AN ABSTRACT OF THE THESIS OF

Micheal H. Zehfus for the degree of Doctor of Philosophy in Biochemistry and Biophysics presented on May 25, 1983

Title: Properties and Conformation of P-form DNA

Abstract approved: Redacted for Privacy

W.C. Johnson Jr.

Work is presented which explores the properties and nature of the P-form of DNA. Initially the P-form represented an unknown state of DNA which occurred when this molecule was in a solvent of 47.5% methanol, 5% water, and 47.5% ethanol, and which showed properties of both native and denatured conformations. In the first part of this thesis data are shown that (1) demonstrate that the P-form also exists as an irreversible function of temperature in solvents containing only 5% water but ranging from 95% to 47.5% methanol and 47.5% to 0% ethanol, (2) extend the circular dichroism spectrum of the P-form into the far UV where its low intensity indicates a lack of base stacking, and (3) demonstrate that the P-form in these solvents has an unusual and unexpectedly high heat stability. These data are interpreted to show that the P-form must be caused by a structural change within the DNA molecule, and several structural models are proposed that may account for the data acquired.

The latter part of this work presents experiments which prove

that the P-form structure is a combination of collapsed tertiary and denatured secondary structures. The presence of a collapsed tertiary structure is first indicated in viscosity data and is then demonstrated by the actual visualization of the condensed state with electron microscopy. The denatured state of the molecule is first hinted at in a delayed 10.2 to P-form transition in covalently closed circular DNA, and then proved when infrared spectroscopy shows that there is no hydrogen bonding in the P-form structure.

This condensed-denatured structure for the P-form fully explains all of the seemingly contradictory properties of this state.

Properties and Conformation of P-form DNA

by

Micheal H. Zehfus

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Completed May 25, 1983

Commencement June 1984

APPROVED:

Redacted for Privacy

Professor of Biochemistry and Biophysics in charge of major

Redacted for Privacy

Chairman of department of Biochemistry and Biophysics

Redacted for Privacy

Dean of Graduate School

Date thesis is presented May 25, 1983

Typed by Valerie L. Palmer for Micheal H. Zehfus

ACKNOWLEDGEMENTS

I would first like to thank my wife Betty for her continued support, patience, and understanding throughout an overly long graduate career.

I would also like to thank W. Curtis Johnson Jr., my graduate advisor, whose lessons into the nature and art of science extended well beyond the laboratory.

TABLE OF CONTENTS

LIST OF FIGURES

THE PROPERTIES AND CONFORMATION OF P-FORM DNA

SECTION I: INTRODUCTION

When DNA is in an aqueous environment it has a B-form structure with 10.4 base pairs in each complete helical turn.[1,2] If this aqueous environment is gradually changed by adding the moderately less polar alcohol, methanol, the twist of the helix relaxes, and the DNA structure changes smoothly into a form with only 10.2 base pairs per turn.[3] In contrast to this behavior, if the water is replaced with the slightly more nonpolar alcohol, ethanol, the same kind of continuous change will occur only until 65% of the water is replaced with ethanol.[4,5] At this point the DNA abruptly changes to the A-form which has a more tightly twisted helix containing 11 base pairs per turn.

Since it is rather puzzling that such similar solvents as methanol and ethanol should produce such different DNA forms as the 10.2 and the A-form, Johnson and Girod decided, in 1972, to study this system further.[6] In their work they used circular dichroism (CD) to trace the occurrence of these forms and their intermediates in a tertiary solvent containing methanol, water, and ethanol. Much to their surprise, they found that when placed in a solvent of equal parts methanol and ethanol and only 5% water, the DNA changed in form, not to some intermediate state between the 10.2 form and the A-form, but instead into an entirely different form. This form had a low conservative CD spectrum and exhib-

ited a hyperchromic absorption at 260 nm. From these properties, which closely resembled those of denatured DNA, they concluded that they had unexpectedly found a novel way of denaturing the DNA molecule.

About two years later Ernest Kay examined this system and observed basically the same results.[7] However, he also found that the DNA which had undergone this transition could be easily transformed back into native DNA by simply adding water to the solvent. Because he was working with large complex DNAs which could not renature under these conditions, he concluded that the DNA in this mixed solvent was not denatured, but was still in a native state. His explanation of why the circular dichroism and optical density of the DNA closely resembled that of denatured DNA was simply that some other undefined interaction had produced these changes.

In 1978 Seth and Nordén also examined this system, this time using linear dichroism as well as CD to study the DNA's properties.[8] After confirming both of the other groups' results, they did further experimentation which suggested that this unknown state of the DNA might be due to a tertiary structural condensation of the molecule, and called this state the P-form.

In this thesis I will describe work which I have done both to further define the properties of the P-form, and to discover the exact nature of its structure. Section II of this work contains the text of a paper that was published in BIOPOLYMERS in

1981 in which I used circular dichroism to study the properties of the P-form transition.[9] From these properties I prove that the P-form must be a structural transition within the DNA molecule, and propose several structural models which can account for its observed properties. Section III of this work describes two unpublished experiments in which I first tried to eliminate some of these models as possible P-form structures. This was done first by examining the P-form transition in covalently closed circular and linear DNA molecules, and second by measuring the viscosity of the P-form state. Section IV contains the text of a manuscript which will be submitted for publication shortly, and describes the details of two critical experiments which resolve the nature of the P-form structure. Through the use of infrared spectroscopy and electron microscopy these experiments prove that the P-form is actually a curious combination of both secondary structure denaturation and tertiary structure condensation. Finally, in Section V, all the significant experiments and results are reviewed to see how they fit with this collapsed-denatured structure. The physical cause of the P-form collapse is also discussed in an attempt to gain new insight into the structural dynamics of DNA itself.

SECTION II: PROPERTIES OF P-FORM DNA AS REVEALED BY CIRCULAR DICHROISM

SYNOPSIS

Investigations of DNA using circular dichroism spectroscopy show that the P-form is available in a wide variety of methanol-ethanol mixtures when the water content is low. Increasing the temperature or the ethanol content of a 95 percent methanol solution causes DNA to undergo a cooperative transition to the P-form. However, this transition cannot be reversed on cooling, or on adding methanol. Thus P-form DNA appears to be stable at high methanol concentrations, but it is usually not observed because the DNA is trapped by a kinetic barrier. P-form DNA will instantaneously assume the native B-form on addition of water, confirming earlier reports that P-form DNA is not strand separated.[7] CD spectra extended to 190 nm show that there is no base-base interaction in the P-form. However, the P-form is extremely stable to heat denaturation in solvents which promote hydrogen bonding between the base pairs.

A number of models that can account for the properties of P-form DNA are discussed.

INTRODUCTION

When DNA is packed into the head of a virus or closely associated with protein as it is in chromatin, it is no longer

in a truly aqueous environment. Instead, the amount of water around the DNA molecule has become severely restricted, and may even have been partially replaced with protein moieties.

Since many of the physical parameters dealing with DNA structure and conformation are derived in dilute aqueous solutions, the application of these parameters to partially aqueous biological systems is questionable. One way of investigating this problem is to study DNA in partially or completely nonaqueous solvents, and then to use this information as a model for the in vivo environment.

A good first choice for such a model system would seem to be different amounts of low molecular weight alcohols and water. Using such a system one could obtain large ranges of water concentration and polarity, with few problems of solubility.

Work using alcohol solvents has not been straightforward. Even closely related alcohols like methanol and ethanol have shown distinctly different properties.[4,5] As water is replaced with methanol, the DNA goes smoothly from the 10.4 base pair per turn "B-form"[1,2] to a more tightly twisted form with 10.2 base pairs per turn (10.2 form).[3] The ethanol system behaves similarly up to 65-70% ethanol, at which point the DNA cooperatively shifts to the more loosely twisted A-form, a structure also seen in isopropanol and dioxane.[4,5]

In an attempt to study this difference between methanol and ethanol, Johnson and Girod[6] used a mixed methanol-water-ethanol system and found yet another anomaly. As the water was replaced

with a solvent of equal parts methanol and ethanol, the DNA went smoothly from 10.4 toward 10.2 base pairs per turn, but at 12.5% water, the circular dichroism (CD) of the solution changed abruptly to one which closely resembled that of DNA heat denatured in aqueous solution (henceforth called denaturation). Accompanying this shift in the CD was an increase in the optical density (OD) of the solution, which also corresponded to that seen in denaturation. They further found that if they attempted to reverse the transition by adding either more ethanol or more methanol, they could not obtain the CD spectrum expected for that addition. These properties led them to conclude that the DNA had undergone a rather novel denaturation.

