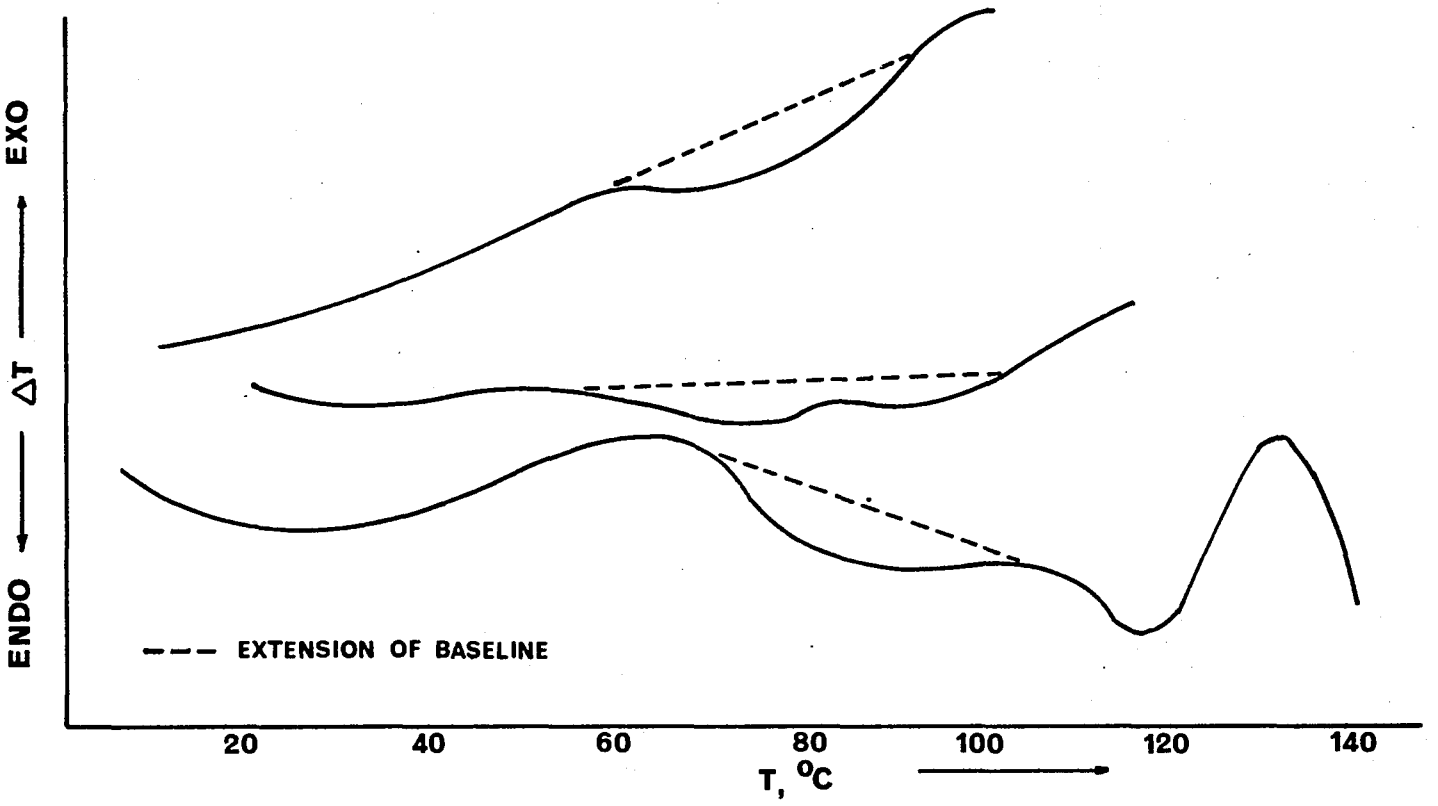

Figure 8. Thermograms of DNA in water from Calorimeter cell of 900 DTA.

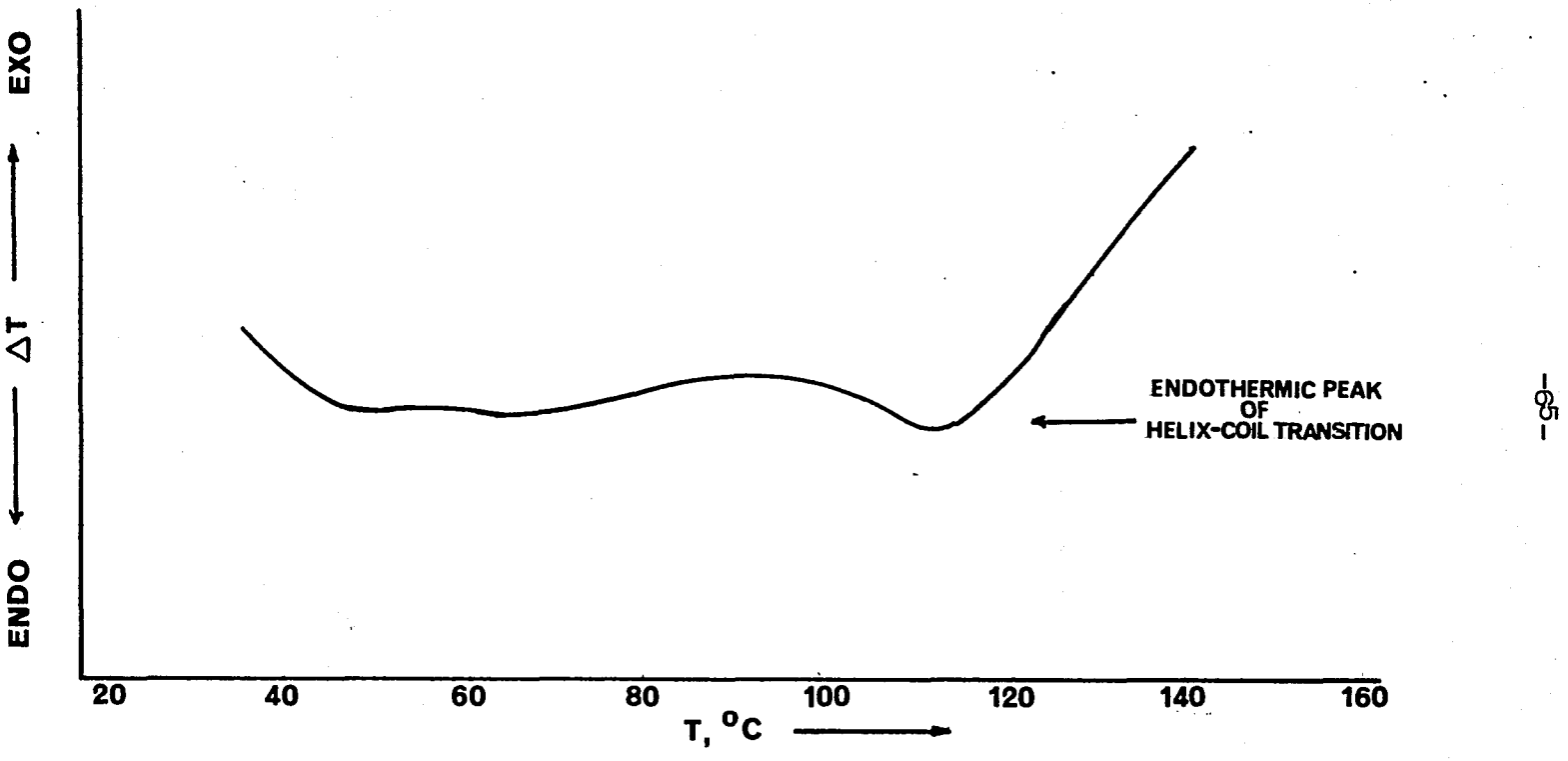

Figure 9. Thermogram of DNA in water from Calorimeter cell, illustrating thermal behavior of DNA solutions.

2) DNA Thermograms - DSC Cell

The operating principles and the configuration of the DSC cell were described in chapter II of this paper. The thermograms obtained in this cell were much more reproducible, sufficiently so, that it was possible to do the quantitative studies that are the aim of this project. When using this cell, the sample and reference materials are placed in small aluminum cups which are in turn placed on the sample and reference platforms of the cell, directly above the thermocouples. These aluminum cups can be used alone or with matching covers. A Hermetic Sealing Die Press, manufactured by DuPont, was used to seal the materials being studied. The sealing was usually sufficiently effective to withstand the pressures built up by the heating of the aqueous solutions to approximately 120 to 130°C. The effective capacity of these holders is between 15 and 20 microliters of gel or solution, (see figure 10).

When using the DSC cell and the hermetically sealed aluminum capsules the performance was, as previously mentioned, greatly improved. The baselines on most thermograms were sufficiently level to permit analysis of the thermogram for area of the peak. Reproducibility became extremely good. The two remaining

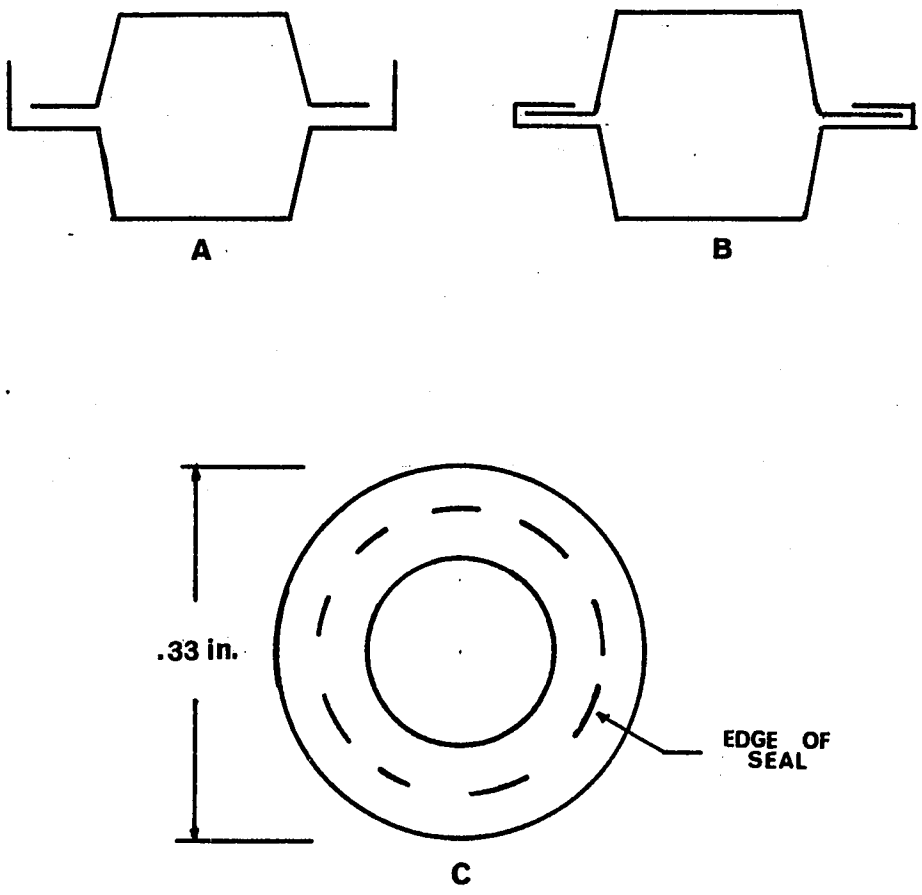

Figure 10. Sample holders used with DSC cell. (A) Side view, unsealed. (B) Side view, sealed. (C) Top view.

problems were leaking capsules and instrument noise. The noise problem was sporadic but fairly infrequent. When the reference capsule was improperly sealed and leaking badly the pen moved sharply in the same direction as a response to an exothermic reaction. A badly leaking sample capsule caused the pen to move sharply in the opposite direction. When the seals on the capsules held to 120°C or better it was usually possible to determine the point of return to the baseline and therefore the actual shape and size of the area enclosed by the curve and the extended baseline. Leaks that became apparent after this temperature caused little or no problem. The thermogram in figure 12 is an example of the shape of the curve obtained when the reference begins to lose water (and absorb heat) only after reaching a temperature a few degrees higher than the temperature corresponding to the end of the transition being studied. When water loss from either sample or reference capsule was apparent at lower temperatures there would be considerable distortion of the shape of the thermogram. Since the calorimetric data is based on size of the area of the peak, distortion of the shape of the curve would render the particular thermogram useless for the calorimetric study. In many instances where the thermograms were badly distorted, the capsules were found after heating to have accumulated small droplets of water along the seal. Cases of very minor amount of water loss from the

capsules introduced some uncertainty into the exact location of the baseline. This in turn introduced some experimental error into the area measurements. Estimates of the location and slope of the baseline and the shape of the final portion of the curve are made particularly difficult by the asymetry of the endothermic peak obtained with the DSC cell for the helix-coil transition of the DNA polymer. Figure 11 shows several examples of distorted thermograms.

Uncertainties or irregularities in the slope of the baseline were of greater concern when attempting the determination of activation energy for the transition by either the Piloyan or the Borchardt and Daniels method. The same thermograms used for the determination of reaction enthalpy can also be analyzed for activation energy by either of the methods mentioned above. In practice, it was necessary to select for kinetic study the thermograms with the most regular and level baselines from among those used for the calorimetry study.

C. Transition Midpoint Temperatures (Tm)

The thermograms of solutions of calf thymus DNA in phosphate buffer showed a single, slightly asymetric, endothermic peak. This endothermic peak is clearly due to the heat induced transition of the double stranded polymer to the random coil form. The onset

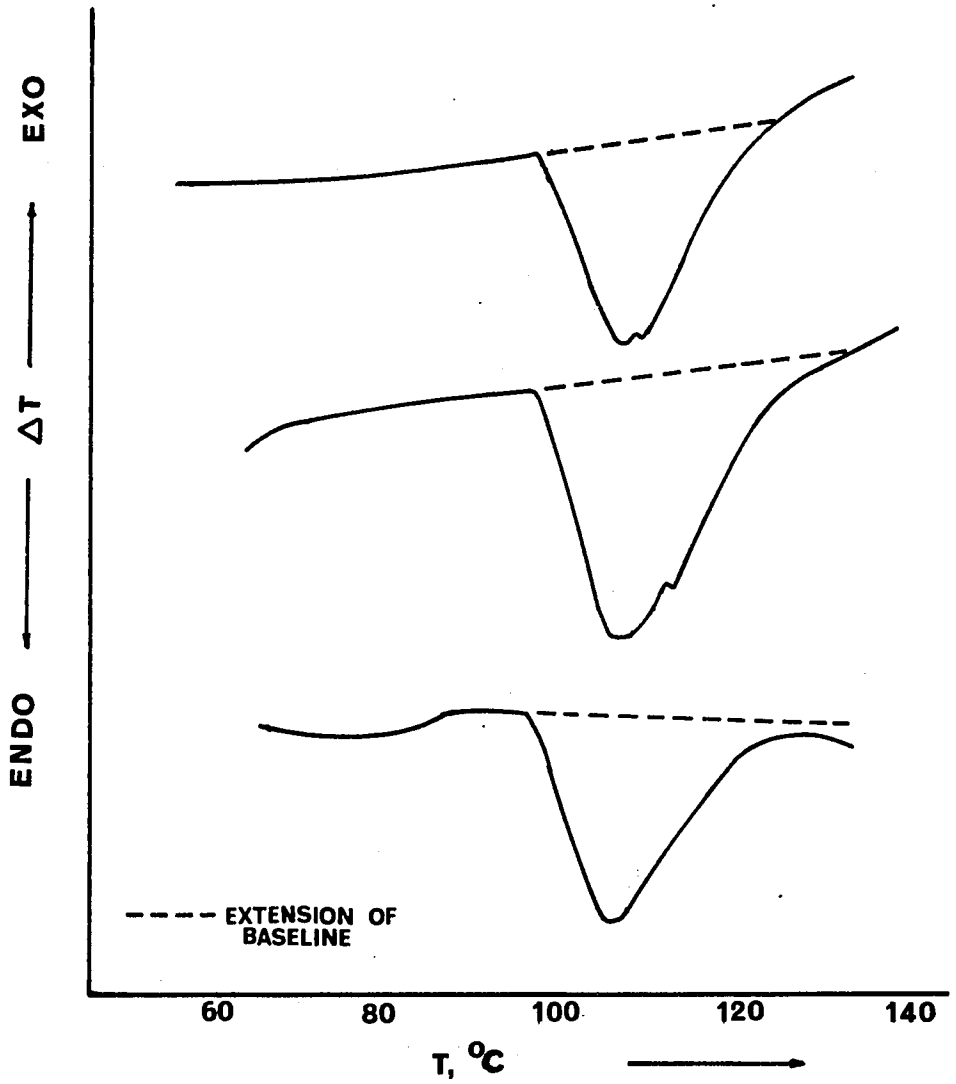

Figure 11. Thermograms of DNA in phosphate buffer, from DSC cell.

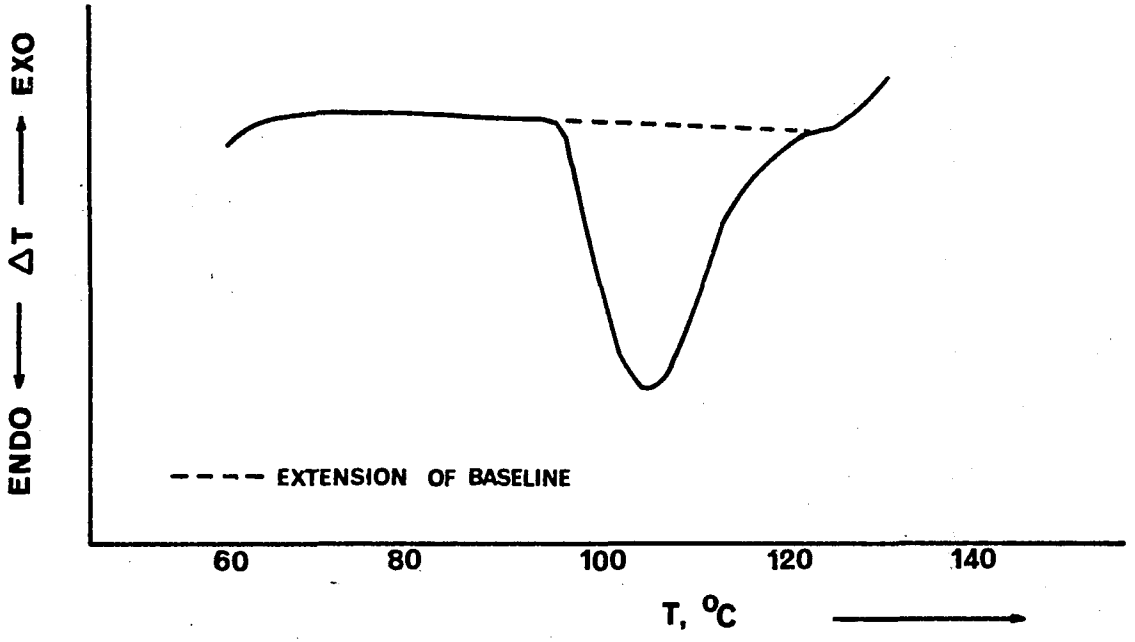

Figure 12. Thermogram of DNA in phosphate buffer, from DSC cell, illustrating the thermal behavior of DNA solutions.

temperature of the transition occured at 94-96°C. The asymetry involved a drawing out of the transition near the end rather than a sharp return to the baseline comparable to the fairly steep slope from onset to the peak temperature. The peak temperatures occured at 102-106°C. The gradual return to baseline, or tailing effect, carried the transition to temperatures slightly above 120°C. Midpoint temperature (T_m) of the transition as followed by DTA is taken to be the temperature at which the partial area under the curve to that point of the temperature axis (calculated by numerical integration) is equal to 50 percent of the total area under the curve. The values of T_m were found to be 106°C for pH 5.4 to 7.4 and 104-105°C for pH 7.8 and 8.2. The finding that T_m is slightly higher than the peak temperatures is a further reflection of the asymetry of the endothermic peak.