Kay[7] reexamined this system and showed basically the same results, but he added an experiment where the DNA which had been exposed to the alcohol solvent was dialyzed back to 100% water. After this treatment he obtained the original 10.4 form CD spectrum, indicating that denaturation had not occurred. He confirmed this result by using neutral CsCl density gradient centrifugation to show that no strand separation had occurred.

Recently, Nordén et al[8] have used linear dichroism in the presence of the dye, methyl green, as a probe of this system. Their results confirmed the experimental findings of the other groups, and further showed that the presence of the dye helped to delay the transition. They concluded that the DNA was undergoing a strutural change, probably a tertiary condensation, and have called it "P-

form" DNA.

In this paper we report an extensive CD study of P-form DNA. We find that DNA assumes the P-form conformation in a wider variety of solvents than was hitherto recognized, and that P-form DNA is particularly stable to heat denaturation. While the stability of P-form DNA could argue for a condensed structure, other evidence argues against this. We discuss how this and other models could account for these properties.

MATERIALS AND METHODS

Calf thymus DNA was obtained from Worthington Biochemical Corporation and used without further purification.

To avoid confusion when referring to mixed alcohol solvent systems, we have adopted the same convention used by Johnson and Girod.[6] A three number code will refer to the percent concentration (by volume) of the solvents in the order methanol-buffer-ethanol. Thus 75-5-20 is a solvent system composed of 75% methanol, 5% buffer, and 20% ethanol by initial volume. The final buffer concentration considering the entire solvent mixture was 0.005 x SSC (SSC is 150 mM NaCl, 15 mM citrate, pH 7.5). The change in volume of the solvent upon mixing is not significant.

For work at wavelengths longer than 220 nm, a DNA stock solution of 1 mg/ml was made in 0.1 x SSC. This was diluted by a factor of 20 with the appropriate solvents at 5° C in the order water-methanol-ethanol, since it was found that mixing with ethanol first could precipitate the sample. Heat denatured DNA stock was pre-

pared by taking an aliquot of the native stock, heating it to 100°C for 10 min, then cooling it on ice. The standard pathlength was one centimeter, except in mixing experiments where longer pathlengths were used to accommodate more dilute samples.

For work at wavelengths shorter than 220 nm, a solution of 0.88 mg/ml was prepared by dissolving the DNA in a 50-50 buffer-methanol solution; the resulting gel was then diluted with the remaining methanol or ethanol over the course of several days to prevent precipitation. This solution is 1.0 OD at 190 nm in a 200 μm cell.

CD work above 220 nm was done on either a Jasco-Durrum J-10, or a Jasco J-40. In both cases an electronic heat exchanger was used to control cell temperature to within 0.1°C. Below 220 nm the CD work was performed on a McPherson vacuum UV spectrophotometer modified for CD work.[10] Here temperature was controlled to ±0.5 C with a thermostated circulating water system. All CD spectrometers were calibrated daily with a (+)-10-camphosulfonic acid standard, using $\Delta\varepsilon$ at 290.5 nm = 2.37.[11]

Concentrations of calf thymus DNA were calculated using ε ($l\ mole^{-1}cm^{-1}$) at 260 nm of 6600 for aqueous solution and 6720 for 100% ethylene glycol.[12] Values of ε at 260 nm were determined to be 6920 in 95-5-0 at 8°C, 9580 in 95-5-0 at 33°C, and 9590 in 47.5-5-47.5 at 8°C for calf thymus DNA.

RESULTS

Since the major difference in interpretation of the 47.5-5-47.5

spectrum stems from the presence or absence of reversibility, our first task was to duplicate the reversibility experiments of both Johnson and Girod,[6] and Kay[7]. Our work (not shown) confirms the results of both groups: (1) DNA in the P-form in 47.5-5-47.5 may not be reversed to the 10.2 form usually seen in 85-5-10, and (2) the conformation may be reversed to "B-form" by dialyzing into water.

Since dialyzing solutions is both time consuming and subject to changes in concentration, we have simplified the procedure by taking DNA in a solvent mixture of low water content and adding it directly to buffer to get a solvent mixture of high water content. Thus DNA in 47.5-5-47.5 solvent (P-form) was mixed with buffer to produce a 5-90-5 solvent. As Fig. 1 demonstrates, this solution has the same CD (within experimental error) as a DNA sample originally made in the 5-90-5 solvent (10.4 form) which has not undergone the transition to P-form. Furthermore, this CD is distinctly different from the CD of heat denatured DNA in 5-90-5 (Fig. 1).

This denatured DNA spectrum was obtained by two methods. In the first, a heat denatured DNA stock was exposed to the 47.5-5-47.5 solvent mixture, and then mixed with additional buffer until it was 5-90-5, exactly as was done with the native sample. In the second, a native DNA sample in the 5-90-5 solvent was simply heated until it denatured. In both cases the same denatured spectrum was obtained.

The same results, that DNA exposed to P-forming solvent and re-

Fig. 1 The CD spectra of calf thymus DNA in the 5-90-5 solvent system at 8°C. (--) native DNA, (——) DNA which has been reversed from 47.5-5-47.5, and (····) heat denatured DNA.

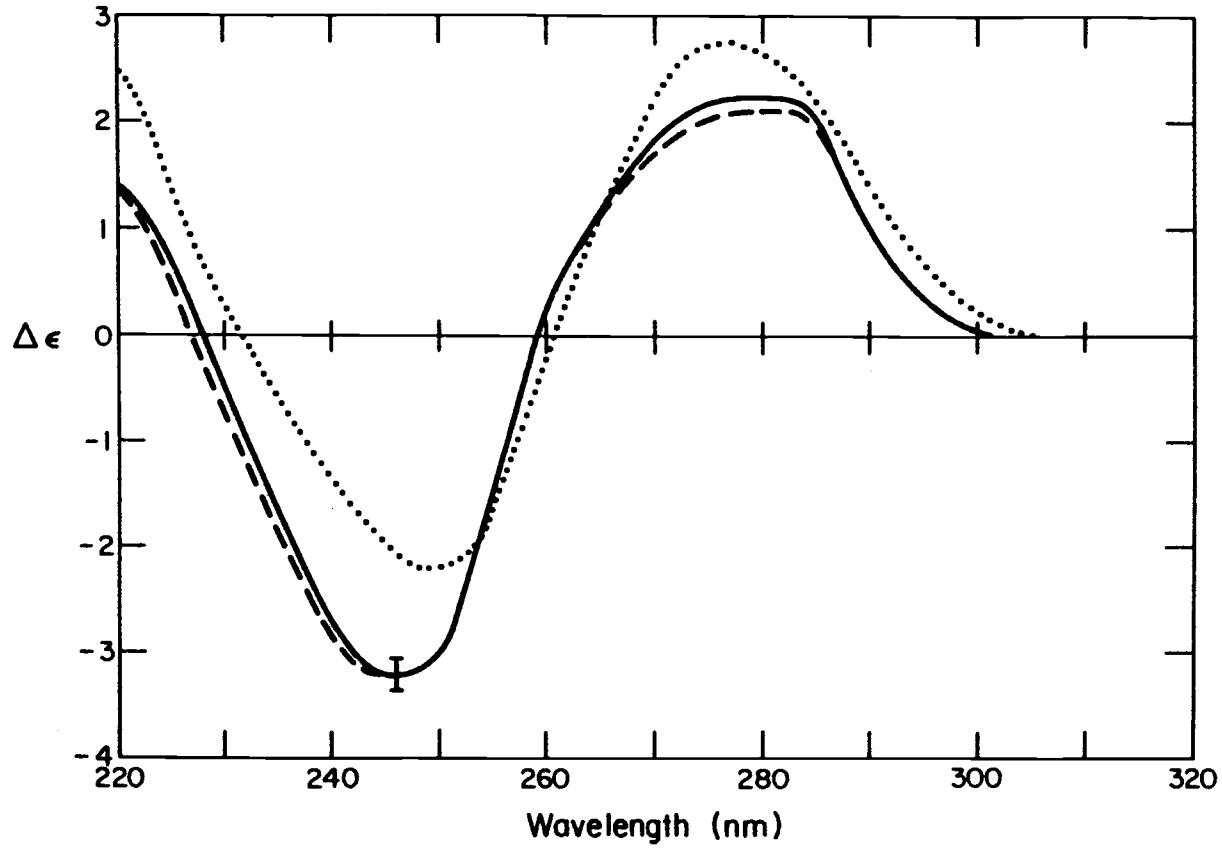

Fig. 1

versed to a solvent of higher water content showed native, and not denatured conformation, could be obtained using a reversal to 25-50-25. The resulting CD spectra in this case are similar to those shown in Fig. 2.

This technique of exposing DNA to a P-forming solvent, mixing it with buffer to reverse it to a solvent of high water content, then comparing spectra with known native and denatured samples will be used in other experiments as a probe for denaturation.

At low temperature DNA in 95% methanol is in the 10.2 form. However, above 26°C it undergoes a transition, and its CD changes to one which could be either a P-form CD or a denatured form CD.[4] Thus we have carried out reversibility experiments similar to those just described to see if this is a transition to P-form, or to denaturation. Fig. 2 compares the CD of DNA shifted from 95-5-0 to 50-50-0 for both the low temperature (10.2 form) and higher temperature (unknown) form. The CD spectrum of the unknown form does not match the denatured CD, but is identical to non-denatured DNA, within experimental error. This indicates that the higher temperature form is not heat denatured. Similar results are obtained for the shift to 10-90-0, although the CD spectrum is close to the "B-form" in this nearly aqueous solvent, and the spectra resemble those in Fig. 1. Presumably, the temperature dependent transition in 95-5-0 is also a transition to the P-form.

Taken together, the results for the 47.5-5-47.5 and 95-5-0 solvent systems indicate that the temperature for transition to the P-form must depend on solvent composition. Thus we have examined

Fig. 2 The CD spectra of calf thymus DNA in the 50-50-0 solvent system at 8°C. (--) non-denatured DNA which has been reversed from 95-5-0 at 8°C, (—) DNA which has been reversed from 95-5-0 at 35°C (P-form), and (····) DNA that has been heat denatured.

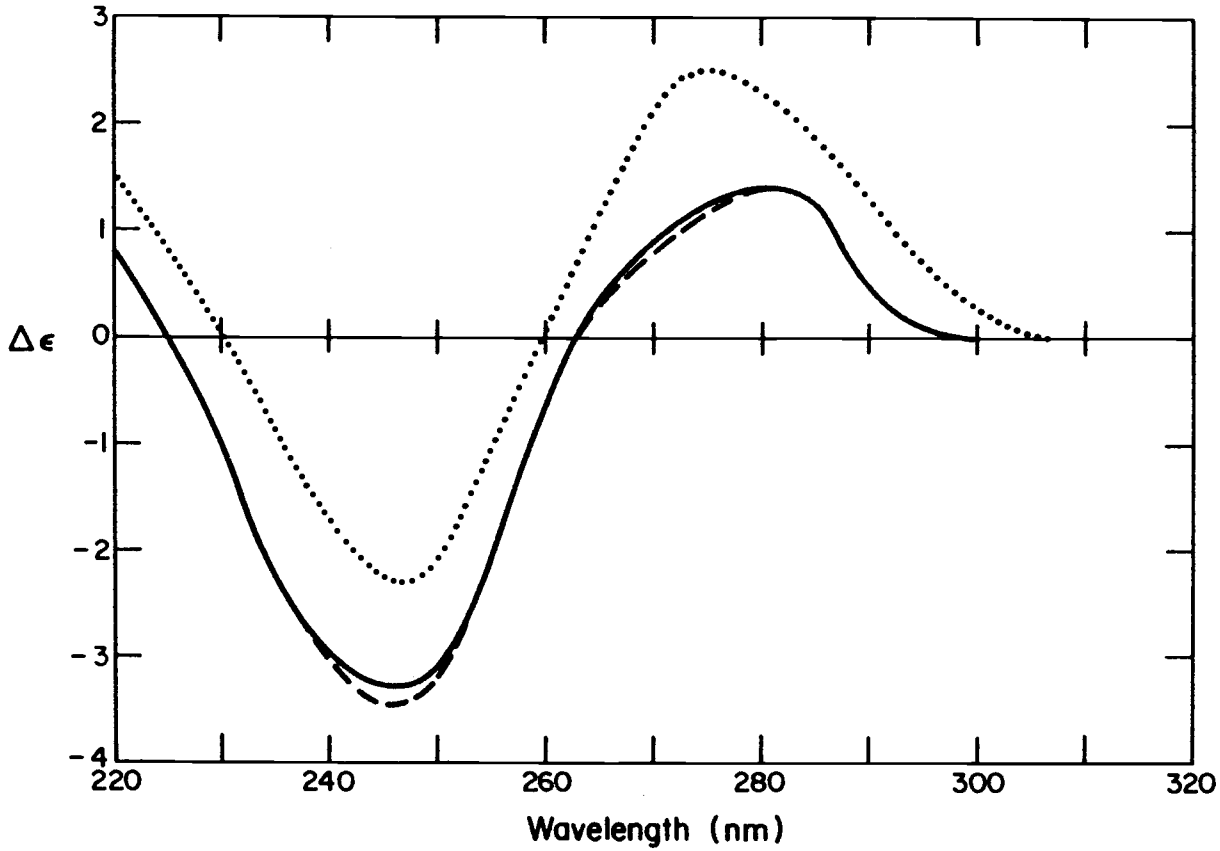

Fig. 2

the relationship between temperature and relative methanol-ethanol concentration in our solvent mixture. To do this we made solutions of 65-5-30, 75-5-20 and 85-5-10, then followed the transition to the P-form as a function of temperature by monitoring the CD. In these experiments the DNA was first mixed with alcohols at -15°C. The solution was then placed in a pre-chilled cell and brought to the lowest temperature in the run. The solution was then allowed to equilibrate for at least 1.5 hours before each measurement. After the experiment was finished the DNA solution was mixed with the appropriate solvent at 20°C to reverse it to a 5-90-5 solution, and its CD was again taken to confirm that denaturation had not occurred. A typical run is shown in Fig. 3.

The results clearly show that there is an interrelationship between the temperature of transition and the relative alcohol concentration. We find that the temperature at the midpoint of the transition to P-form is -4°C for 65-5-30, +9°C for 75-5-20, and +20°C for 85-5-10. Apparently we could obtain the 10.2 form of DNA in 47.5-5-47.5 if we prepared the solution at a low enough temperature.

As final evidence for the thermal transition in methanol being a P-form transition, we have investigated the 190 nm CD band of DNA on our vacuum UV spectrograph since this CD band is particularly sensitive to variations in base-base interactions.[13] Fig. 4 compares the CD of DNA in 47.5-5-47.5 to DNA in 95-5-0, both before and after the 26°C transition. Both DNA in 47.5-5-47.5 at 8°C and

Fig. 3 The CD spectra of calf thymus DNA in 85-5-10 as a function of temperature.