D. Enthalpy of the Helix-Coil Transition of DNA

1) Maximum Value

The transition enthalpy was measured with the DSC cell under a variety of experimental conditions with regard to pH, ionic strength and polymer concentration in order to obtain information about the effect of changing conditions on the binding forces in the DNA molecule. A maximum value for the transition enthalpy was found at pH 7.0, buffer concentration 0.75 moles per liter and a polymer concentration of approximately 5 percent DNA by weight. It is 9.4 + .2 Kcal per mole base pair.

2) Dependence of transition enthalpy on pH.

Variation of transition enthalpy with changes in pH was studied at a fixed ionic strength and a narrow range of polymer concentration. The pH range was 5.4 to 8.2, at intervals of 0.4 pH units. The enthalpies were calculated from the thermogram areas, weights of DNA used and the average weight per base pair of calf thymus DNA. For the detailed description of the calculations see appendix 1. The average values for the enthalpy and the standard deviations are summarized in table I. Figure 13 gives the enthalpy dependence on pH.

As previously stated, the maximum enthalpy value was found at pH 7.0. The minimum values are found at the highest and the lowest pH values of the range. Greatest stability at neutrality was not surprising since it is known that DNA can be denatured by acid or alkali as well as by heat. The curve (figure 13) is fairly symetrical about pH 7 and the arrangement of points suggests a stepwise decrease as the pH is changed. The standard deviations of these values are large compared to the actual size of the differences between the values, thus making it difficult to determine the precise shape of the curve.

In the course of studying the effect of pH changes it was advisable to check on the degree of ionization of the bases of the monomer units. Percentage ionization was calculated by applying the Henderson-Hasselbalch equation to the bases, using the

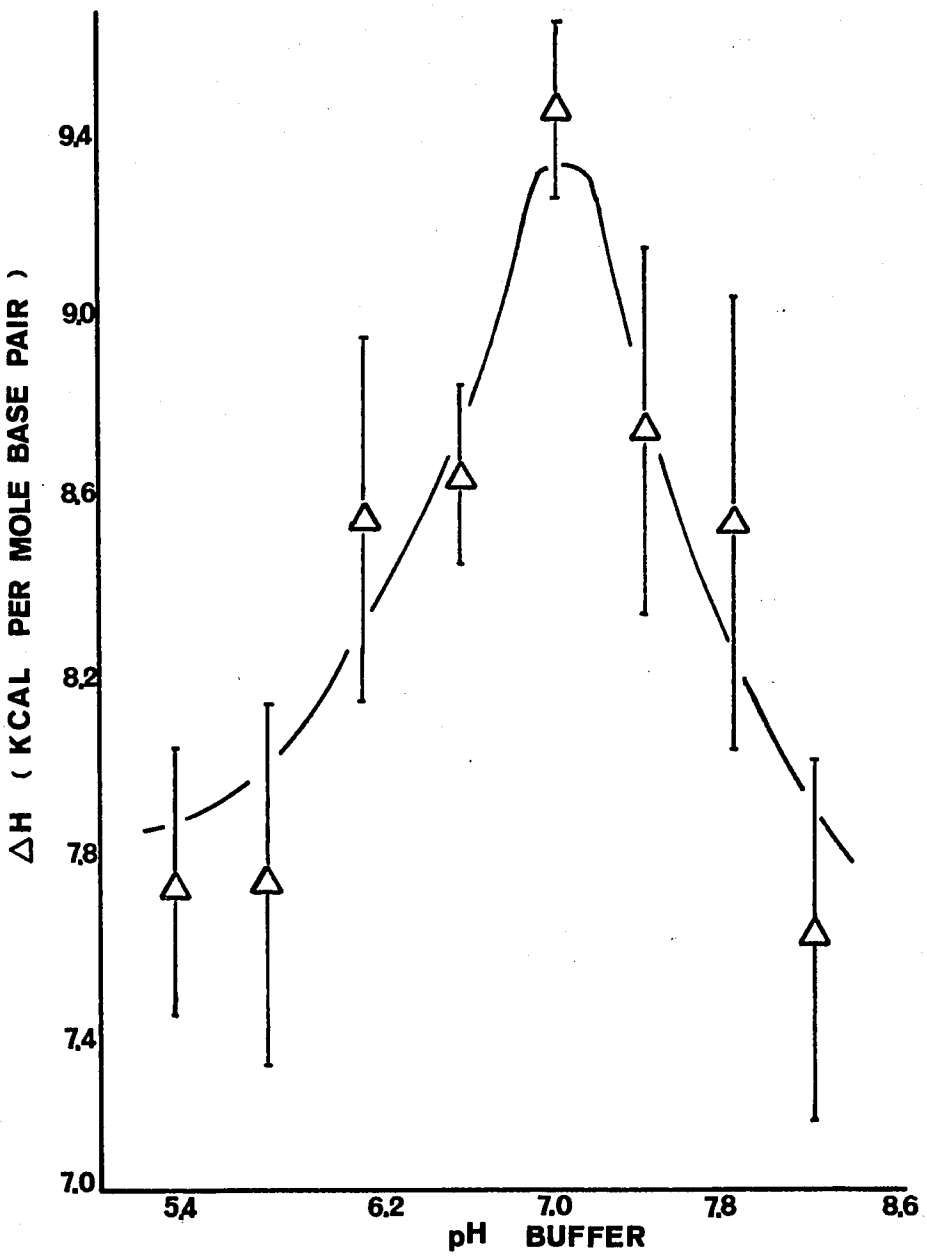

Figure 13. Enthalpy of helix-coil transition of DNA as a function of pH. Phosphate buffer, 0.75 moles (liter).$^{-1}$

TABLE I

Changes in Enthalpy of Helix-Coil Transition with Change in pH

pH	Enthalpy (Kcal. per mole base pair)
8.2	7.6 ± .4
7.8	8.5 ± .5
7.4	8.7 ± .4
7.0	9.4 ± .2
6.6	8.6 ± .2
6.2	8.5 ± .4
5.8	7.7 ± .4
5.4	7.7 ± .3

TABLE II

Percentage Ionization of Bases

pH	Percentage decrease of ΔH from maximum	Percentage Ionization of Bases C	A	G	T	Total**
8.2	19	--	--	.2*	neglig.*	neglig.
7.8	9.5	--	--	--	---	---
7.4	7.4	--	--	--	---	---
7.0	---	--	--	--	---	---
6.6	8.5	1	1	--	---	1
6.2	9.5	3.6	1	--	---	2
5.8	18	10	1	--	---	5
5.4	18	23	2	1	---	13

* Proton removal.
** Divided by 2 to reflect percentage of base pairs effected.

experimental pK_a values shown in the literature[38] for the bases in the polymeric form of calf-thymus DNA rather than as free bases. Percentage ionization of the bases as a function of pH is shown in table II. Although the ionization (protonation) in acid solution is large enough to account for a substantial part of the enthalpy changes, the calculations do not indicate any significant amounts of deprotonation of the bases even at the highest pH used here.

3) Effect of Polymer Concentration

The effect of changes in DNA concentration on the enthalpy of the helix-coil transition was studied over the range of one to eight percent polymer by weight. When the polymer weights are corrected for water present in the polymer, the concentrations of actual DNA present are calculated to vary from approximately 0.6 to 6.0 percent by weight. The graph of enthalpy as a function of DNA concentration is shown in figure 14. Each data point represents an enthalpy value calculated from the area of the endothermic peak of a single thermogram. Concentrations were difficult to reproduce precisely, making averages over small concentration ranges less meaningful than a least squares treatment of the set of individual data points. Clearly, there is more scatter of points at the upper concentration part of the graph. For this reason, the least squares treatment was done twice. The solid line represents the slope and intercept calculated for all of the points except the one circled. That point was rejected as it had a deviation more than twice the standard deviation (including the point). The

dotted line represents the second statistical treatment, in which the other four points at the highest concentations were omitted. In addition to the increased scatter of points in this region, there appears to be a possibility of a leveling of the curve in this region, as indicated by the continuation of the seoond line.

The average enthalpy at pH 7.0, from the pH study, supplies addition evidence for de-emphasizing the points in the high concentration region of the graph. This value (triangle on the graph) can be seen to lie on the hand drawn curve which neglects these points. Nothing definitive can be said about extending the graph beyond the data points. Logically, the reaction enthalpy will not increase indefinitely and the leveling off (as shown by the curved line) can be considered a likely possibility based on the data.

The correlation coefficient for the solid line on this graph is 0.840 and the correlation coefficient for the interupted line is 0.925 (excluding the last four points). The correlation coefficients for these lines of non-zero slope are high enough to be considered as evidence for a dependence of transition enthalpy on the concentration of polymer in solution. However, the picture is not entirely clear when taking into account transition enthalpies reported by other workers for DNA and for poly(A + U) at pH 7 but at much lower

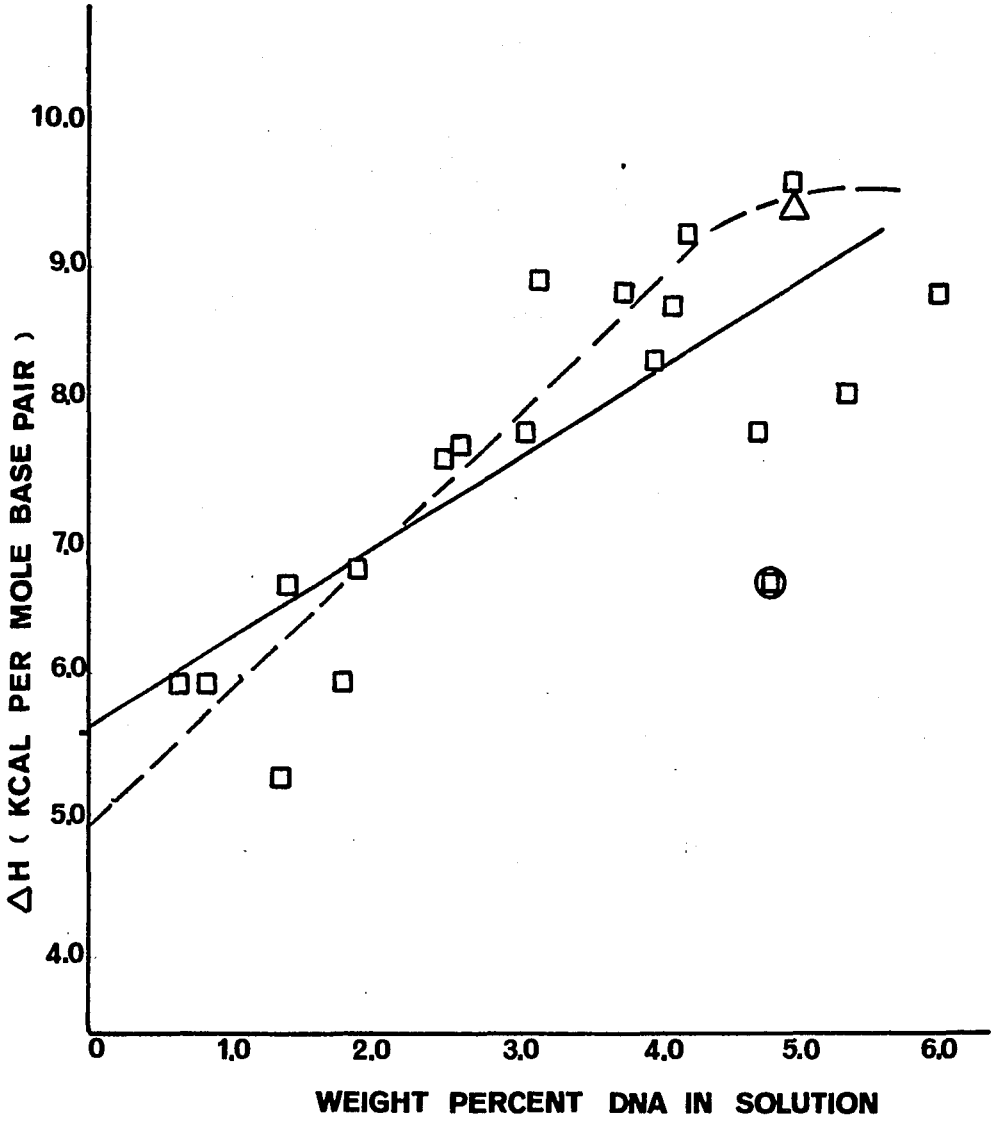

Figure 14. Enthalpy of helix-coil transition of DNA as a function of DNA concentration. Conditions: buffer concentration 0.75 moles $(liter)^{-1}$ (phosphate buffer), pH=7.0.

———— Least squares analysis, all points.

– – – – – – – – – Least squares analysis to 4 percent by weight DNA, hand drawn extension.

△ From results of pH study.

◎ More than twice standard deviation.

polymer concentrations. Shiao and Sturtevant's[8] reported value of 7.2 Kcal for the transition enthalpy of calf thymus DNA (equivalent to 8 or 8.5 Kcal at 95°C) is the one value low enough to allow for a concentration dependence when comparison is made to the results obtained in this laboratory. The lower value can also be considered to be due to the lower counterion concentration used by Shiao and Sturtevant in their work.

3) Effect of Buffer Concentration

One of the preliminary experiements was the measure of peak areas of thermograms of DNA in solutions of different buffer concentrations. The high polymer concentrations were believed to require relatively large buffer concentrations. The largest peak areas were found for DNA in buffer solution for which the concentration was 0.75 moles $\cdot$liter.$^{-1}$ The results of this preliminary experiment are shown in graph form in figure 15. The results are reported simply as peak area (converted to calories by a calibration coefficient) per gram of weighed polymer. As can be seen, the standard deviations are very large relative to the differences between the data points. Nevertheless, some trend was indicated and, as a result, the subsequent work was done

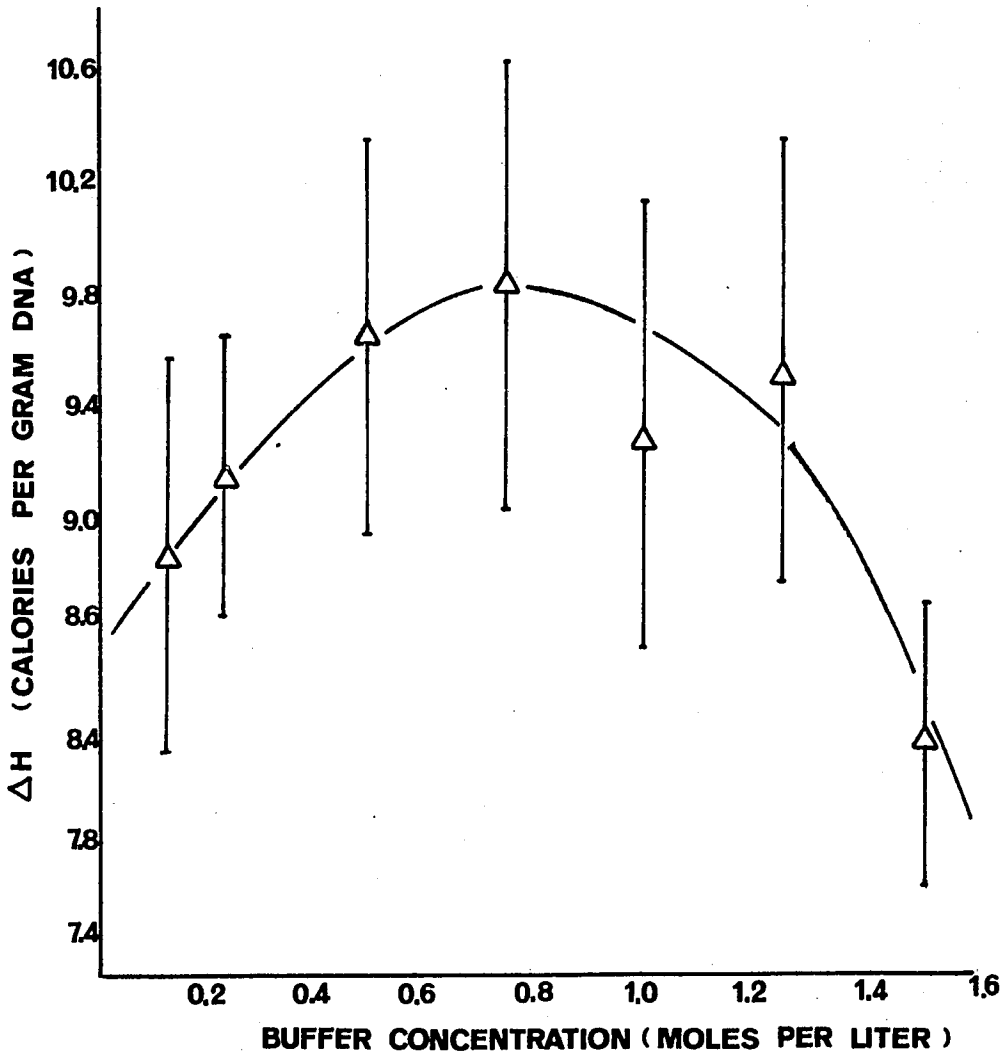

Figure 15. Enthalpy of helix-coil transition of DNA as a function of buffer concentration. Reported in calories per gram of DNA polymer. Buffer pH=6.8.

at the buffer concentration 0.75 moles·liter^{-1}. It is interesting to note the sharp drop in peak area that occurs at the highest buffer concentration used. This drop was initially attributed to an interference by the high salt concentration with the solubility of DNA in aqueous solution.

E. Kinetics of the Helix-Coil Transition of DNA

In this work, the measurement of the energy barrier to the thermal denaturation of DNA was undertaken using two different methods; the method of Piloyan[14] and the method of Borchardt and Daniels.[13] Due to the complexity of the reaction studied, the latter method led to results more amenable to analysis.