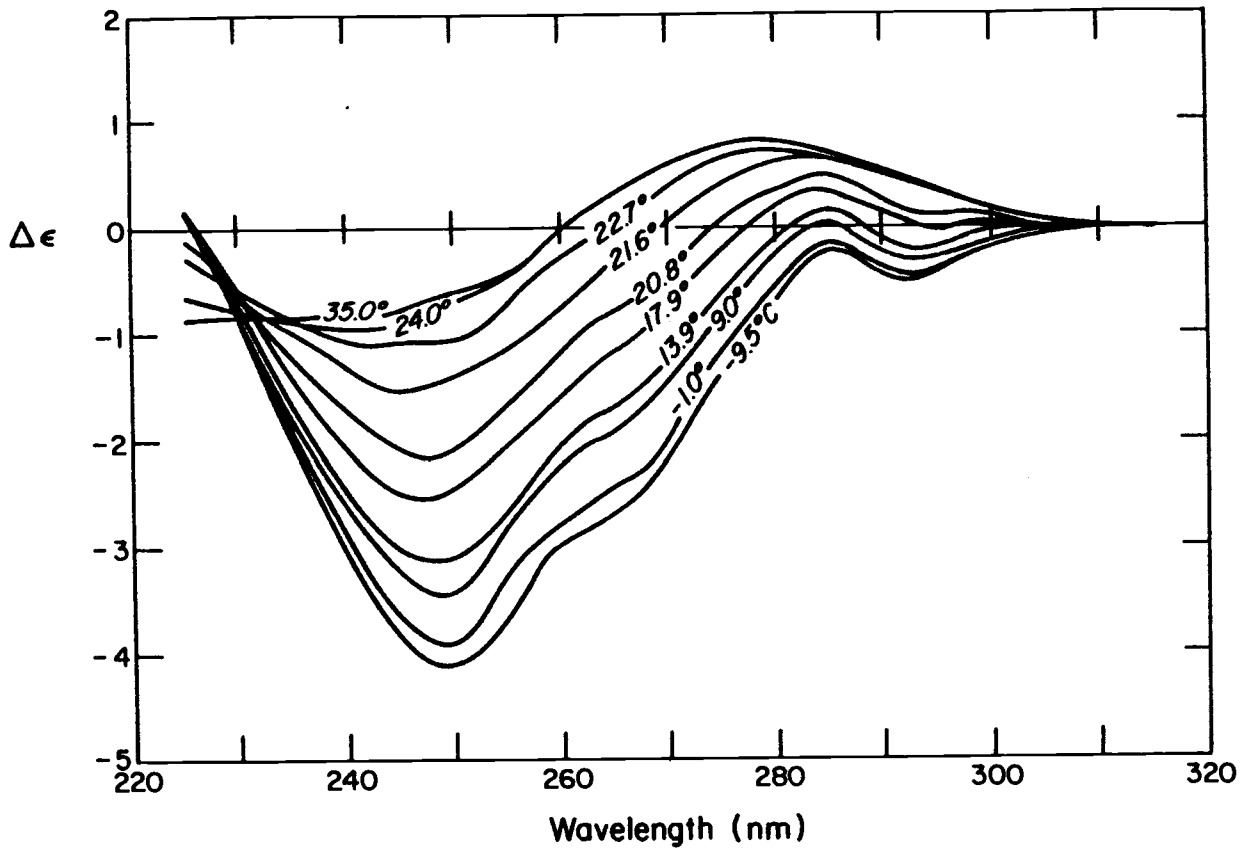
2
1
0
-1
-2
-3
-4
-5
Δε
220
240
260
280
300
320
Wavelength (nm)
35.0°
24.0°
22.7°
21.6°
20.8°
17.9°
13.9°
9.0°
-1.0°
-9.5°C

Fig. 3

Fig. 4 Extended CD spectra of calf thymus DNA. (——) DNA in 95-5-0 at 8°C, (— —) in 95-5-0 at 33°C (P-form), and (····) in 47.5-5-47.5 at 8°C (P-form).

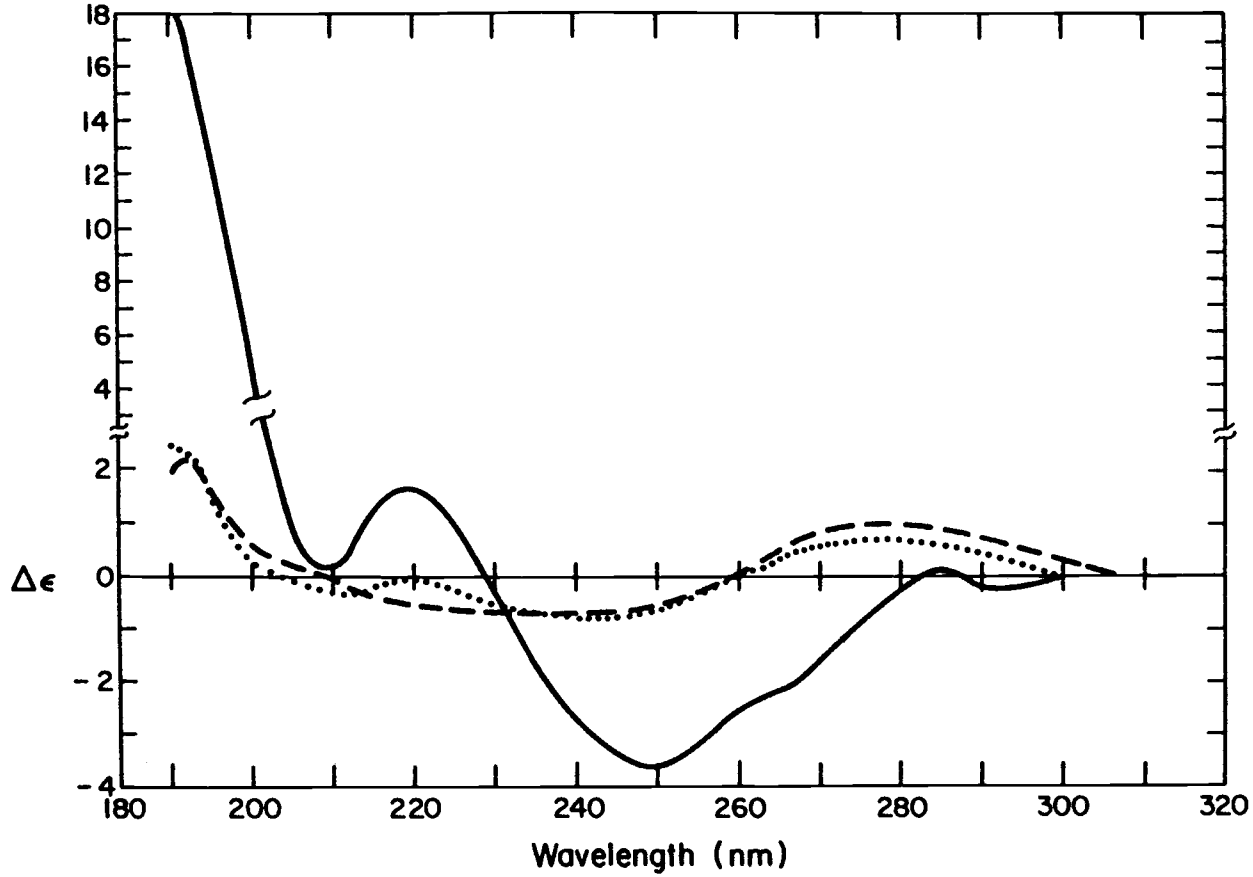
18
16
14
12
10
8
6
4
2
Δε
0
-2
-4
180
200
220
240
260
280
300
320
Wavelength (nm)

Fig. 4

DNA in 95-5-0 at 33°C have almost the same CD spectrum, demonstrating that P-form DNA exists in both solvents. These CD spectra are different from the denatured DNA spectrum measured previously.[14] However, the low intensity of the 190 nm CD band for P-form DNA, like the low intensity of this band in heat denatured DNA, indicates that there is no base-base interaction in the P-form.

Previous work has shown that DNA in the P-form may not be reversed to the 10.2 form by the addition of methanol.[6] A similar situation exists with respect to the heat-induced transition. Once DNA is changed to the P-form by heating, simple cooling will not reverse the transition. We have heated DNA in 95-5-0 to 33°C to change it to P-form, then cooled it back to 8°C, and monitored for the return of the 10.2 form. Even after three weeks at this temperature, we found no evidence of reversibility. It must be emphasized that at the same time the conformation may be reversed to "B-form" by simply adding buffer. Thus, as long as the water content in the solvent remains low, the transition to P-form brought about by either solvent or temperature change appears irreversible, but in reality it is reversible when the water content is increased.