1) Piloyan Method

Initially, an attempt was made to study the kinetics of the transition; more precisely, to find the activation energy of the reaction by the Piloyan method.[14] A detailed description of this method including the equations used, their derivation and justification as well as a discussion of the limitations of the method is found in chapter II of this dissertation. To briefly summarize, the size of the deflection of the pen from the baseline (equal to the temperature difference, Δt, between sample and reference) is

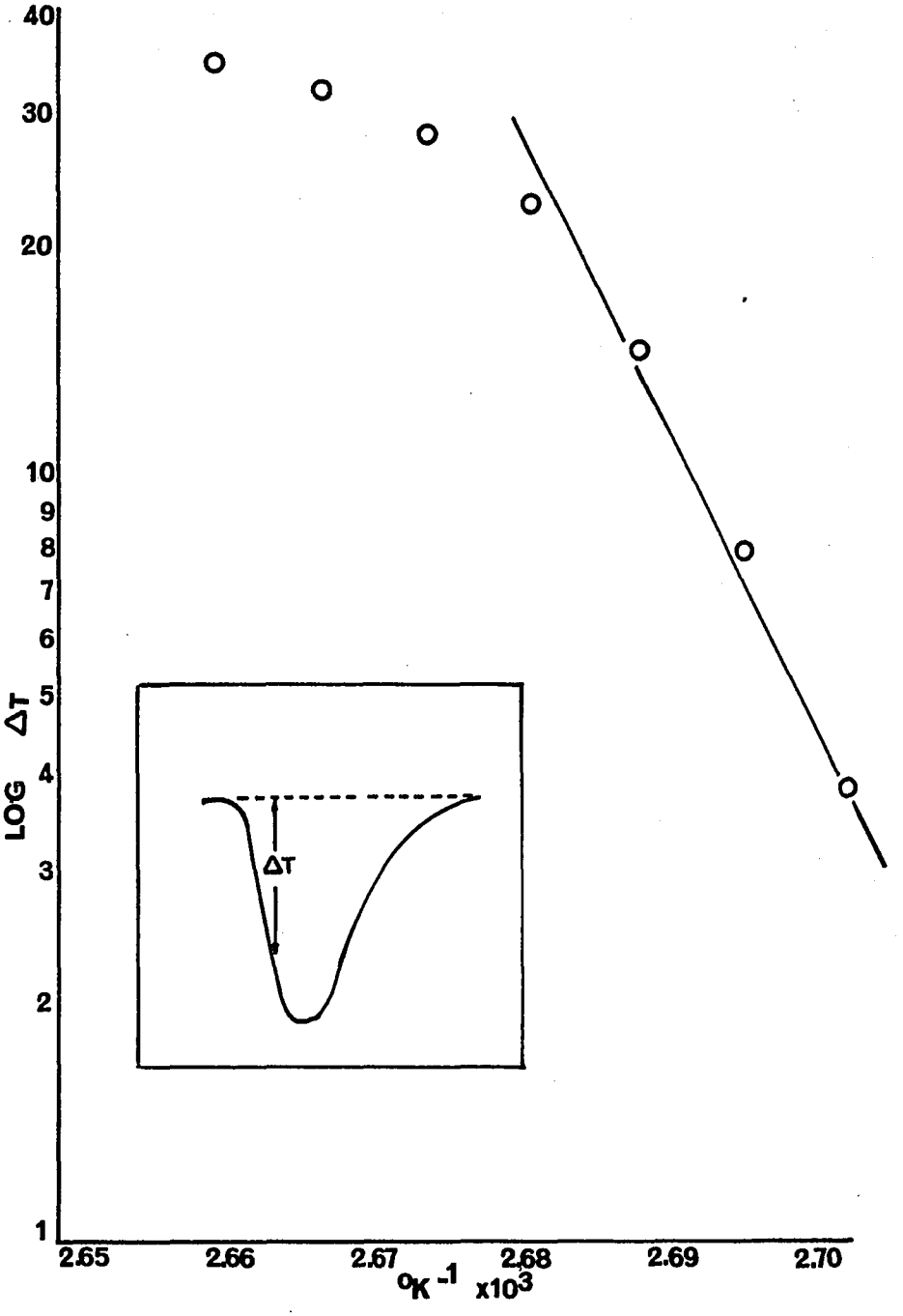

Figure 16. Activation energy plot, by method of Piloyan; for helix-coil transition of DNA in phosphate buffer. Insert: trace of thermogram showing measurement of Δt.

proportional to the reaction rate and therefore, to exp (-E/RT). Under these conditions, a plot of log Δt as a function of the reciprocal of absolute temperature should give a straight line for which the slope is equal to -E/R. Figure 16 is a sample plot. The insert is a tracing of the thermogram peak from which the Δt values were measured. As this graph indicates, there is a rounding off of the curve after the first few points (reasons for this are also discussed in chapter II). The "straight line," then, is the best fit to the first few points.

Almost all of the thermograms used for the enthalpy study were analyzed for activation energy by this method. A few thermograms had to be omitted because of uncertain baselines or because of excessive instrument noise. The activation energy values were averaged at each experimental pH value. Figure 17 shows the results of this tabulation. This graph indicates a possible dependence of activation energy on pH value but the differences between points are not much outside the standard deviations from the means. A change of one pH unit (in either direction) from pH 7.0 gives a decrease of about 25 percent, while the deviations are from 8 to 14 percent. The method allows for much possibility for error. The lines from which activation energy is calculated (based on the slopes) are determined by only a few

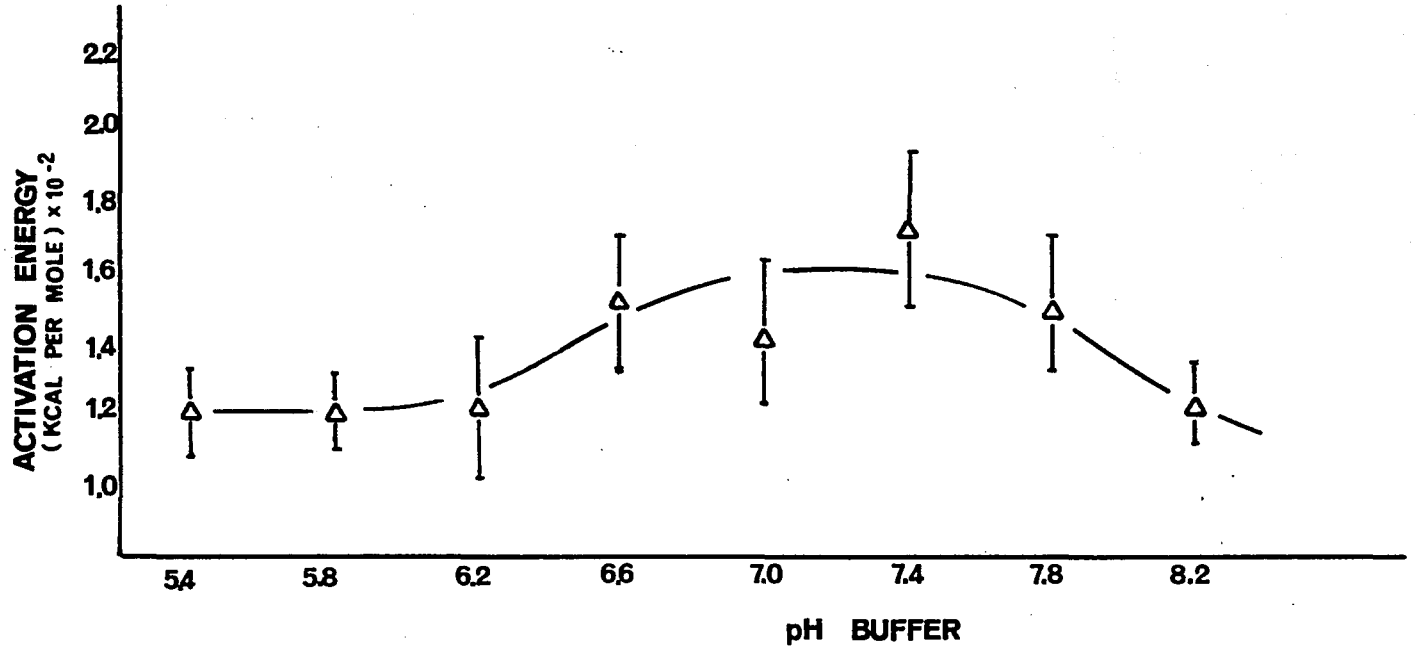

Figure 17. Activation energy of helix-coil transition of DNA, determined by method of Piloyan, as a function of pH.

points. For this and other reasons, the Piloyan method was found to be less than satisfactory for the study of the kinetics of the DNA uncoiling.

The reported results of a study of the kinetics of the DNA uncoiling by entirely different methods[12] show that the helix-coil transition is a series of consecutive steps. This can lead to a complex kinetic picture[39] and, therefore, difficulties with an Arrhenius-type plot such as used in the Piloyan method. With the possibilities of a complex kinetic picture, it is desirable to be able to analyze the entire thermogram curve.

2) Borchardt and Daniels Method

Another method of analysing DTA thermograms has been developed by Borchardt and Daniels.[13] This method uses the entire thermogram curve for data points for an Arrhenius-type activation energy plot. Complexities can possibly be recognized when they exist. A detailed analysis of this method is also found in chapter II.

In the Borchardt and Daniels method, rate constants are calculated from the expression given by equation 31, listed again

here, where

$$k = \frac{\left(\frac{AV}{n_o}\right)^{x-1}\frac{dH}{dt}}{(A-a)^x} \quad .$$

To summarize: In this equation, A is the total area of the endotherm; dH/dt the instantaneous rate of heat transfer to the sample (y-axis deflection); V/n_o the reciprocal of the initial concentration; a is the partial area under the curve at that temperature and x is the assumed reaction order. The term "mole" as applied to a molecule such as DNA is somewhat vague. Also, initial concentration is difficult to determine precisely. Omitting the constants in the first term (AV/n_o) will not effect the slope of a graph of log k as a function of 1/T. A new constant k' was therefore used, where

$$k' = \frac{dH/dt}{(A-a)^x} \qquad (32)$$

Since the calculations involved a great deal of repetitive arithmetic a computer was used to evaluate k' at each T and for each trial value of x from the lengths of the y-axis deflections at each temperature. The computer program and the explanation of its use is given in appendix 2.

The three or four best thermograms (most certain baseline and most free of noise) were selected at each pH value. A number of Arrhenius-type plots using k' were obtained for each thermogram, one for each assumed reaction order. Initially, reaction orders zero to three in steps of 0.5 were tried. Since a complex reaction sequence was expected, non-integer experimental rate orders could not immediately be ruled out. Figures 18 to 35 show the resulting graphs for each value of x for the reactions at pH 7.0, 5.4 and 8.2.

Estimates of $(AV/n_o)^x$ for the appopriate values of A, V/n_o and x are shown with the kinetic curves.

It can be seen that change in pH does not make a noticeable difference in the pattern when comparing the graphs of any given value of x. All of the plots, except at x=0 follow a similar pattern; an ititial straight line segment followed by a fairly abrupt change in slope. The final portion of all of the plots (except zero order) involves a moderate to sharp tailing upward. The extent of trailing upward was found to be sensitive to changes in the value of total area by as little as one or two percent. Looking at the equation this becomes very understandable. As (A-a) approaches zero, small errors in A cause very large errors in 1/(A-a) and

still larger errors in $1/(A-a)^2$. For this reason, this final portion of the graphs was not considered in the evaluation of the Arrhenius plots.

The literature mentions the analysis of non-linear Arrhenius plots as being the intersection of two straight lines due either to two competing[42] or to two sequential reactions[41]. If an activation energy plot can be composed of two intersecting lines, a plot involving the intersection of more than two straight lines can also be considered a possibility. However, the best approximation to any small number of straight lines occurs with the second order plots. Shown on the graphs in figures 18-35 is the percentage completion of the reaction as several temperatures as calculated by the rato a/A. Since the area under the curve is proportional to the heat absorbed, the percentage of total area should be a good approximation to the extent of the reaction at a given temperature. The second order plots show an initial, sharply sloped straight line segment to approximately the temperature at which a/A is equal to 0.1. This segment is followed by an abrupt change in slope. The second straight line segment extents to the region of the curve where a/A is equal to 0.9. After this, the graph begins to curve upward. As discussed before, this final

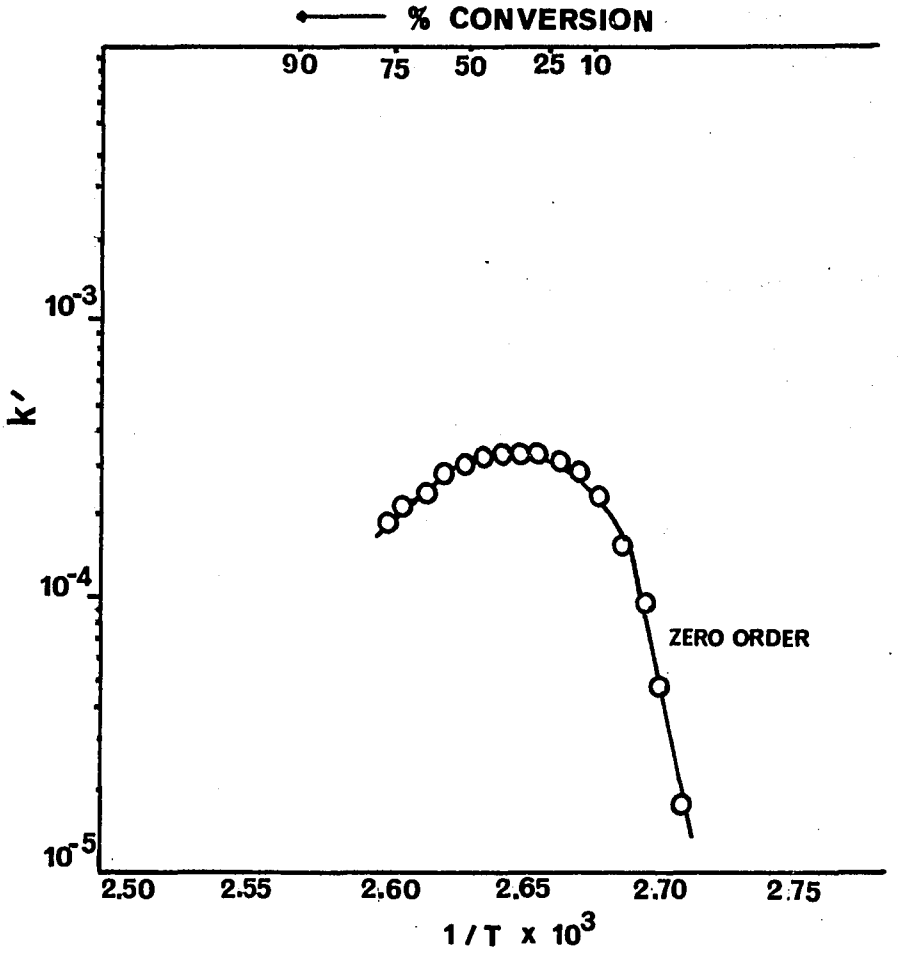

Figure 18. Zero order kinetic plot, by method of Borchardt and Daniels, for helix-coil transition of DNA in phosphate buffer, pH 7.0. Buffer concentration 0.75 moles(liter).$^{-1}$ Rate constant k, from equation 31 is equal to $k\ (AV/n_o)^{-1}$ or, k=0.34 k'.

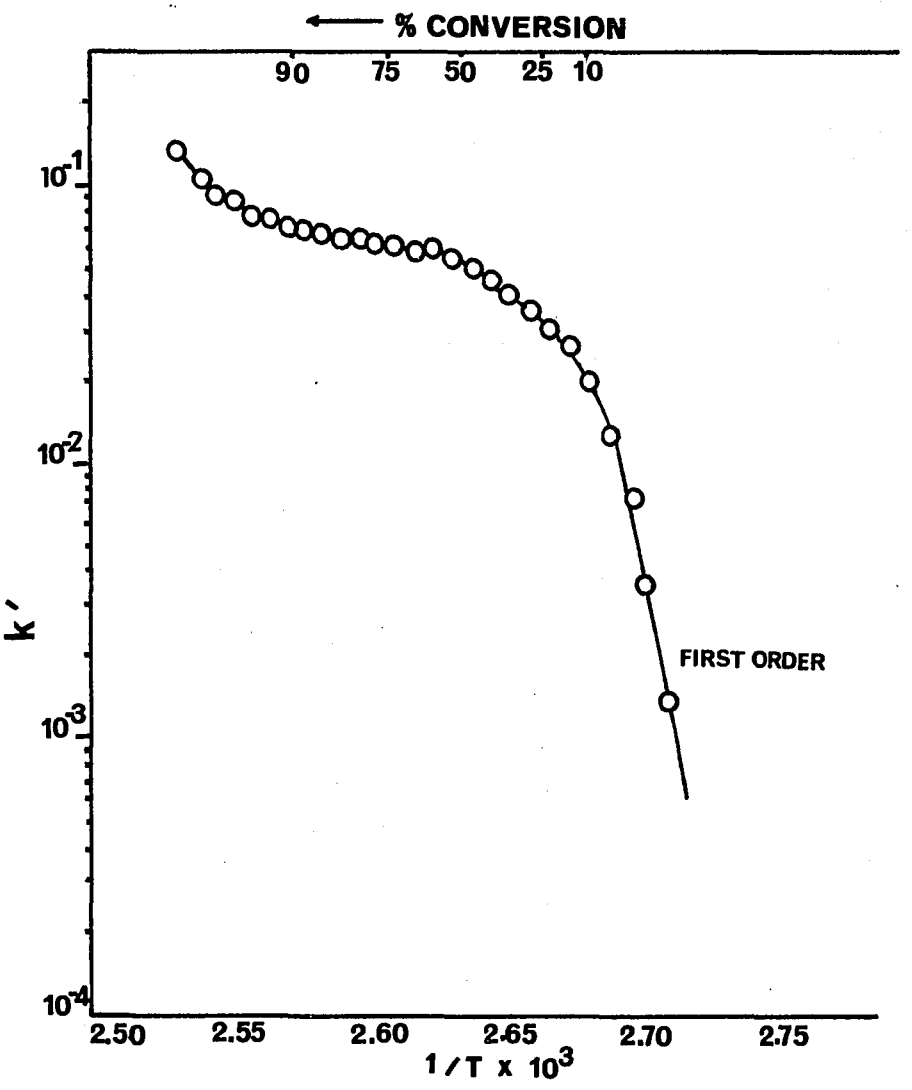

Figure 19. First order kinetic plot, by method of Borchardt and Daniels, for helix-coil transition DNA in phosphate buffer, pH 7.0. Buffer concentration is 0.75 moles(liter).$^{-1}$ Rate constant k, from equation 31 is equal to k'.

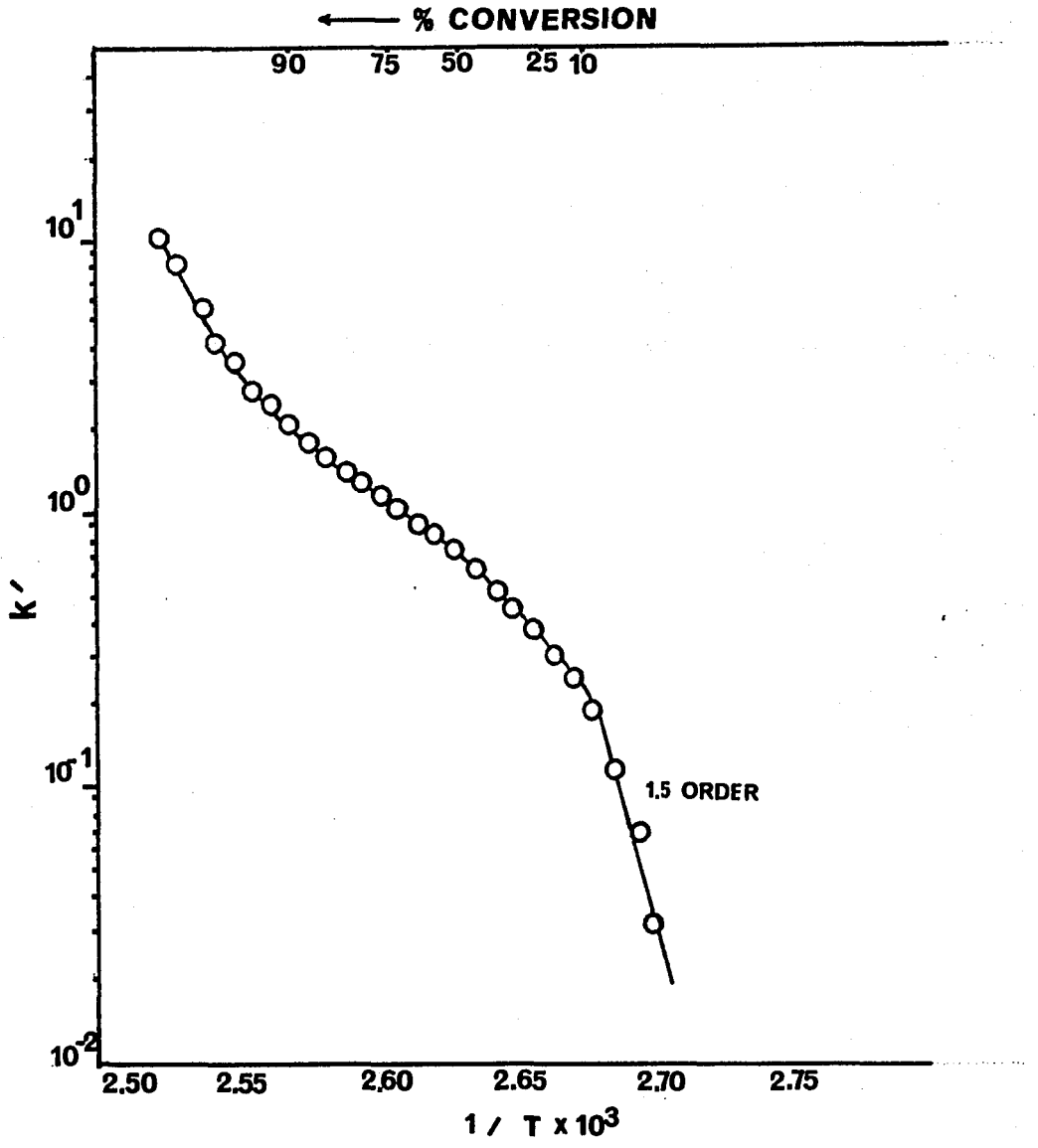

Figure 20. Kinetic plot (1.5order), by method of Borchardt and Daniels, for helix-coil transition of DNA, in phosphate buffer, pH 7.0. Rate constant k, from equation 31, is equal to $k' (\Delta V/n_o)^{1/2}$ or, $k = 1.7k'$. Buffer concentration is 0.75 moles(liter)$^{-1}$.

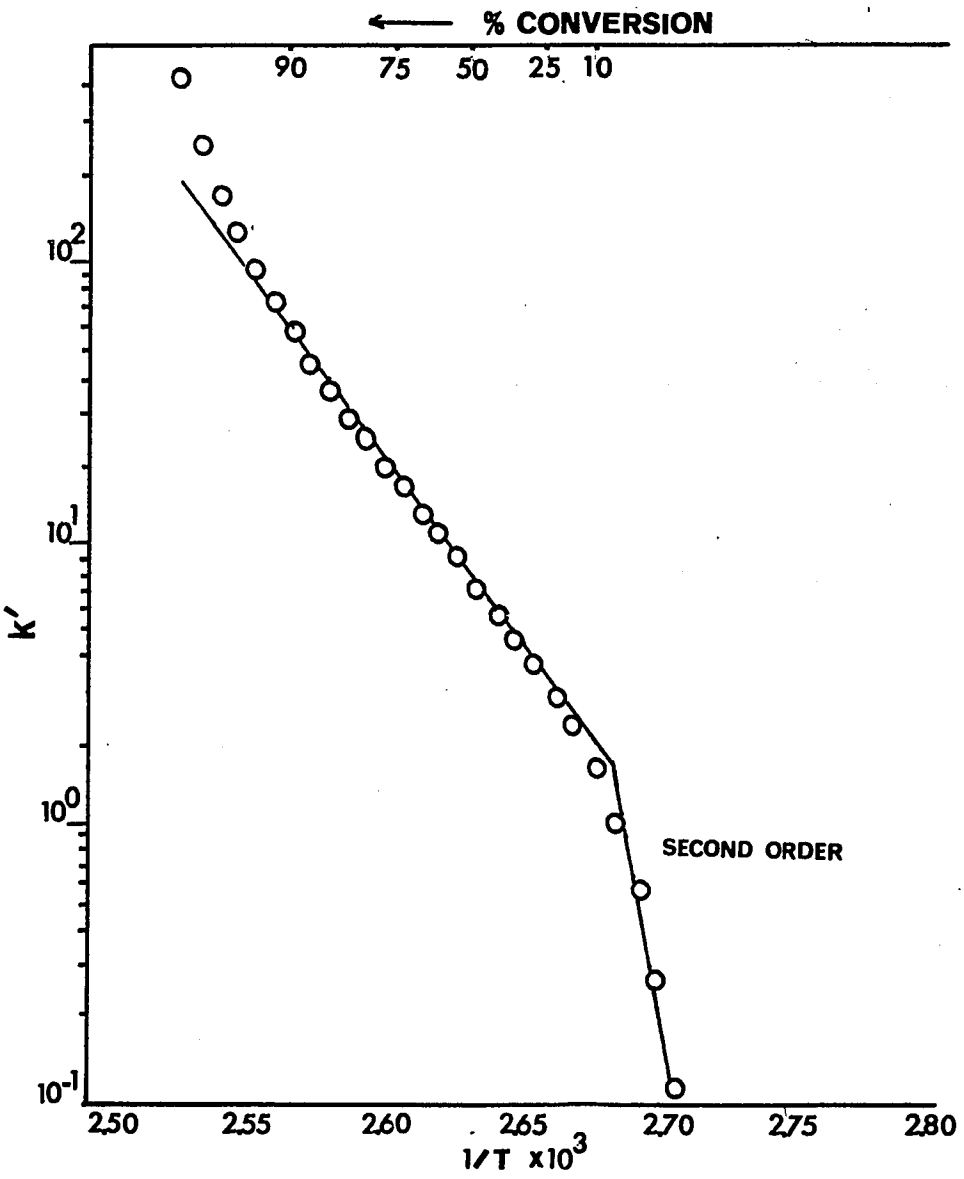

Figure 21. Second order kinetic plot, by method of Borchardt and Daniels, for DNA in phosphate buffer, pH 7.0. Buffer concentrations 0.75 moles(liter).$^{-1}$ Rate constant k, from equations 31, is equal to $k'(AV/n_o)$ or, $k=3.0\ k'$.

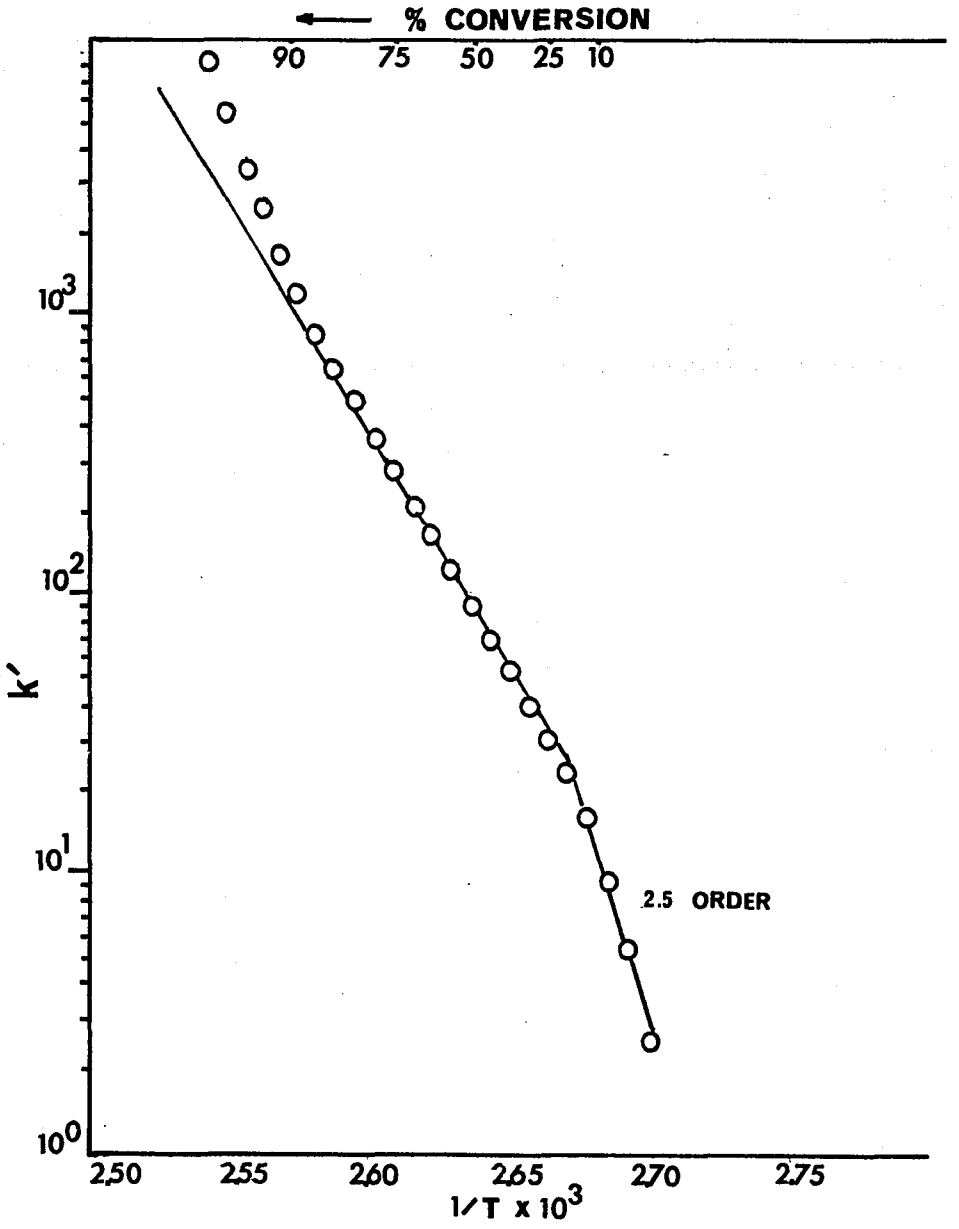

Figure 22. Kinetic plot (2.5 order), by method of Borchardt and Daniels, for helix-coil transition of DNA in phosphate buffer. Buffer concentration is 0.75 moles $(liter)^{-1}$. Rate constant k, from equation 31, is equal to $k' (AV/n_o)^{1.5}$ or, $k=5.1\ k'$.

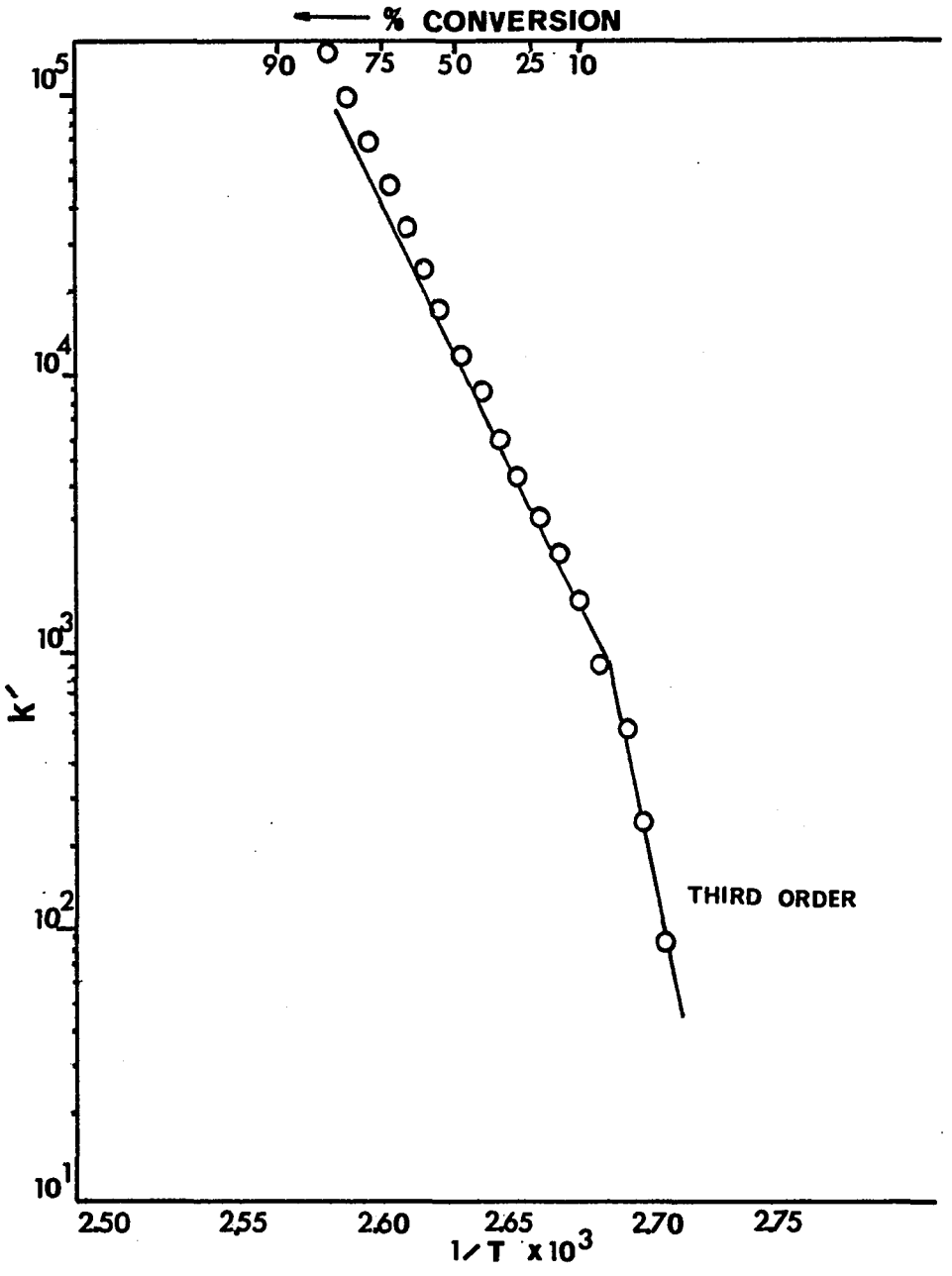

Figure 23. Third order kinetic plot, by method of Borchardt and Daniels, for helix-coil transition of DNA in phosphate buffer, pH 7.0. Buffer concentration is 0.75 moles(liter).$^{-1}$ Rate constant k, from equation 31, is equal to k' $(AV/n_o)^2$ or, k=9.0 k'.

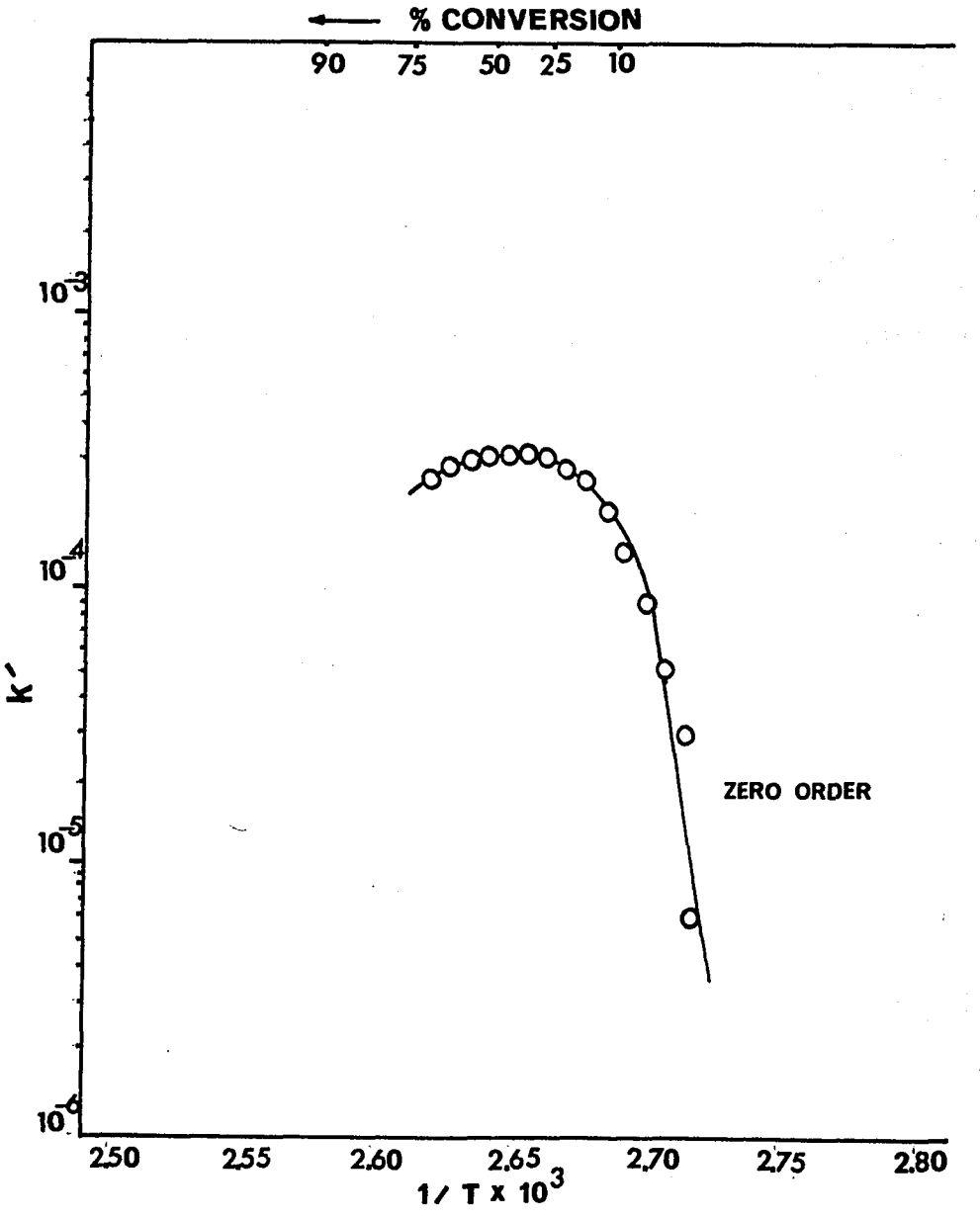

Figure 24. Zero order kinetic plot, by method of Borchardt and Daniels, for helix-coil transition of DNA in phosphate buffer, pH 5.4. Buffer concentration is 0.75 moles(liter).$^{-1}$ Rate constant k, from equation 31, is equal to $k'(AV/n_o)^{-1}$ or, $k=0.43\ k'$.