Having established that DNA in 47.5-5-47.5 at 8°C or 95-5-0 at 33°C is not denatured, it would seem that at higher temperatures we should be able to detect true denaturation. The first experiment to test this hypothesis was to place DNA in the 47.5-5-47.5 solvent and to observe its CD as it was heated to 59°C, almost the boiling point of the solution. This experiment gave the puzzling

result shown in Fig. 5. As the DNA was heated, the 280 nm CD band decreased in intensity and stayed the same width, while the 240 nm band broadened into the 220 nm region. Concomitantly the OD of the solution decreased slightly. We would expect the 280 nm CD band to increase slightly and broaden into the 305 nm region and the 240 nm band to remain constant for heat denatured DNA (Fig. 5).

Since this type of experiment could give no clear indication of denaturation, we looked for denaturation using the solvent reversibility technique described earlier. Here the DNA exposed to the P-forming solvent mixture was first heated to a specific temperature before reversing it to a solvent mixture of high water content where denaturation could be observed. To extend the range of the experiments above the boiling point of the solvent mixture, we sealed the solutions in screw-top vials. These sealed samples could be exposed to 100°C with no boiling of the solvent. After the solution was heated for 10 minutes at a given temperature it was then rapidly cooled on ice, and mixed with water to reverse it to either a 25-50-25 or a 5-90-5 solvent in which denaturation, if it occurred, could be detected.

Fig. 6 shows that even when heated to 100°C for 10 minutes, DNA in 47.5-5-47.5 seems stable and shows no signs of denaturation. The DNA in 95-5-0 does not seem quite as stable. As shown in Fig. 7, when DNA in this solvent mixture is heated above 70°C some irreversible change must take place, since shifting the solvent back to 50-50-0 or 10-90-0 does not give a full recovery of a "B-form"

Fig. 5 The CD spectra of calf thymus DNA in 47.5-5-47.5. (——) 8°C, (--) 59°C, and (····) heat denatured at 8°C.

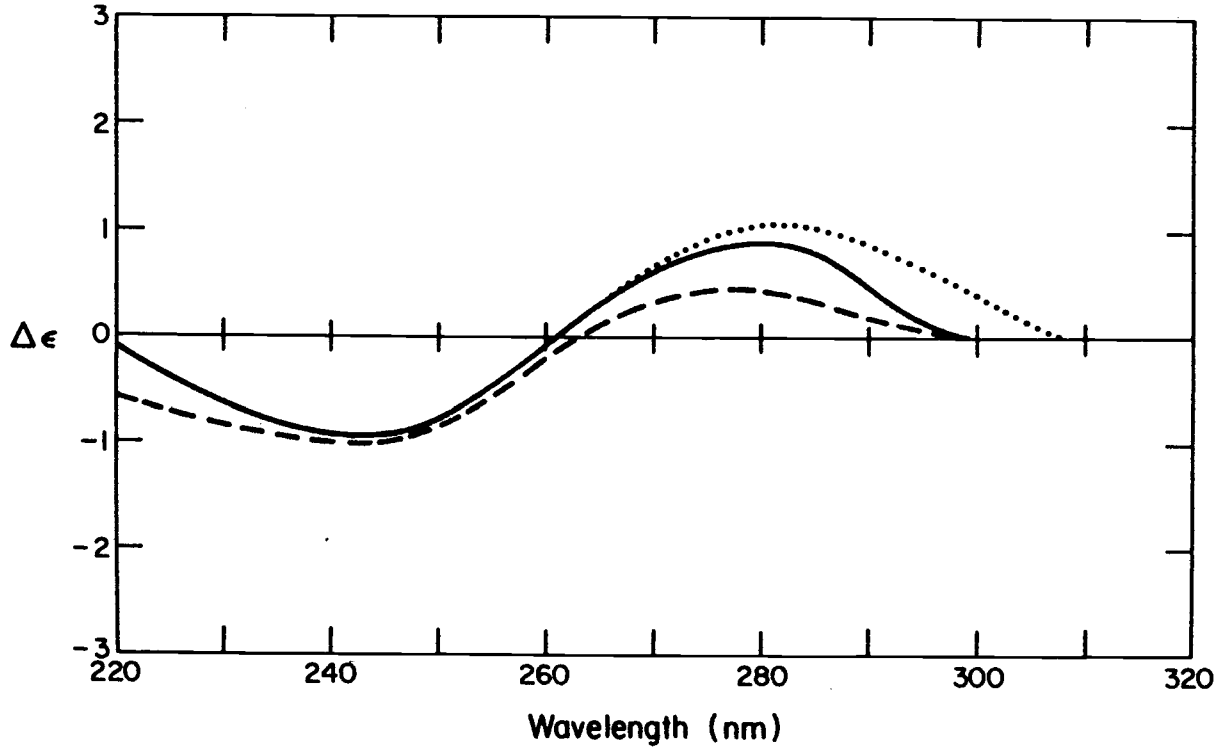

Fig. 5

Fig. 6 The CD spectra of calf thymus DNA heated to 100°C for 10 minutes in the 47.5-5-47.5 solvent and reversed to a solvent of higher water content. (--) both heated sample and non-heated control reversed to 25-50-25, and (····) heated sample and (——)non-heated control reversed to 5-90-5. Spectra were run at 8°C.

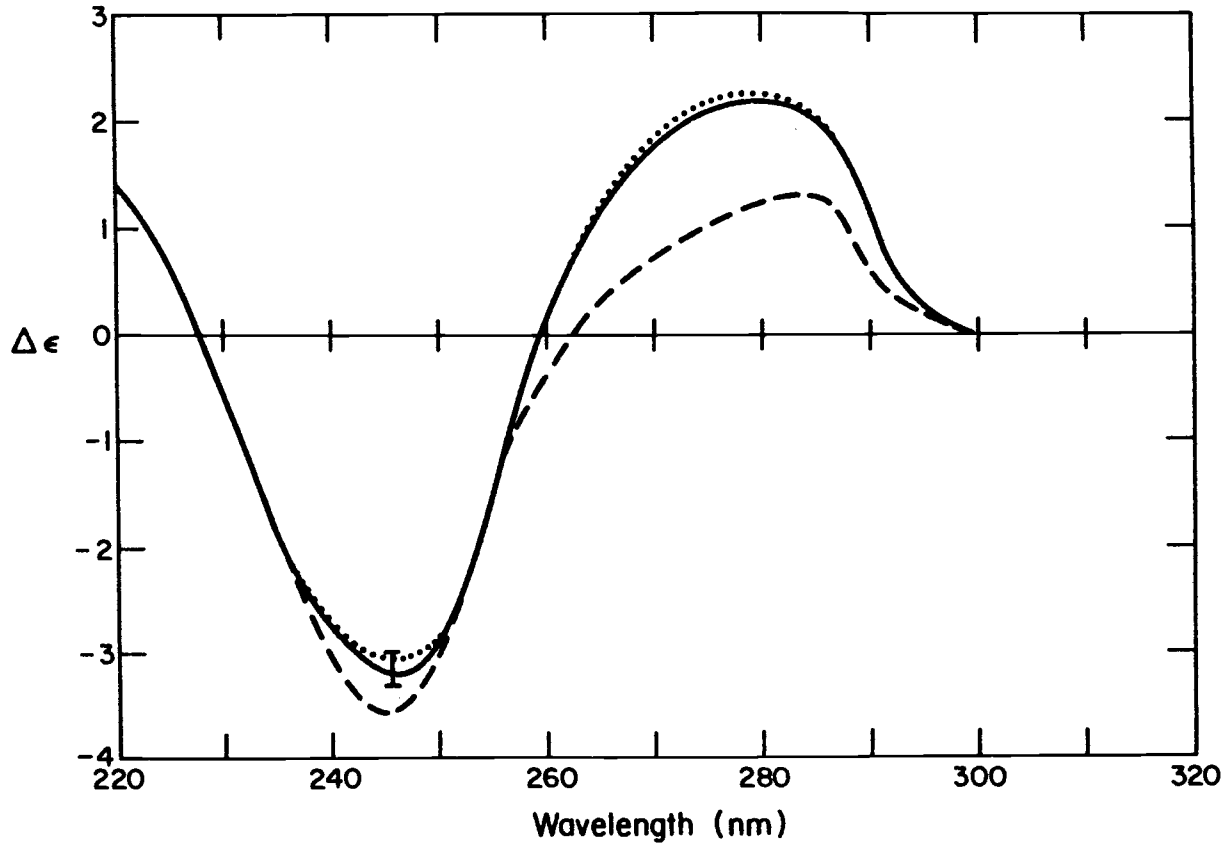

Fig. 6

Fig. 7 The CD spectra of calf thymus DNA heated to different temperatures and reversed to (a) the 50-50-0 solvent at 8°C, and (b) the 10-90-0 solvent at 8°C. (——) 40°C, (---) 80°C, and (····) 100°C.