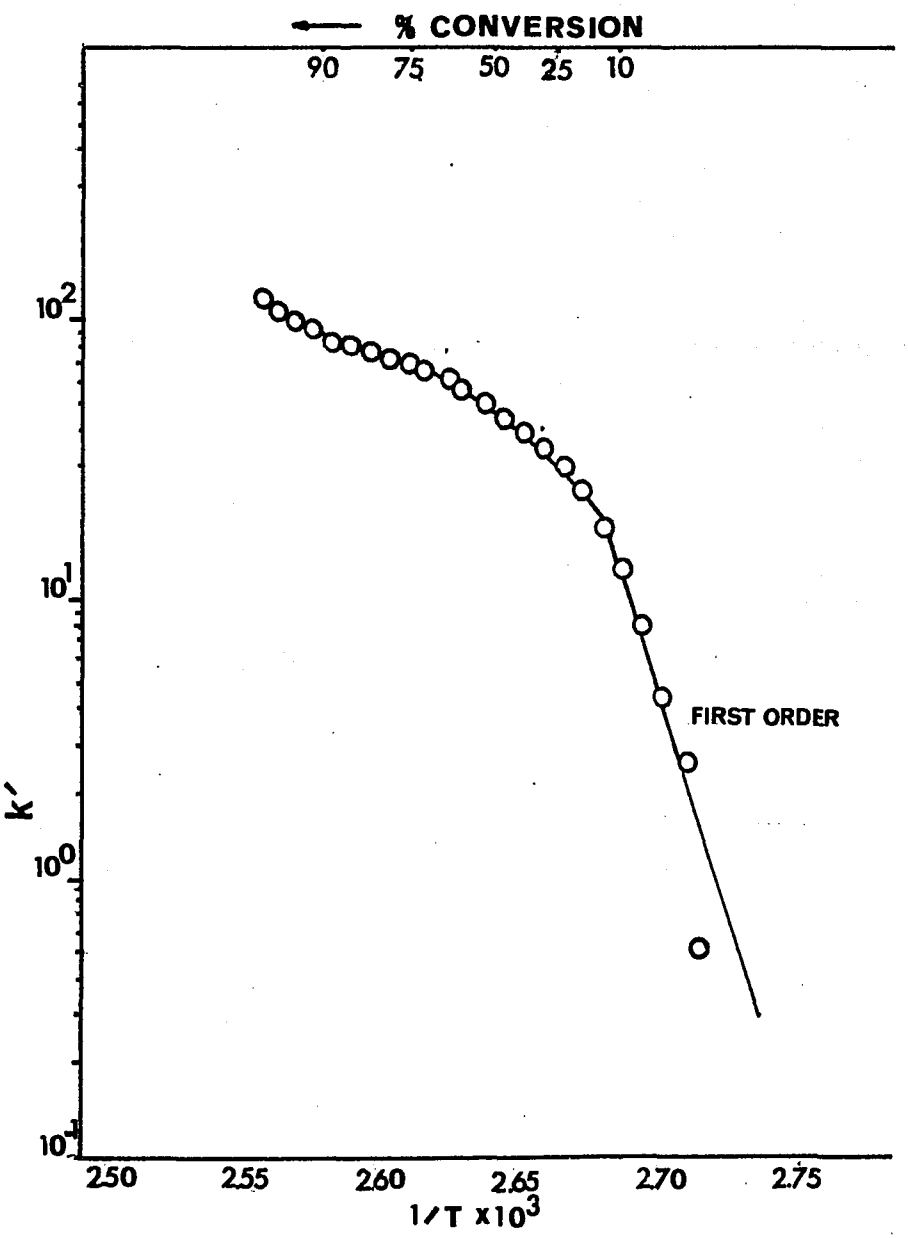

Figure 25. First order kinetic plot, by method of Borchardt and Daniels, for helix-coil transition of DNA in phosphate buffer, pH 5.4. Buffer concentration is 0.75 moles (liter)$^{-1}$. Rate constant k, from equation 31, is equal to k'.

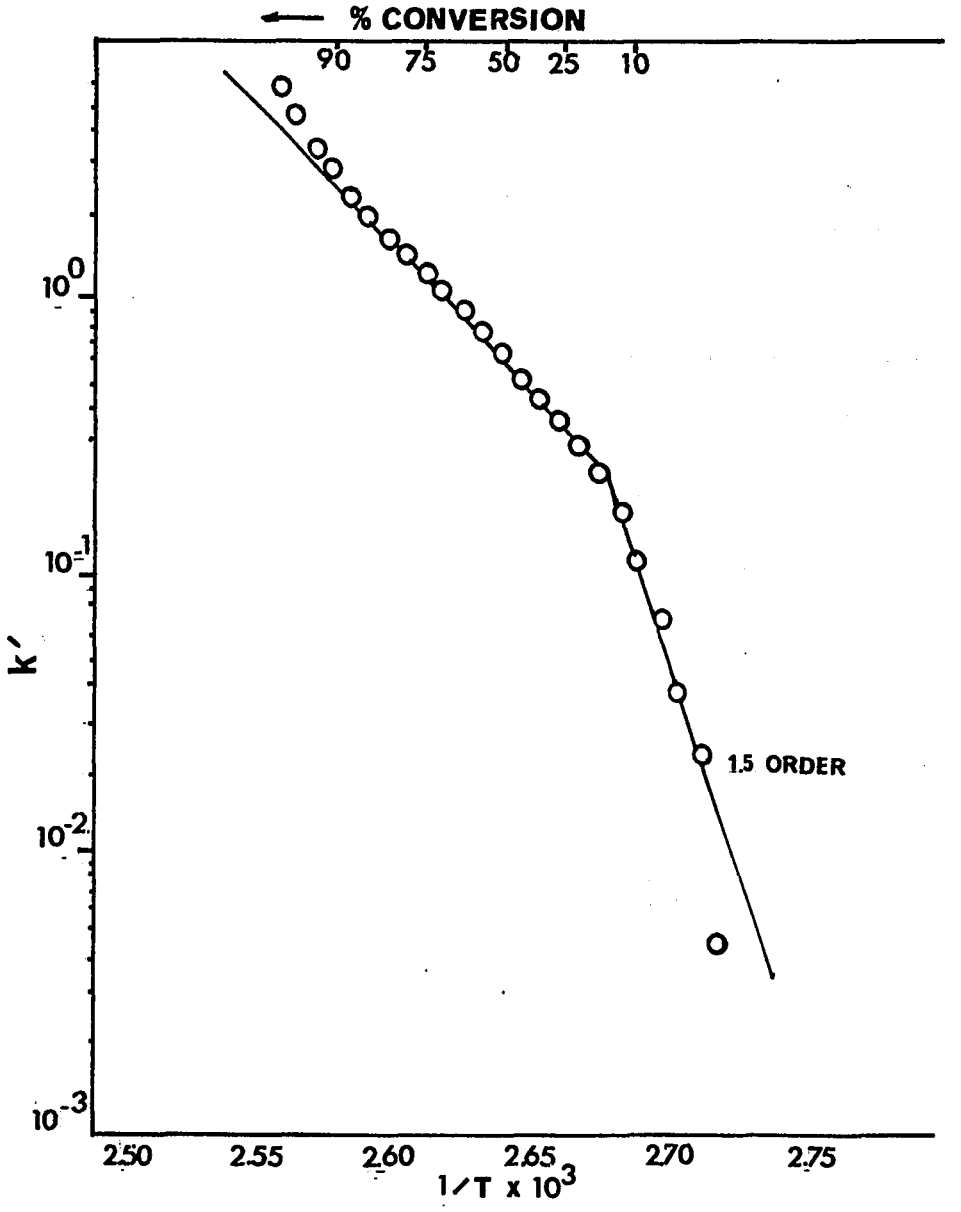

Figure 26. Kinetic plot, (1.5 order) by method of Borchardt and Daniels, for helix-coil transition of DNA in phosphate buffer pH 5.4. Buffer concentration is 0.75 moles(liter).$^{-1}$ Rate constant k, from equation 31, is equal to $k'(AV/n_o)^{\frac{1}{2}}$ or, $k=1.5\ k'$.

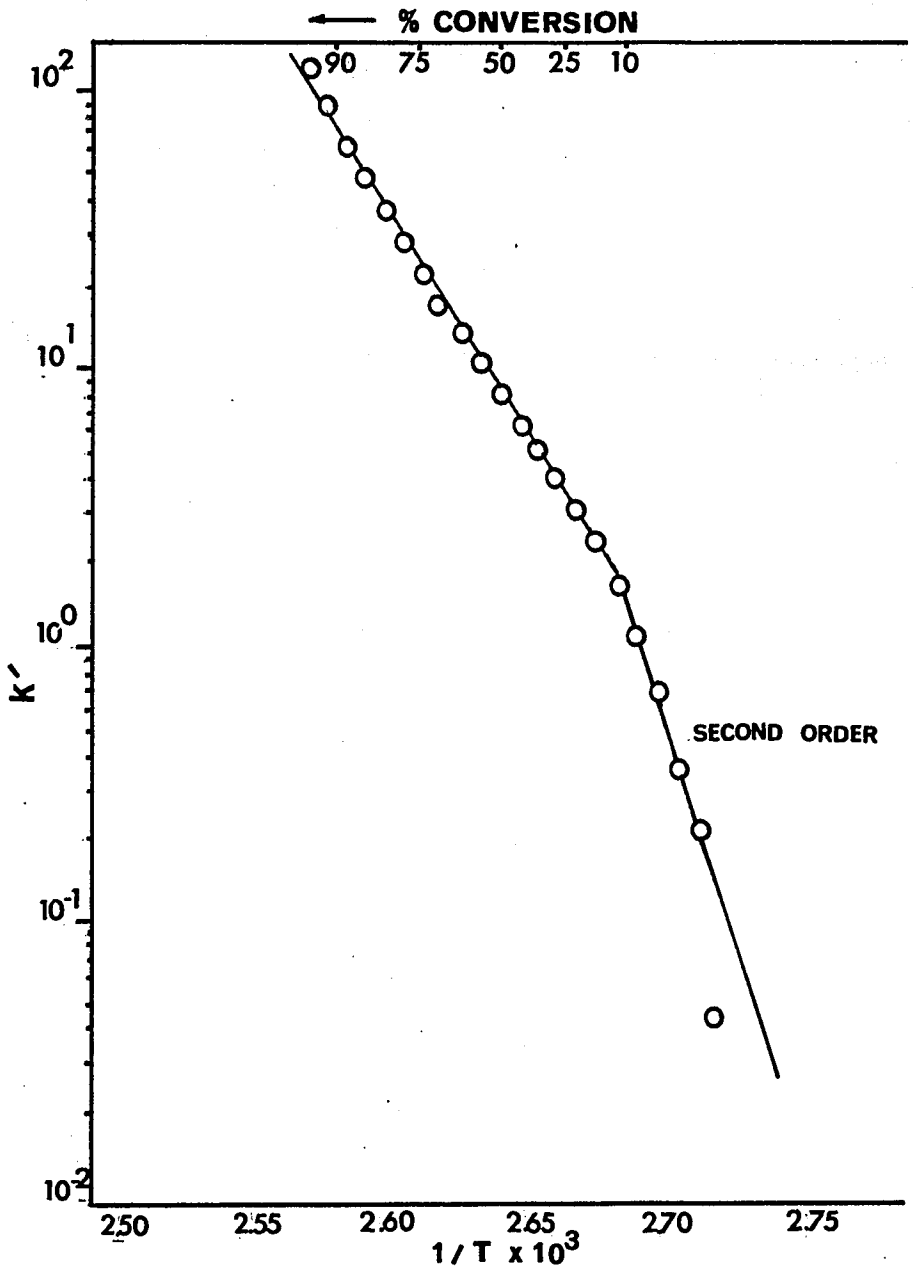

Figure 27. Second order kinetic plot, by method of Borchardt and Daniels, for helix-coil transition and of DNA in phosphate buffer, pH 5.4. Buffer concentration is 0.75 moles(liter).[-1] Rate constant, from equation 31, is equal to $k'(AV/n_o)$ or, $k = 2.3\ k'$

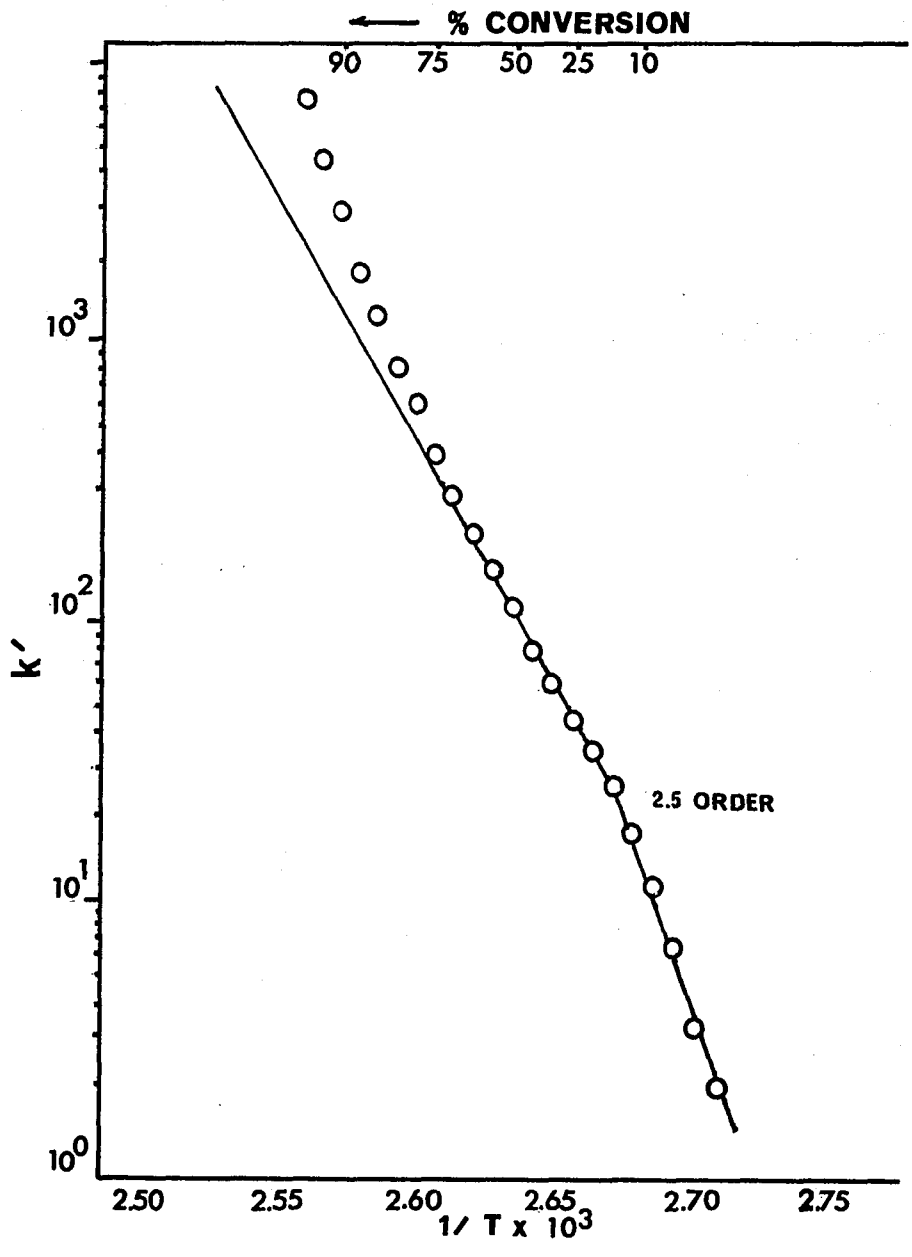

Figure 28. Kinetic plot (2.5 order), by method of Borchardt and Daniels, for helix-coil transition of DNA in phosphate pH 5.4. Buffer concentration is 0.75 moles(liter).$^{-1}$ Rate constant, from equation 31 is equal to k' $(AV/n_o)^{1.5}$ or, k=3.5k'