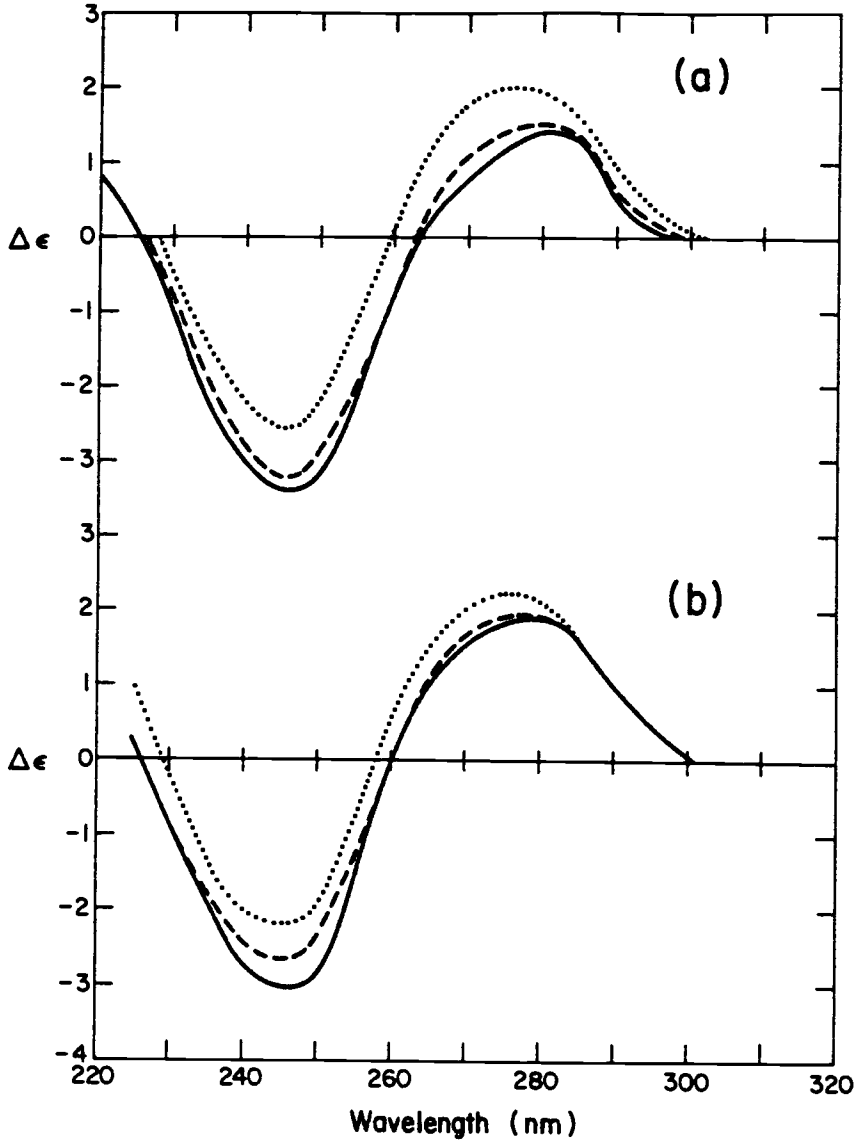

Fig. 7

spectrum. We believe that this second irreversible transition represents the true denaturation of DNA in this solvent. Our data indicate that even though denaturation seems to be occurring, it is not complete. When we compare the 100°C spectrum in Fig. 7a with the native and denatured spectra in Fig. 2, we find that at 100°C the CD is between the two extremes.

As a control for these experiments DNA was dissolved in buffer (0-100-0), sealed in a tube, and subjected to the conditions just outlined. When this solution was cooled and mixed with the appropriate alcohol mixture it showed complete denaturation above 60°C. We then used this denatured spectrum and the native spectrum as two endpoints with which to examine the 95-5-0 results. Analyzing the 100°C spectrum as a linear sum of native and denatured components, we found that at this temperature about 40% of the DNA was still in the native conformation, a rather unexpected result for DNA which should have denatured in a sharp transition in buffer at about 60°C.

One final question to be answered by CD spectroscopy is whether the transition to P-form is unique to the methanol-ethanol solvent system, or if it may be seen in other solvents giving the 10.2 form. 100% ethylene glycol (0.1 M KF, 0.01 M Na_2EDTA) is such a solvent, and DNA in this solvent undergoes a transition at about 34°C to a conservative CD spectrum of low intensity which could denote either denatured or P-form DNA.[12] However, when this DNA is heated above its transition temperature, only the denatured spectrum may be obtained when 50% or 90% buffer is added. We have also duplicated

this experiment using 95% ethylene glycol and 5% of our standard buffer with the same result. Furthermore, the 10.2 form of DNA under the high salt conditions of 5.4 M NH_4Cl and 6.2 M LiCl gave the same negative result. Based on this information we conclude that neither ethylene glycol nor these high salts creates a stable P-form.

DISCUSSION

To help visualize the domains of DNA conformation in mixed alcohol solvents we show in Fig. 8a a triangular phase diagram adapted from Johnson and Girod.[6] The data presented in this paper essentially take the line between 47.5-5-47.5 and 95-5-0 and extend it upward in an added dimension of temperature, as shown in Fig. 8b. In this figure, the distinct transition between the 10.2 form and the P-form is represented as a line, while the gradual onset of denaturation is shown by shading.

One important feature of the P-form transition that these diagrams do not convey is its polarity. The transition from the 10.2 form to the P-form, whether induced by heat or solvent change is not reversible if the water content remains unchanged. Thus a P-form DNA may exist in a region of the phase diagram marked 10.2 form, if the DNA had previously been exposed to P-forming conditions. So far the only way we have found to change a P-form DNA to another form is to add more water to the solvent, to bring the DNA toward the 10.4 form.

Any model proposed for the P-form must be consistent with all

Fig. 8 The domains of DNA conformations: (a) as a function of alcohol and buffer content at constant temperature (8°C)* and (b) as a function of alcohol content and temperature at a constant buffer concentration (5%).

*Adopted from reference 6.

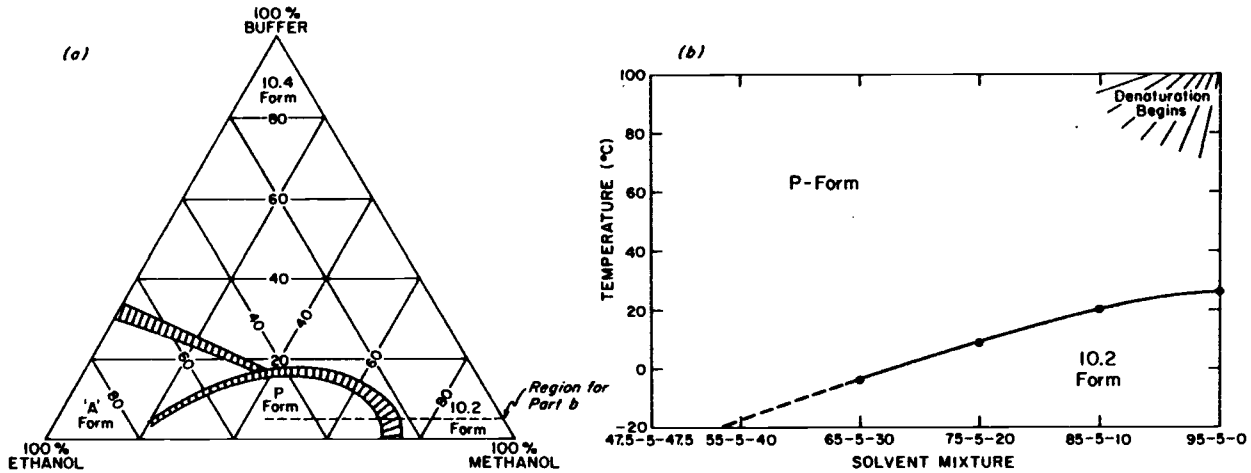
(a)
100 %
BUFFER
10.4
Form
80
60
40
40
40
20
60
80
80
'A'
Form
P
Form
10.2
Form
Region for
Part b
100 %
ETHANOL
100 %
METHANOL
(b)
100
80
60
40
20
0
-20
TEMPERATURE (°C)
Denaturation
Begins
P-Form
10.2
Form
47.5-5-47.5
55-5-40
65-5-30
75-5-20
85-5-10
95-5-0
SOLVENT MIXTURE