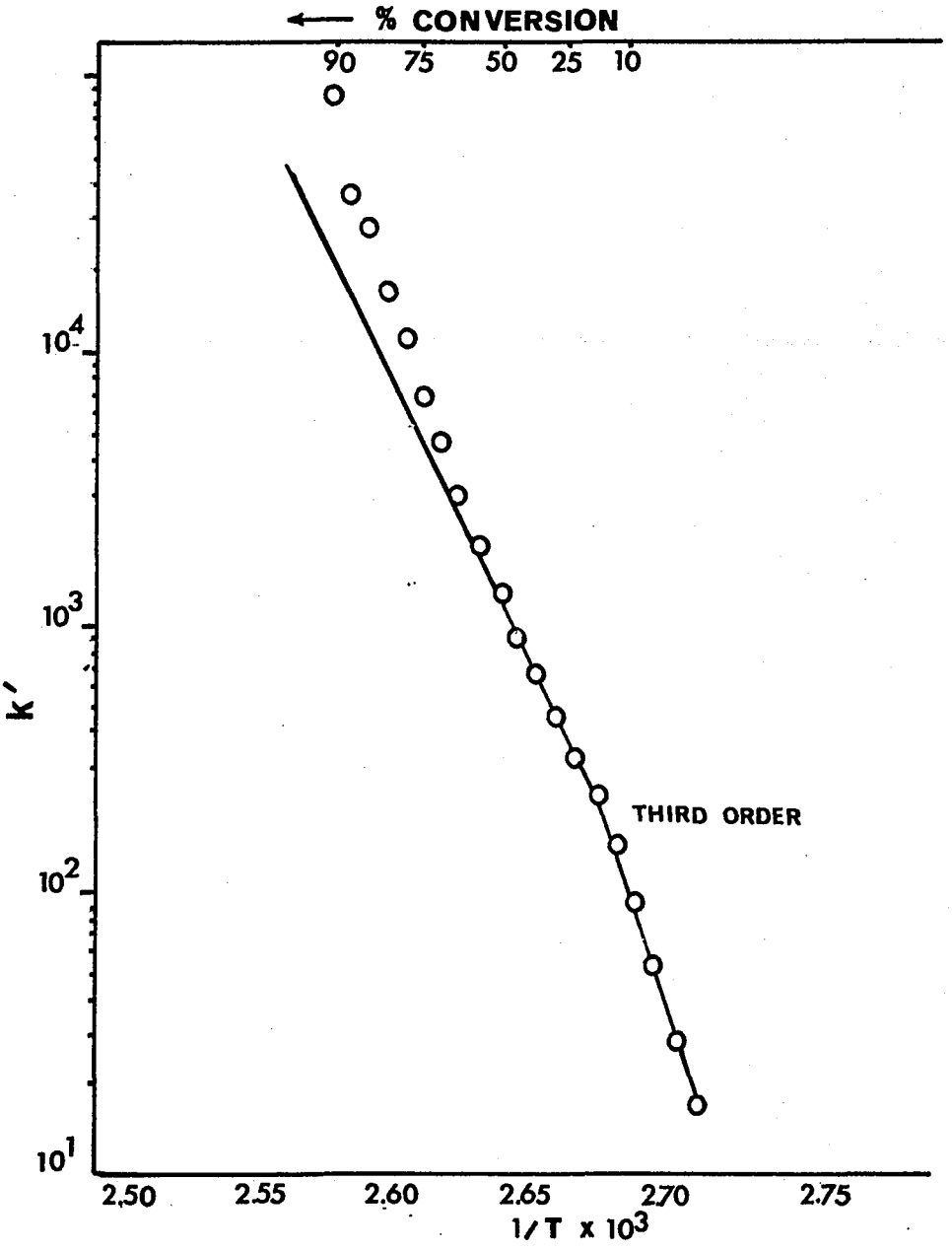

Figure 29. Third order kinetic plot, by method of Borchardt and Daniels, for helix-coil transition of DNA in phosphate buffer, pH 5.4. Buffer concentration is 0.75 moles(liter).$^{-1}$ Rate constant, k, from equation 31 is equal to $k'(AV/n_o)^2$ or, $k=5.3\ k'$.

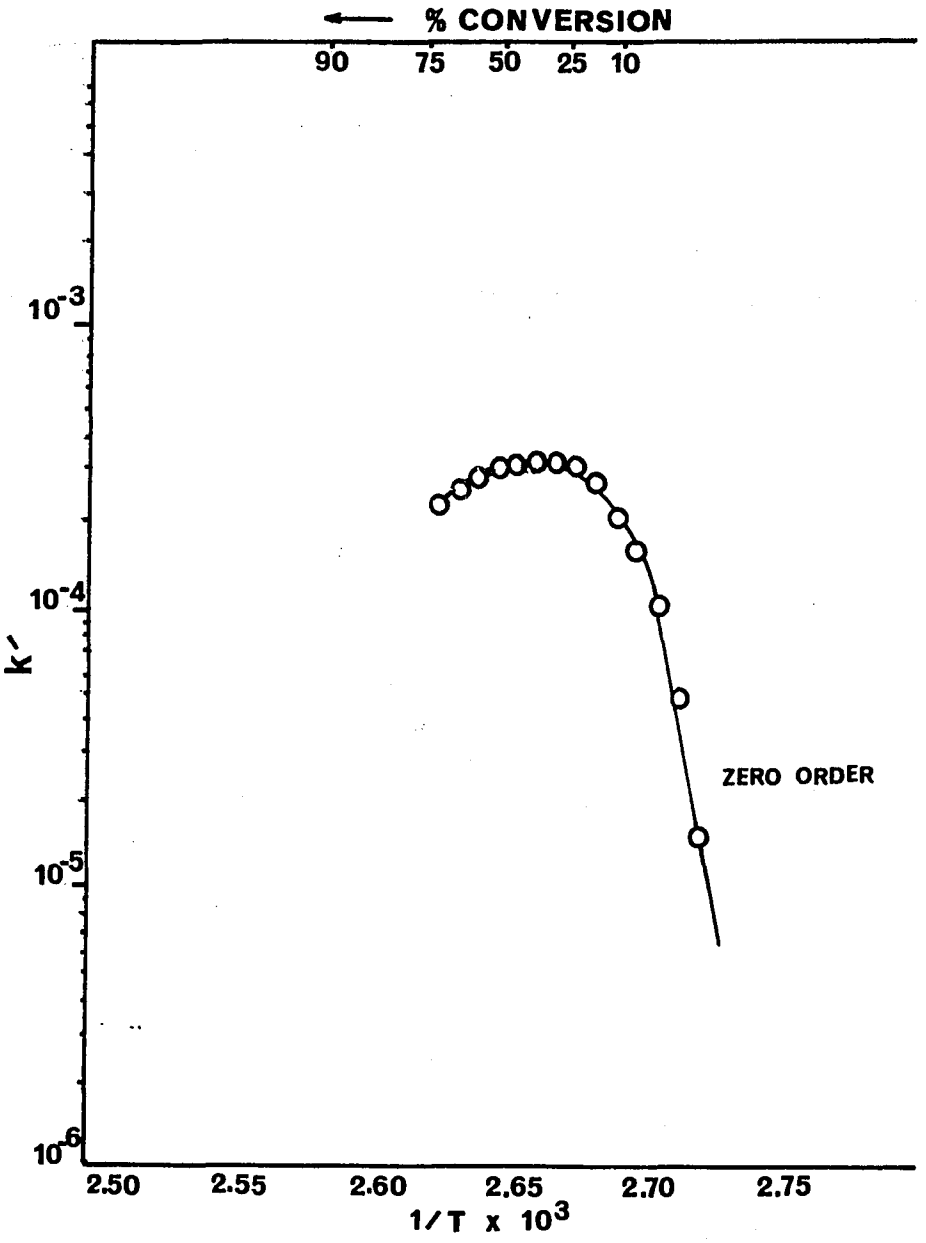

Figure 30. Zero order kinetic plot, by method of Borchardt and Daniels, for the helix-coil transition of DNA in phosphate buffer, pH 8.2. Buffer concentration is 0.75 moles(liter).$^{-1}$ Rate constant, k, from equation 31, is equal to $k'(AV/n_o)^{-1}$ or, k=0.36 k'

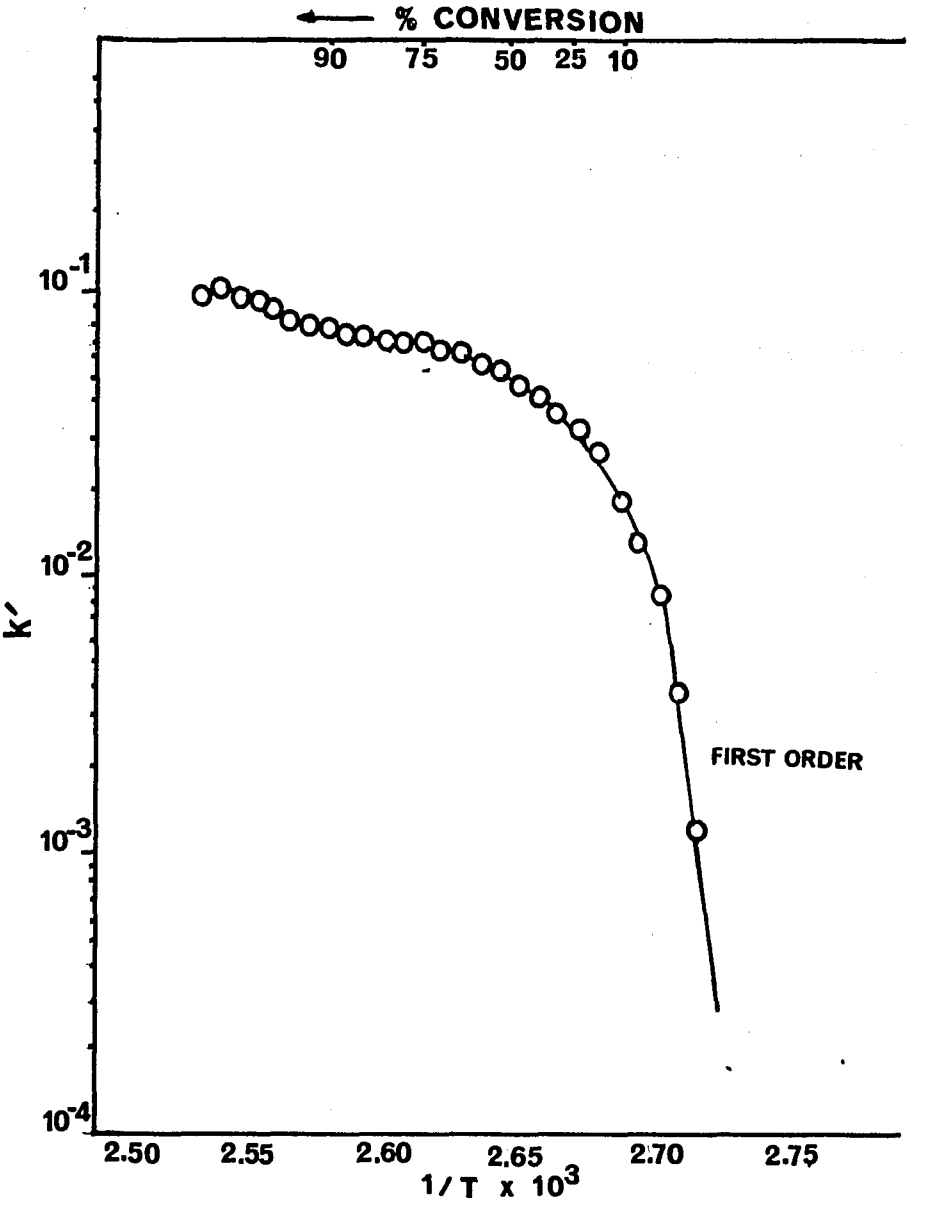

Figure 31. First order kinetic plot, by method of Borchardt and Daniels, for the helix-coil transition of DNA in phosphate buffer, pH 8.2. Buffer concentration is 0.75 moles(liter)$^{-1}$. Rate constant, k, from equation 31 is equal to k!

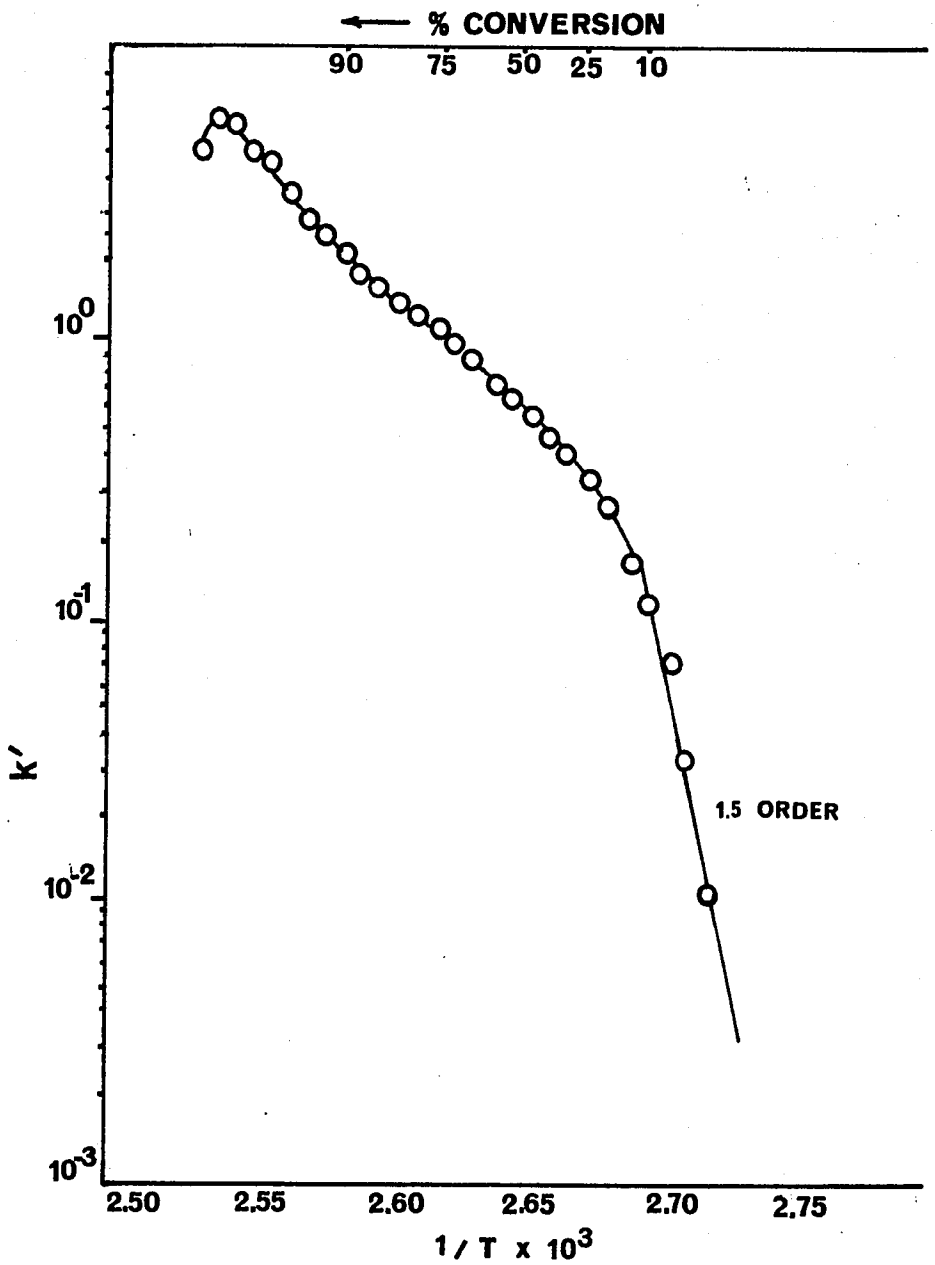

Figure 32. Kinetic plot (1.5 order) for the helix-coil transition of DNA in phosphate buffer pH 8.2. Buffer concentration is 0.75 moles(liter).$^{-1}$ Rate constant k, from equation 31 is equal to $k'(AV/n_o)^{1/2}$ or, k=1.9 k'

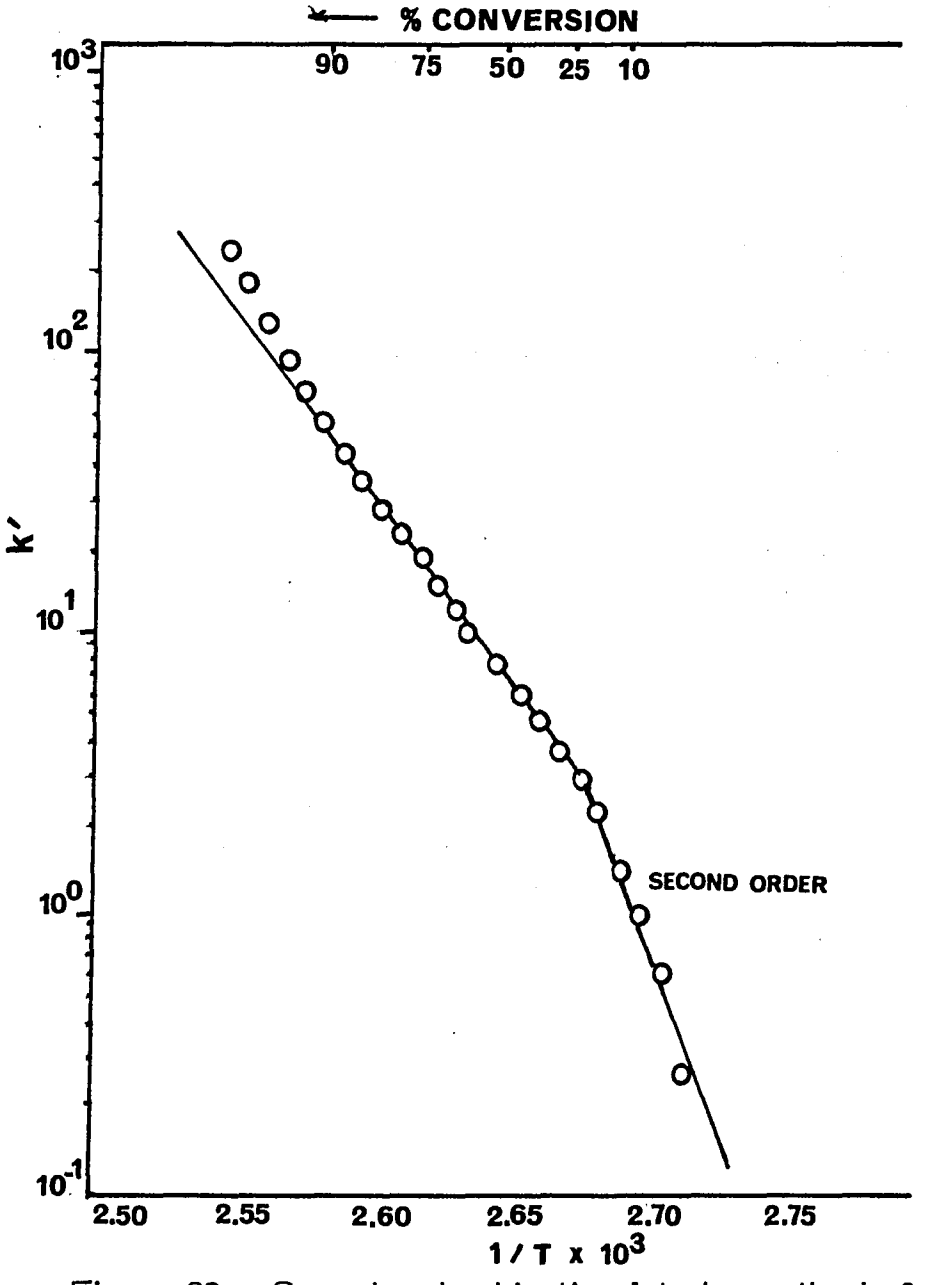

Figure 33. Second order kinetic plot, by method of Borchardt and Daniels, for the helix-coil transition of DNA in phosphate buffer, pH 8.2. Buffer concentration is 0.75 moles(liter).$^{-1}$ Rate constant k, from equation 31 is equal to $k'(AV/n_o)$ or $k=3.6\ k'$