Fig. 8

of these properties. Primarily it must account for the high heat stability of the DNA and provide a rationale for both the non-reversibility of the transition when the water content of the solvent is low, and the free reversibility of the transition when the water content is increased. Further, it must be consistent with both the CD and LD data which provide information on the position of the bases within the P-form DNA molecule.

Denaturation Model

It seems quite apparent now that normal denaturation involving complete strand separation has not occurred in the P-form transition. In earlier experiments where dialysis was used to reverse the P-form to "B-form" transition,[7] there was a slim possibility that renaturation was occurring during the period of dialysis. However, in our experiments the DNA in alcohol solvent is rapidly mixed with buffer and we find that the "B-form" spectrum appears immediately. This rapid change in a DNA as heterogeneous as calf thymus simply could not happen if the P-form involves complete strand separation.

Solvent Effect Model

The possibility of a solvent effect that does not involve a structural change may also be dismissed. We have shown DNA in a given methanol-ethanol solvent may be placed into the P-form by heating, and that once this is done the DNA remains in the P-form even when it is cooled back to its original temperature. This co-

existence of two forms in the same solvent at the same temperature is not possible unless some structural change has occurred to give the molecule a "memory" of its previous exposure to the conditions that caused the transition.

Structural Models

Nordén et al.[8] suggest that P-form DNA could be a tertiary condensation similar to condensation of A-form DNA seen in solutions of high ethanol content. We would like to point out that three other models will account for all the properties we have observed. First, the helix might unwind to an unstacked but hydrogen-bonded double-stranded DNA. Second, it might unwind to a stacked and double-stranded DNA with a roughly equal mixture of right and left handed[15] helical sections. Finally, the DNA might denature with strand separation for all but some short region that is exceptionally stable.

Thermal Stability of Structural Models

The resistance of P-DNA to thermal denaturation may be explained by all four structural models. If the condensed particle itself were heat stable, a large amount of strand separation could occur within the particle as it was heated, but the overall particle structure would hold the Watson and Crick strands close together. When the solution is cooled the close positioning of the strands would allow them to quickly snap back in register as if no denaturation had occurred. In this instance our mixing experiment,

which only detects permanent denaturation, would give a negative result. One problem with this model is that the behavior just outlined does not occur in the A-form condensed structure seen in high ethanol concentrations. Here the condensed structures seem to disappear at about 50°C, indicating that these structures are not very stable.[4] Similarly, exceptional stability of a small region would keep the DNA strands in register.

To have the unwound model or the model with a mixture of helicities account for the heat stability of P-form DNA, we must postulate a second property for DNA in low water solvents. We would propose that the DNA bases would have a greater tendency to hydrogen bond with each other, rather than hydrogen bond with the solvent, if the solvent is of low polarity. This would make interbase hydrogen bonding relatively stable in the 47.5-5-47.5 solvent (dielectric constant, about 31) somewhat less stable in the 95-5-0 solvent (dielectric constant, about 35) and unstable in ethylene glycol (dielectric constant, about 41) or buffer. This would give the same hierarchy of stability seen in our results.

Non-reversibility of Structural Models

The non-reversibility of the transition at low water concentration may be explained in all four models if there is a fairly high activation energy for the transition, and the P-form occurs at a lower overall energy. This would mean that the P-form was actually more stable than the 10.2 form, but that a kinetic barrier existed to hold the DNA in the 10.2 form. This energy bar-

rier could be overcome by heating the solution above the transition temperature, or by adding ethanol to lower the transition temperature, below the temperature of the sample. Once in the P-form it would take even more energy to return the DNA to the 10.2 form. Cooling the solution would instead supply less energy, while adding methanol would raise the amount of energy required for the reverse transition by raising the transition temperature. In either case the DNA would now be locked in the P-form.

CD Data

The main feature of the P-form CD is its overall low intensity. This is especially interesting in the 190 nm band, since low intensity here indicates that there is little or no base-base interaction. These data match best with the unwound model, since the physical act of unwinding the helix would move the bases further apart, thereby decreasing their interactions.

A model of DNA stacked in left and right handed helices would have strong base-base interactions, but it is possible that the interactions in a right handed helical section would cancel out the interactions arising from the left handed helical section. The net result might be a CD spectrum of low overall intensity.

The mostly denatured model for the P-form also fits the CD data, since it is already known that the P-form and the denatured form have very similar CD spectra. If the sections of DNA which remained hydrogen bonded comprised less than 1% of the molecule,

they would not be seen in the CD simply because the CD signal from the remaining 99% of the denatured molecule would swamp out the small signal from the non-denatured section.

It is hard to make a condensed DNA model fit the observed CD, because we do not expect a condensation to decrease base-base interactions. In its simplest case a condensation will not change base-base interactions at all, and will leave the CD of the condensed molecule the same as the CD of the free solution molecule. This type of behavior is seen in the condensation of "A" form DNA from ethanol.[16]

In more complex cases condensation may give rise to unusual long range interactions, or to scattering artifacts.[16] In either case the CD of the condensed molecule becomes much greater in overall magnitude than the CD of the free solution molecule. Obviously this is not happening in the P-form transition, since here we have a change to a CD of much lower overall magnitude. This leads us to conclude that a condensed structure alone will not account for the P-form spectrum.

LD Data

The weak positive LD of P-form DNA[8] tells us that the angle of the average transition moment of the bases is less than 54.7° away from the orientation axis.[17] This kind of behavior has already been observed for denatured DNA,[17] and again the mostly denatured model could be applied since the signal from the 99% of the molecule which is denatured could swamp out the signal from the 1%

which isn't.

These data have also been fit to the condensed model by simply hypothesizing that the condensed structure holds the bases in the correct geometry with respect to the long axis of the condensed molecule.[8]

An unwound model may also be consistent with the LD data. In unwinding the phosphate backbone which - normally has about a 70° angle with the molecular axis - must be straightened until it is roughly parallel with this axis. This would have the effect of moving the bases farther apart, allowing them more freedom to twist in place. Moving the bases farther apart would also increase exposure of the bases to the solvent. Since this might not be thermodynamically favored, the bases might compensate by tilting excessively. This would bring them back into contact with each other and thereby decrease their solvent exposure. In any case the unwound model could have dramatic changes in angles of both twist and tilt, either of which would be enough to move the average transition moment to the angle required by the LD data.

The mixture of helicities model is not consistent with the LD data, if the helices used resemble those already seen in X-ray work. In both left and right handed helices, the bases are roughly perpendicular to the molecular axis with very little twist in the base. The most extreme case for either sense of the helix is the left handed "A" form. Here the base is tilted about 20° away from the perpendicular with a -4° twist. The result is that any planar transition

within the base has an angle of about 70° with the molecular axis and is not within the limit of 54.7° required by the LD data. Thus, as long as no radical changes of structure have occurred, the mixture of helices model may be eliminated by the LD data.

We have found then that the properties of the P-form so far uncovered do not clearly favor any one of the potential models over the others. Work must continue both to more clearly define the P-form domain, and to define the P-form's properties so that its structure may be deduced.