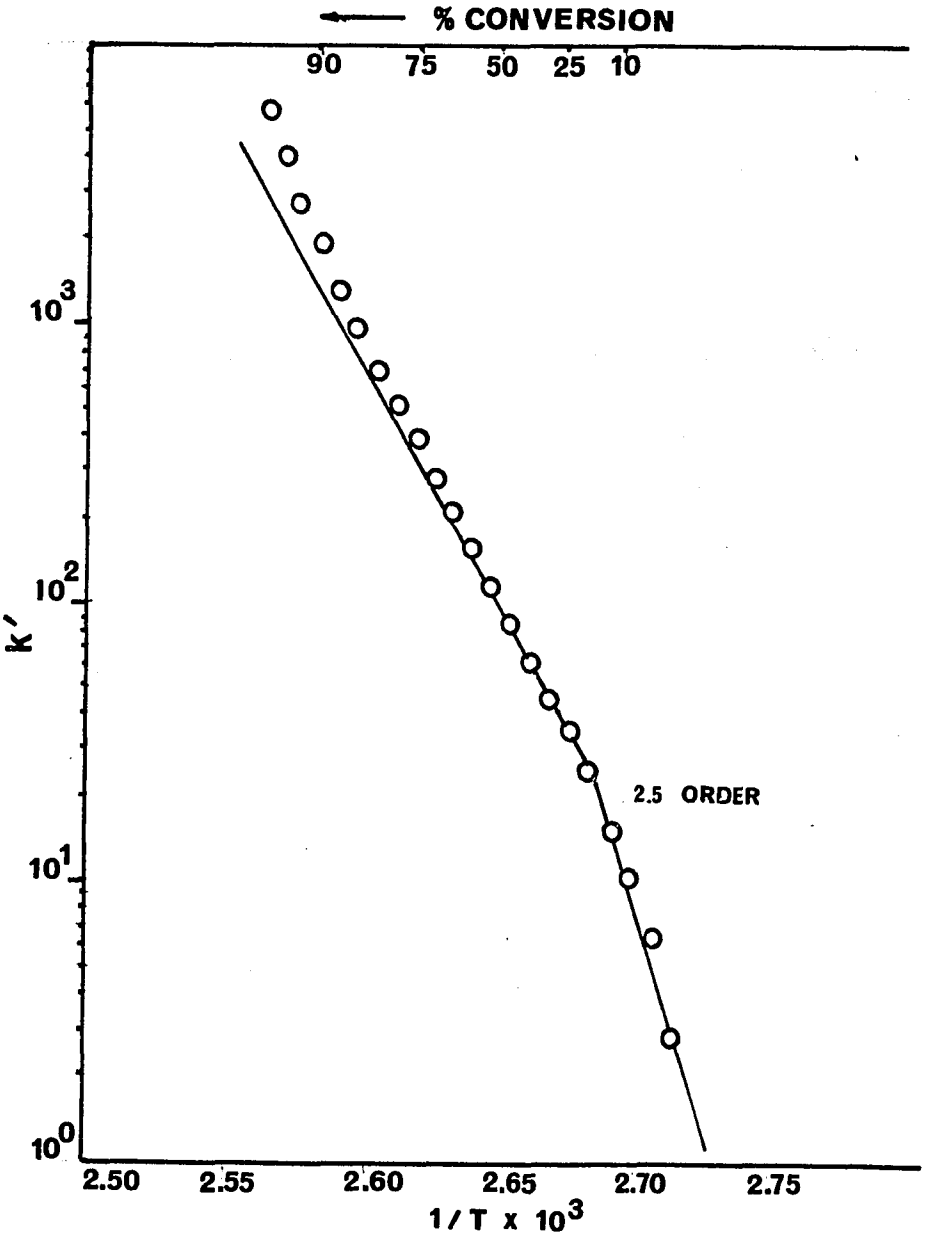

Figure 34. Kinetic plot (2.5 order), by method of Borchardt and Daniels, for the helix-coil transition of DNA in phosphate buffer, pH 8.2. Buffer concentration is 0.75 moles(liter).$^{-1}$ Rate constant k, from equation 31 is equal to $k'(AV/n_o)^{1.5}$ or, $k=6.8\,k'$.

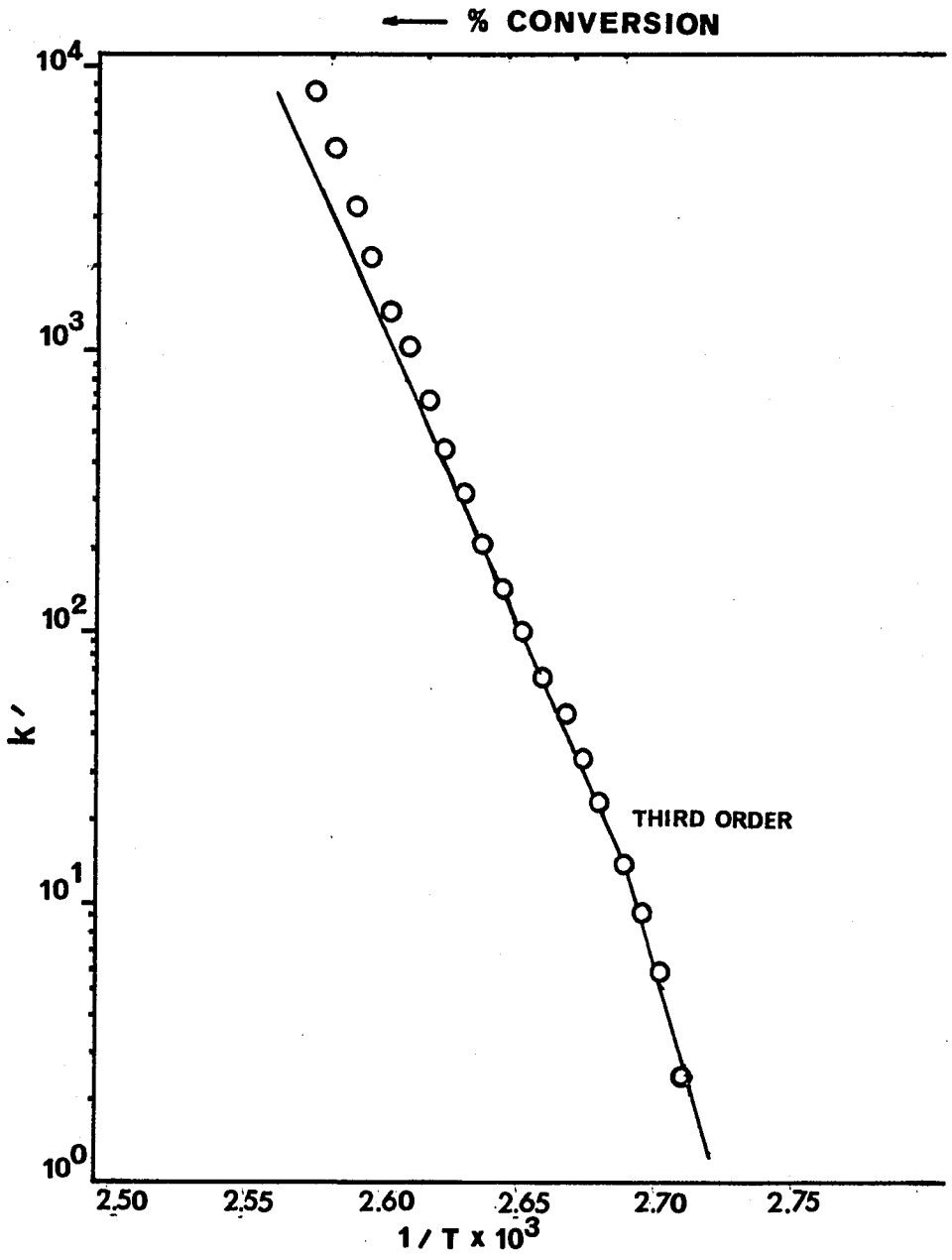

Figure 35. Third order kinetic plot, by method of Borchardt and Daniels, for the helix-coil transition of DNA in phosphate buffer, pH 8.2. Buffer concentration is 0.75 moles(liter)$^{-1}$. Rate constant k, from equation 31 is equal to $k'(AV/n_o)^2$ or, k= 13 k'

segment is considered to be due to difficulties with the equations. The analysis of the series of thermograms by the method of Borchardt and Daniels therefore suggests a two or more step sequential reaction with apparent second order kinetics. Second order kinetics for the helix coil transition was, to say the least, unexpected. The explanation for this may be in the high concentration of the solutions used.

The activation energies calculated from these graphs at the different pH values are listed in table III. For the initial segment of the graphs, the calculated activation energy is high - on the order of 200 Kcal/mole, regardless of the value of x. There is some trend towards lowering of the activation energy of this initial step by increase in acidity of the solution.

Apparent second order activation energies were calculated from the slopes of the segment of the graphs (second order) that is linear over 80 percent of the plot. The average activation energy was found to be 69 ± 5 Kcal.

TABLE III

Results of Activation Energy Study

pH of Buffer	Average Activation Energy - First Stage		Average Activation Energy - Final Stage	
	First Order (Kcal/mole)	Second Order (Kcal/mole)	First Order (Kcal/mole)	Second Order (Kcal/mole)
8.2	220	220	uncert.	70
7.8	240	210	uncert.	70
7.4	210	200	uncert.	72
7.0	230	250	10-15	60-70
6.6	180	190	19	74
6.2	190	170	15	60-70
5.8	185	uncert.	20	70
5.4	uncert.	175-180	15-20	70

CHAPTER V

DISCUSSION

A. Introduction

Observed transition temperature is discussed here as it is highly relevant to the subject of transition enthalpy. The values of the other thermodynamic functions, entropy and free energy, calculated from the enthalpy values are presented in this chapter. Discussion of the kinetics of the reaction is concerned with the results obtained from calculations by the Borchardt and Daniels method.[13]

B. Transition Temperatures

The transition midpoint temperatures of approximately 106°C were higher than those obtained by Neumann and Ackermann,[2] Krakauer and Sturtevant,[3] Privalov[6] and Shiao and Sturtevant.[8] The results of these studies also clearly show that transition temperature is a function of the concentration of counterion in solution. The ion concentration used in the study by differential thermal analysis was many times higher than used in the studies previously mentioned. Gruenwedel, Hsu and Lu[40] did a detailed study of the dependence of T_m on salt concentration for DNA from several different organisms, among them calf thymus DNA. They found a linear dependence of transition temperature on ion concentration up to counterion concentrations in the order of 1 mole(liter)$^{-1}$. At higher counterion

concentrations T_m decreased. The linear dependence was found to be expressed by the following equation

$$T_m = A - B\,pM \qquad (33)$$

where A and B are experimentally determined constants for each variety of DNA and pM is the negative of the logarithm of the counterion concentration. For calf thymus DNA, the value of A is 100°C and the value of B is 17.3°C. Assuming that the sodium ion concentration used is 1.5 times the molarity of the salt the predicted value of T_m would be approximately 101°C. The high salt concentration then accounts for most, although not all, of the increase in T_m observed in this laboratory. Several thermograms were tried on solutions of DNA in distilled water (but containing some sodium ion present in the dried polymer). Transition midpoints were not determined but the onset of transition was approximately ten degrees lower than in the high salt concentrations. The calculated ion concentration from the salt associated with the dried polymer is approximately 0.1 moles(liter).$^{-1}$ This gives an estimated midpoint of approximately 83°C. The higher transition temperatures we observe are most likely due to the high concentration of the polymer in solution. At high polymer concentrations lateral aggregation

must be assumed to be a possibility.

It should also be noted here that Gruenwedel and co-workers[40] attributed the lowered T_m values at very high counterion concentration as possibly being due to an interference with the binding of water to the DNA molecule and believe that an interference with the binding of water may interfere with the H-bonding in the G-C pairs.

C. Enthalpy of the Helix-Coil Transition

The value for ΔH of 9.4 Kcal(mole base pair)$^{-1}$ at pH 7.0 should be compared only to the estimation of enthalpy at 95°C of 9.3 Kcal[2] for the helix-coil transition of poly(A + U) and the value of 9.65 Kcal for the DNA transition measured by Privalov[2] at 85°C along with the value 7.6 Kcal·(mole base pair)$^{-1}$ measured by Shiao and Sturtevant[8]. Some of the earlier works involved measurements at much lower temperatures. Ackermann[2] showed that the results of the earlier work of Krakauer and Sturtevant[3] were in agreement with his when taking temperature dependence into account. The results of Barber[16] and of Shiao and Sturtevant[8] involved substantial corrections for heat of ionization on the calorimetric measurements while those of Rialdi and Profumo[17] are difficult to compare directly due to the presence of the concentrated urea

solution. The value obtained in this lab tends to support the data of Privalov.[2]

The dependence of enthalpy on pH agrees in form but not in magnitiude with the results of Privalov.[6] Barber's[16] report of change in pK values for the G and C residues with increase in ion concentration is in the wrong direction to explain the results obtained in this laboratory. The enthalpy decrease with increasing acidity may be at least in part explainable by the ionization of the bases by protonation. It may be possible that a change in pH interferes with the stacking interactions of the bases, especially polyA, in the alkaline region. Under these circumstances the double helical form of the DNA may be partly denatured, or at least partly destablilized by solutions of higher pH. At higher pH values there is an apparent, but not statistically significant, decrease in the T_m which may be a reflection of the destabilization of the helical form.

The apparent dependence of transition enthalpy on polymer concentration is not explainable in view of the agreement of the enthalpy value measured at high polymer concentration with values obtained by other workers at much lower polymer concentrations than any used in this study.

The effect of salt concentration on the areas of the endothermic

peaks can be explained by the study by Gruenwedel and co-workers[40] on the effects of salt concentration on transition temperatures. If increase in salt concentration stabilizes the helix until there is so much counterion that it interferes with the binding of water molecules to the polymer (causing destabilization), then the transition enthalpy could be expected to increase with increasing salt concentration to a peak value and then decrease.

D. Estimation of Entropy and Free Energy of Reaction

Using the assumption that the helix-coil transition can be considered thermodynamically as a phase change, and that at the transition temperature free energy will be zero, the entropy can be estimated. Making the further assumption that the enthalpy and entropy of the helix-coil transition are independent of temperature, the free energy of this reaction can be calculated from equation 7. The validity of this assumption is discussed in chapter I. From these relationships entropy and free energy of the transitions were calculated at each pH. Values for T_m were obtained by finding the temperature at which the computer determined value of the ratio a/A is 0.5 (see chapter II for explanation of terms). Values for ΔG_{298^o} and ΔG_{310^o} were calculated. Entropy values are listed in table IV and free energy values are given in table V. Entropy was found to vary between

20 and 25 cal·deg^{-1} mole^{-1} The entropy appears to decrease with change in pH in either direction from pH 7. If the structure is partially disordered before heating, due to acidity or alkalinity of the solution, then the entropy change for the reaction would be lower. The magnitude of the calculated entropies is similar to those reported by other workers (Neumann and Ackermann[2] and Shiao and Sturtevant[8]). The former team reported the transition enthalpy for the disordering of the Poly(A + U) double helix as 25 cal·deg^{-1} mole^{-1}, at pH7. The latter team reported entropies for the transition of calf thymus DNA is alkaline solution as being between 27 and 30 cal·deg^{-1} mole^{-1} and in neutral solution as 19 to 21 cal·deg^{-1} mole^{-1} It must be remembered that the enthalpy values reported in that study for alkaline solutions involve substantial correction factors for heat of deprotonation. Privalov[6] did not report entropy values but when equation 7 is used with the values of enthalpy and transition temperature reported, ΔS would be 27 cal·deg^{-1} mole^{-1}.

Free energy of the transition is found to vary with pH in much the same manner as the transition enthalpy. Again, the change with change in pH is greater that that observed by Privalov[6], especially with regard to decrease in free energy at pH 5.4 with respect to the free energy at pH 7.0. Other workers (Neumann and Ackermann[2] Shiao and Sturtevant[8]) also report values for the free energy of the

TABLE IV

Entropy of the Helix-Coil Transition of DNA

pH	ΔH (kcal·mole^{-1})	ΔS (cal·deg^{-1}mole^{-1})	T_m * (^oK)
8.2	7.6 ± .4	20.3 ± 1.1	104
7.8	8.5 ± .5	22.4 ± 1.3	105
7.4	8.7 ± .4	23.0 ± 1.1	106
7.0	9.4 ± .2	24.8 ± 1	106
6.6	8.6 ± .2	22.7 ± 1	106
6.2	8.5 ± .4	22.4 ± 1.1	106
5.8	7.7 ± .4	20.3 ± 1.1	106
5.4	7.7 ± .3	20.3 ± 1	106

* Temperature at which partial area under the curve is equal to one half of the total area.

TABLE V

Free Energy of Helix-Coil Transition of DNA

pH	ΔG_{298° (cal·mole^{-1})*	ΔG_{310° (cal·mole^{-1})*
8.2	1,600	1,350
7.8	1,800	1,530
7.4	1,800	1,570
7.0	2,000	1,700
6.6	1,800	1,540
6.2	1,800	1,530
5.8	1,700	1,400
5.4	1,700	1,400

*Calculated from equation 7

helix coil transition. The former workers reported 1800 cal(mole base pair)$^{-1}$ for the free energy of the transition of poly(A + U) at pH 7.0 and the latter report 800 cal(mole base pair)$^{-1}$ for the free energy of DNA at 298°K. Privalov[6] calculated free energy at pH 7.0, 310°K as 1200 cal(mole base pair)$^{-1}$. The free energy values listed in table 5 are somewhat high compared to the values mentioned above. There may be a relationship between the high free energy values and the high transition temperatures. One of the reports (Shiao and Sturtevant[8]) gives an equation for a temperature correction for ΔH in the equation used to calculate free energy. Unfortunately it requires values of enthalpy of at least two temperatures in order to calculate a ΔC_p term and that information is not available from the DTA data of this present work. To estimate the magnitude of the error introduced by uncorrected ΔH values, the temperature correction term was applied to Privalov's[6] enthalpy value and the calculated free energy di d not change.

E. Kinetics of the Helix-Random Coil Transition

The assumptions that are made in the analysis of the Arrhenius type plots using the method of Borschardt and Daniels[13] are as follows:

1. The reaction is complex and, having the possibility of being

a multistage reaction, a "temperature break," or sharp change in slope of an Arrhenius plot, can be valid (see Hulett,[39] Kumanoto,[41] Frost and Pearson[42]). Kumanoto[41] attributes this type of phenomena to phase changes in biological systems. It is possible that the loss of bound water molecules in sufficient number can have the kinetic properties of a phase change.

2. The final portion on the graphs of rate constant as a function of temperature are distortions inherent in the equations, i.e. dH/dt divided by (A-a) is mathematically equivalent to 1/x which asymptotically approaches infinity as x goes to zero. As the value of a approaches that of A the value of (A-a) approaches zero. It approaches zero even faster if there is an undervaluation of A, even by a very small percentage. The shape of the final portion of the curve was found to change as the value of A was changed.

Based on these assumptions, and noting that the second order plots are linear over 80 percent of the extent of the reaction, the reaction can be said to show apparent second order kinetics. It was believed unlikely that an Arrhenius plot could be justifiably analyzed as a series of four or five straight line segments as would be necessary to find any linearity in the plots of first order rate constants. Another consideration is that the first order plots are seen to curve

downward and the third order plots are seen to curve upward. The seond order plots represent the change of direction. By itself, this consideration would have little meaning but it gives some support to the conclusion of apparent second order kinetics.

When the experiment was planned, the expectation was that kinetic study would show first order kinetics if the reaction was in fact amenable to analysis by methods ordinarily used for analyzing simpler reactions. In consideration of the results three possibilities become apparent. First is that the reaction is truly too complex for analysis by this method and the linear second order plot is a coincidence. This possibility cannot be entirely eliminated but the close conformity of the plots to linearity over so large a portion of the reaction makes pure coincidence unlikely. If the assumptions on which the foregoing analysis is based are valid then the second order result is fairly clearcut. A second possibility is that the reaction is second order with respect to the species in solution capable of independently absorbing energy that species being something other than a pair of polymers tied together. That species could be the separated single stranded helixes of the polymer. For such a case the transition state would have to be one in which the strands are sufficiently separated to absorb energy independently and yet are

interacting or at least still linked in the transition state. This is, frankly, difficult to visualize. The third possibility is that the second order effects arise out of the relatively high concentration of polymer in the solutions studied, and that in a sufficiently dilute solution the kinetics would be first order. The high concentration could cause the second order effects either because of lateral interactions, the closest visualization being a sort of super "crystal" in which the helices are loosely bonded to their nearest neighbors, or to the high concentration resulting in a high viscosity and the reaction rate being dependent on the viscosity of the solution.[43] Massie and Zimm[11] found relaxation times dependent on concentration and on bulk viscosity of the solution. They attribute the concentration dependence as being largely due to viscosity effects. However, simultaneous dependence on concentration and on bulk viscosity (in turn dependent on concentration) could conceivably result in second order kinetics. It has also been suggested[43] that the frictional resistance to unwinding effecting the individual molecule can be expressed as the sum of two terms; the first being the intrinsic viscosity of the solution and the second being proportional to the intrinsisc viscosity to the seond power, and that the second term becomes more important at higher viscosities.

The activation energy calculated from graphs of second order rate constants is 69± 5 Kcal. There is no indication of pH dependence for the second order activation energy in either the alkaline or acidic pH region. If the second order effect is due primarily to the viscosity of the solution the "true" or first order activation energy would probably be found to be lower. The initial ten percent of the graphs show an apparent activation energy of approximately 200 Kcal/mole. There is an apparent decrease in activation energy at lower pH values (relative to activation energy at pH 7), but no observable decrease at high pH values. Spatz and Crothers[12] observe a non frictional limited process with an apparent activation energy of at least 100 Kcal(mole)$^{-1}$. They ascribe this to nucleation, the disordering of small regions of the double helix. No results of this study are in contradiction to this. However, if the apparent decrease in activation energy of this initial phase of the reaction is real, then a large part of the activation energy must be due to forces other than poly A stacking interactions since poly A is known to be more ordered in moderately acidic solutions than in neutral solutions.[4]

CHAPTER VI

Mechanism for the Thermal Denaturation of DNA - An Hypothesis

A. Bound Water

The kinetic data presented in this thesis points to certain possibilities concerning the mechanism of DNA denaturation. The apparent break (i.e. intersection of two straight lines) in the Arrhenius plots suggest that the mechanism involves at least two steps. The slopes of the lines indicate that the initial phase of the reaction is characterized by an activation energy of approximately 200 Kcal while the second phase has an apparent second order activation energy of 69 Kcal(mole).$^{-1}$ The phenomenon of non-linearity in Arrhenius plots has been variously ascribed to consecutive steps in a reaction,[39] the existence of pre-equilibria[39] and to phase changes in the system.[41] Any one of these terms might be used in a description of a mechanism for the thermal denaturation denaturation of DNA that involves the removal of a layer of bound water as the initial phase of the reaction; the completion of this initial phase being required before further disordering can occur. Krakauer and Sturtevant[3] proposed that the overall reaction for the disordering of the poly(A + U) helix includes the removal of bound counterions and bound water molecules (equation 2, chapter I).

Gruenwedel[40] attributed the decrease in T_m for DNA at very high salt concentration to an interference with the binding of water molecules.

The problem of water bound to, or associated with, biopolymers has been studied previously by differential thermal analysis. Certain observed endothermic peaks on the thermograms of biopolymers have been attributed to changes in the degree of association of water molecules to the biopolymers.[44,45,46] The problem of bound water also been studied by neutron scattering,[47] x-ray diffraction,[48] and by NMR techniques.[49,50,51] Although at present there is not complete agreement on the subject of bound water,[50] the results of the previously mentioned studies[47-51] are generally interpreted as evidence of the existence of an ordered layer of water molecules oriented around and/or tightly bound to the DNA molecule. It has been proposed that this ordered layer of water molecules has a role in maintaining the ordered structure of the DNA molecule.[48,49,50] Furthermore, evidence from NMR studies on DNA indicates that changes in the state of hydration of the helix occur before any of the charges in structure generally associated with the thermal denaturation of DNA.[49]

In a recent study, Alvarez and Biltonen[10] measured the heat of

solution of thymine in water and in ethanol, to test for the existence of hydrophobic forces as they are classically pictured.[52] The study showed almost identical heats of solution of thymine in the two solvents and a strong dependence of heat of solution, in both solvents, on temperature. The stabilization of the ordered structure of DNA by solute-solvent hydrogen bonding was proposed as an explanation for the observed phenomena. Lubas and co-workers[49] used NMR techniques to study the changes in DNA that occur before and during thermal denaturation. They found that the relaxation times of water protons increased rapidly with increases in temperature, up to the onset of denaturation. At higher temperatures the relaxation times remained constant or decreased. Signals associated with non-rotationally bound water ceased at temperatures approximately 10^{o} below the temperature at which denaturation begins.

B. Proposed Model for the Mechanism of Thermal Denaturation of DNA

The hypothesis that the first step in the thermal denaturation of DNA is the removal of bound water molecules is consistent with the results of the studies mentioned here and is definitely supported the findings of Lubas.[49] The second phase of the reaction is believed by this author to be the friction limited unwinding of the double helix.

Friction limited uncoiling is generally accepted as a rate determining step in the denaturation of DNA. It is consistent with the data presented in this study. It is a possible explanation for the observed second order kinetics.

The mechanism proposed by Spatz and Crothers[12] contains many elements of similarity to the mechanism presented in this paper. Their model is based on the results of a detailed study on the thermal denaturation of DNA in which the reaction was initiated by temperature perturbations (t-jumps) and followed by monitoring the resultant optical density changes. Several different kinetic responses, or "effects" were observed. A molecular weight independent "slow effect" was identified as having an apparent activation energy of at least 100 Kcal. Spatz and Crothers attribute this high activation energy component as due to the sudden uncoiling of G-C rich regions existing between A-T rich coiled sections. A "fast effect" estimated to have a low activation energy was attributed to the unwinding of the helix. An "instantaneous effect" was also identified. Both mechanisms suggest that the reaction consists of at least two steps, one of which has a very high activation energy, and the other being identified with the unwinding of the helix. The principal difference between the two proposed mechanisms is the suggested nature of the high-activation energy component of the reaction. The mechanism proposed in this paper attributes this

reaction component to the "melting" of a layer of bound water rather than to the "melting" of a G-C rich helical region into a coiled region. It should be possible to test for the possible role of bound water in the stabilization of the helix and the possibility that the removal of a layer of bound water is a first phase in the reaction mechanism.

C. Projections of Further Study

Two procedures, both based on DTA techniques, are believed to offer possibilities for further testing of the reaction mechanism proposed in this paper. The activated complex for the mechanism presented here would be a DNA molecule in helical form, from which a stabilizing layer of bound water has been removed. This complex would be expected to show a decrease in volume relative to the initial state while an activated complex envisioned for the Spatz and Crothers[12] nucleation step would be expected to show an increase in volume relative to the unreacted state. Activation volume, the change in volume of the activated complex relative to the initial state of the reactants, can be obtained from the slope of a graph of the logarithm of rate constant as a function of pressure. The measurement of rate constants at varying pressures should be possible with the use of the Pressure DSC Cell of the DuPont 900 DTA.

It is believed that differences will exist in the enthalpy and activation energy of the helix-coil transition, as measured with the use of DTA, when the DNA is completely dried and then dissolved in D_2O (compared to the values measured for the reaction of DNA in H_2O) if binding of water molecules has a significant role in the stabilization of the helix and their removal is an important step in the denaturation reaction. Differences in enthalpy and activation energy will be more probable if Alvarez and Biltonen are correct in their conclusion that the binding of water is due primarily to solute-solvent hydrogen bonding rather than to hydrophobic interactions. Interpretation of the results of DTA studies of the thermal denaturation DNA in deuterated water would be aided by further NMR studies of the type reported by Lubas.[49]

Further studies are required to resolve the problem of the mechanism of the denaturation of DNA. It is believed by this author that an understanding of the mechanism of the reaction is closely linked to an understanding of the helix-stabilizing forces.

CHAPTER VII

CONCLUSIONS

The enthalpy of the helix-random coil transition of DNA was found to be 9.4± .2 Kcal(mole base pair)$^{-1}$. In view of the possible concentration dependence of the enthalpy and the high concentration of polymer solution the above value is more likely to be a maximum than a minimum value. The entropy of the transition was estimated to be 25 cal·deg^{-1}(mole base pair)$^{-1}$ at pH 7. The value of the free energy change for the transition was estimated at 37°C and pH 7 to be 1700 cal(mole base pair).$^{-1}$

The results of the kinetic study indicates a two-step process for thermal denaturation of DNA. The activation energy for the initial phase is of the order of 200 Kcal. The second stage, showing apparent second order kinetics has a measured activation energy of 69± 5 Kcal. The trend in slopes of the initial segment of the Arrhenius-type plots with change in pH suggest that much of the activation energy is required for the breaking of bonds other than stacking interactions of the bases. In the proposed model for thermal denaturation of DNA, the first stage of the reaction is attributed to removal of bound water and the second to friction limited unwinding of the helix. The problem of the role of bound water in the stabilization of the helix is open to further study.

This study has shown that the DTA is useful for quantitative

calorimetric measurements of biopolymer reactions. The capability of studying reactions over a range of temperatures gives it an advantage over calorimetric techniques which are applicable only at a fixed temperature. It was distinctly helpful in this case to be able to measure the heat absorbed in the thermally induced helix-coil transition directly, rather than having to create conditions which produce a transition at a lower temperature, introducing other effects for which corrections have to be made. The limitation of the DTA has been found to be the size of the sample which can be studied relative to the sensitivity of the instrument. Development of a Differential Scanning Calorimeter cell with a larger sample capacity and the same or higher sensitivity would greatly extend the range of reactions amenable to study by this instrumental technique.

APPENDIX 1

Calculation of Reaction Enthalpy from Thermogram Data

A. Calibration of the DSC cell

Absorption of heat by a sample above that required by the inert reference will cause the pen to move downward. In the case of a sample undergoing a phase change or an endothermic reaction the pen will move downward until the reaction is completed and then trace a return to a baseline, often to the original baseline. The area under the curve, enclosed by the extension of the original baseline to the new baseline, is directly proportional to the heat absorbed in the reaction or phase transition. Within the limits of instrumental accuracy the area will always be the same for a given amount of reactant, a given sensitivity setting and given heating rate. The size of the area traced out in an endothermic peak, per calorie of heat absorbed, is a property of the particular DSC cell. In order to measure enthalpy it is necessary to calibrate the instrument.

Calibration is achieved by measuring the area of the endothermic peak due to the phase transition of a substance whose heat of fusion is known with certainty. Two materials were used for calibration

purposes in this study: indium with a melting point of 156°C; and benzoic acid with a melting point of 122°C. The melting points of these substances are close to the temperature range over which the endothermic peak under study extends (95°C - 125°C). The peak area, in planimeter units, multiplied by heat absorbed, in calories, gives a calibration factor, in calories per planimeter unit.

The calibration factor used in subsequent calculations is the average of nine trials with a standard deviation of 3 percent from the mean. The calibration factor used is 121×10^{-6} calories per planimeter unit (at a fixed planimeter setting).

B. Enthalpy (Mole Base Pair)$^{-1}$

The weights of the nucleotide phosphates found in the literature[1] are listed in table VI. Allowing for the decrease in weight due to water lost in the 3-5 linkage, the weight per base pair is, to the nearest gram, 618 grams.

According to the records of Sigma Chemical Company (private communication) batch number 101C-9520 of calf thymus DNA is 16.8 percent by weight, water and 4.35 percent, by weight sodium. Therefore, there is .788 grams of DNA for each gram of polymer weighed.

TABLE VI

Weight of the Nucleotide Phosphates

Nucleotide Phosphate	Molecular Weight*
Deoxy A 3' Phosphate	331.22 g
Deoxy C 3' Phosphate	307.20 g
Deoxy G 3' Phosphate	347.23 g
Thymidine 3' Phosphate	322.21 g

* reference 1

Area of the endothermic peak, of the helix-coil transition of DNA, in planimeter units per milligram of DNA, multiplied by the calibration factor gives a value for heat absorbed, in calories per gram. The base pair is the simplest, and most logical unit, in which enthalpy values can be expressed. Heat absorbed, in calories per gram, multiplied by the average weight per base pair gives a value for enthalpy in cal(or Kcal) per mole base pair.

APPENDIX 2

Computer Program for Calculation of Rate Constants

A. Explaination of Computer Program

The expression used for calculation of rate constant k, in the Borchardt and Daniels method for activation energy determination is given in equation 31. A term which is constant for each thermogram was dropped from the expression in the calculations used in the study (equation 32). The calculations required evaluation of dH/dt, in calories per second, and partial area under the curve, in calories, at each temperature for which k' is to be calculated.

The computer takes the measured lengths of the y-axis deflections, in units of length, and calculates dH/dt, in calories per second, using a conversion factor. With values of dH/dt and the differences between measured lines, a constant, the partial areas are found by means of numerical integration. Values are determined for k' for each temperature and trial reaction order. The program contains loops for the calculation of slope and intercept of the graphs of log k' as a function of 1/T, as successive points are dropped from the curve. Due to distortion of the last segments of the graphs, this part of the program was not found to be of significant use.

The conversion factor for finding dH/dt, in calories per second was found by measuring, in planimeter units, a box, having a width of one inch and a length equal to the distance traveled by the pen in one minute at a heating rate of 22°C per minute. Area of the box, in calories, was found using the calibration factor for the particular DSC cell Area, in calories divided by the length (60 seconds) gives a conversion factor in calories $second^{-1}$ $inches.^{-1}$ The value of the conversion factor, determined in this way, is 2.18×10^{-4} $cal.seconds^{-1}$ $inches.^{-1}$

The constant used in the numerical integration is the time required for the recorder pen to travel 1°C at a heating rate of 22°C $min.^{-1}$ This was calculated to be 2.727 seconds.

B. Computer Program for Calculation of Rate Constant (Program Language - Basic)

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5     PRINT
6     PRINT "TYPE TOT AREA, FIRST AREA SEGMENT"
10    INPUT, A1, B1
20    DIM C(30),J(30),K(30),L(30),O(30),W(30),Z(30,6),D(30)
25    PRINT "TYPE TOT NUMBER DATA POINTS"
30    INPUT N1
35    PRINT "TYPE VALUES OF T, DELTA T"
40    FOR I1 = 1 TO N1
50    INPUT C(I1),J(I1)
60    NEXT I1
65    PRINT
70    PRINT "1/T", "DH/DT", "R1", "R2", "R3"
75    PRINT
80    FOR I1 = 1 TO N1
85    LET K(I1) = 1/(C(I1)+273)
90    LET L(I1) = J(I1)*.000218
95    IF I1 > 1 GO TO 110
100   GO TO 270
110   LET O(I1) = 2.727*.5*(L(I1)+L(I1-1)
120   LET W(I1) = O(I1)+W(I1-1)
130   LET D(I1) = A1-W(I1)
135   FOR M1 = 2 TO 6
140   LET Z(I1,M1) = 1(I1)/(D(I1)↑ (M1/2))
150   NEXT M1
160   PRINT K(I1),L(I1),Z(I1,2),Z(I1,4),Z(I1,6)
170   NEXT I1
175   PRINT
180   PRINT "1/T", "PART AREA", "AREA DIF", "R1.5", "R2.5"
185   PRINT
190   FOR I1 = 1 to N1
195   PRINT K(I1),W(I1),D(I1),Z(I1,3),Z(I1,5)
200   NEXT I1
202   PRINT
203   PRINT "VALUE OF A(N) IS", M1
210   FOR M1 = 2 TO 6
215   PRINT
216   PRINT "VALUE OF M1 IS", M1
217   PRINT
220   GOSUB 350
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235    NEXT M1
250    GO TO 9999
270    LET W(I1) = B1
275    GO TO 130
350    LET N = N1
355    DIM X(900), Y(900), T(30)
379    FOR I = 1 TO N
383    LET Y(I) = LOG(Z(I,M1))
384    LET X(I) = K(I)
386    NEXT I
387    FOR J2 = 1 TO N-5
389    LET K2 = (N+1-J2)
390    LET E = O
392    LET F = O
394    LET G = O
396    LET H = O
398    LET P = O
400    FOR I = J2 TO N
402    LET E = E + Y(I)
404    LET F = F + X(I)
406    LET G = G + Y(I)↑2
408    LET H = H + X(I)↑2
410    LET P = P + X(I)*Y(I)
412    NEXT I
414    LET Q = (K2*H) - (F↑2)
416    LET M = (K2*P-E*F)/Q
418    LET B = (H*E-F*P)/Q
422    PRINT M,B,J2
424    NEXT J2
450    RETURN
9999   END
```

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