ACKNOWLEDGMENT

We would like to thank Dr. Walter A. Baase for his helpful discussions of this project. This work was supported by NIH grant 5R01-GM21479-06 from the Institute of General Medical Sciences.

SECTION III: PRELIMINARY PROBES OF THE P-FORM STRUCTURE

INTRODUCTION

Up to this point we have seen that the circular dichroic properties of the P-form allow one to trace its occurrence in different solvents, and to uncover some of its properties, but they do not really shed much light directly on the underlying nature of its structure, except to tell us that the DNA has little electronic base-base interaction. While it is theoretically possible to interpret a CD curve to discover the underlying structure which caused a CD spectrum, this theory has not been well enough developed for us to use the CD directly to tell us anything further about the P-form structure. One can see then, that if the structural models just presented in Section II are to be proved or disproved, either the CD technique must be applied in a clever and indirect manner to get new structural information, or an entirely different physical-chemical technique will have to be applied.

Described in this section are experiments in which both approaches are used. As an indirect approach using CD, the P-form transition for both linear and covalently-closed, circular DNAs was first studied. Since the DNAs chosen have indentical primary sequences, and differ only in pre-existing tertiary structure, it was hoped that differences in the P-form transition of these molecules would allow one to differentiate between either a secondary or tertiary structural change mechanism. The results here indicate that the

P-form transition does involve a secondary structural change, but they are not consistent with any of the proposed models involving a large uniform deformation of the DNA's secondary structural twist. While I do not prove the existence of the condensed-denatured P-form structure until later, the results here are shown to be consistent with this structure.

As an entirely different physical-chemical approach the viscosity of native and P-form DNA molecules were measured. This parameter may be linked to the radius of gyration of a molecule, so this measurement can tell one fairly directly whether or not a change has occurred in the DNA's tertiary structure. My data here clearly show that the 47.5-5-47.5 P-form is a collapsed state because it has an intrinsic viscosity more that an order of magnitude lower than any noncondensed DNA structure. The interpretation of the 95-5-0 results are less clear because while the P-form in this solvent has a viscosity which is lower than the 10.2 form's viscosity, it is only lower by a factor of two, and some of this reduction may be attributed to the denaturation of secondary structure rather than the collapse of tertiary structure.

THE P-FORM TRANSITION IN LINEAR AND CCC pBR322

In linear DNA, the molecule may freely twist and untwist its secondary structure in response to changes in its environment because the two ends of the molecule are free. In covalently-closed, circular (CCC) DNA the two free ends are brought together and attached to each other with covalent chemical bonds. This linking of

the two free ends restricts the ability of the molecule to twist and untwist, and it now becomes impossible to change the molecule's total secondary twist without an accompanying compensatory change in the molecule's tertiary supertwist structure. The final result is that the CCC molecule will not undergo extreme changes in secondary structural twist as easily as the linear molecule.

It should be possible to exploit this difference in the abilities of these molecules to undergo secondary structural change to tell if this kind of change is involved in the P-form transition. If this barrier to secondary structural change has no effect on the 10.2 to P-form transition, ie. both the linear and CCC DNA molecules undergo the P-form transition identically, then the transition cannot involve changes in secondary structure, and must be due only to changes in tertiary structure. On the other hand, if the CCC molecule undergoes the P-form transition at a different rate or at a different temperature from that seen with the linear molecule, then a change in the molecule's secondary twist is clearly implicated. In fact, if either of the models presented for secondary structural change are correct, then it should be virtually impossible to get a P-form transition in CCC DNA.

Both the model where the DNA untwists to a ladder-like form, and the model where the DNA is in roughly equal amounts of left- and right-handed helical segments, involve changing the net twist of the DNA molecule to near zero. Because the CCC molecule is physically constrained, this change may not occur unless as many superhelical superturns are placed into the DNA's tertiary struc-

ture as were removed from its secondary structure. This nearly complete unwinding of the DNA would place roughly one superhelical turn into the tertiary structure for every 10 base pairs in the molecule. The resulting superhelical density would be so impossibly high that if a P-form transition did involve such an unwinding, it simply could not occur. We see then, that by simply monitoring this transition in the 95-5-0 solvent as a function of temperature and different DNA types, it should be possible to gain insight into the structural nature of the P-form.

All of the experiments up to this point have been done with calf thymus DNA. This already linear material may not be easily changed to the CCC form, so it is necessary to find a new source of DNA. For this source the pBR322 plasmid of Escherichia coli was chosen for several reasons. First, it had a similar GC content to calf thymus DNA so this would not become an added variable. Second, this DNA was fairly easy to grow and purify in the milligram quantities required for these experiments. Next, this molecule had been extensively mapped with restriction enzymes,[18,19] so it was already known that it could be cleaved with the commercially available restriction enzyme Eco RI to give linear molecules. Finally, the sequence of the molecule had been determined,[20] so if it looked fruitful further studies could be done where additional restriction cuts would be made in the DNA and the P-form transition could be followed in molecules of known sequence.

Plasmid growth and purification

The pBR322 plasmid was grown under fairly standard conditions and was purified using a slightly modified rapid purification technique developed by James Summerton.[21] Using this method one can make 5 mg of pBR322 DNA in three to four days from a two liter batch of E. coli culture. The final yield from this prep was generally 80% covalently closed linear monomer, 10% linear monomer, and 10% covalently closed dimer.

The E. coli cells were gron in a medium containing M9 salts (6g/1 Na_2HPO_4, 3g/1 KH_2PO_4, 0.5 g/1 NaCl, 1g/1 NH_4Cl and 0.1 mM $CaCl_2$ added after separate autoclaving), 4g/1 glucose, 4g/1 casamino acids 1.5mM $MgSO_4$, 20 mg/1 ampicillin, 20mg/1 tetracycline, 4mg/1 thiamine, and 4mg/1 thymine. 100mg/1 of uridine was also added to the bulk medium, but was not included in the medium for the overnight culture.

A 50 ml overnight culture was inoculated with a single loop of E. coli cells containing the pBR322 plasmid and incubated overnight at 37°C on a rotary shaker bath. The growth of the main culture was started when a small aliquot of the overnight culture was introduced into the bulk medium, and the medium placed in a shaking incubator also at 37°C. Since plasmid amplification in the main culture is initiated by the addition of chloramphenicol when this culture's apparent optical density at 590 nm reaches 0.6, the proper sized aliquot of overnight culture was determined so that this event would occur at some convenient time greater than 8 hours later. The size of this aliquot was found by measuring the overnight culture's apparent OD at 590 nm, then calculating how much this would have to

be diluted in the main culture so that the main culture's OD would reach the desired value at the proper time if this optical density would double every 45 minutes. Since this is at best only a rough estimate of cell growth, the actual OD of the solution at 590 nm was periodically monitored, and 100mg/l of dry chloramphenicol was added to the medium when this number reached 0.6. The culture was then returned to the incubator and plasmid amplification was allowed to continue overnight. The following morning the bacteria were harvested by first chilling the medium to 0°C, then centrifuging it at low speed to pellet the cells.

The DNA was then isolated using a modified Summerton rapid purification technique.[21] In this method the cells are gently lysed in high salt with low levels of detergent, and the plasmid DNA separated from the cellular DNA and debris by high speed centrifugation. The DNA is then selectively precipitated from the liquid with the addition of a 1:1 mixture of 4.5 M aqueous sodium trichloroacetic acid (NaTCA) and ethanol. The DNA is next rehydrated, treated with RNAse, and then precipitated a second time with the NaTCA reagent. The published procedure then finished by rehydrating the DNA and passing it through a nitrocellulose filter. In my preparations however, it was found that the DNA at this point still contained a sizable amount of contaminating RNA, so this step was replaced with gel filtration chromatography.

The NaTCA-precipitated DNA was first rehydrated in a buffer of 0.1M sodium acetate, 0.01M Na_2EDTA, pH 8.0. After allowing 24

hours for complete rehydration of the DNA, the solution was centrifuged to remove any insoluble material. The DNA solution was then passed through a 2.5 x 30 cm column of Bio-Rad A-15m which had been equilibrated with the same buffer used to rehydrate the DNA. In this step the large plasmid DNA comes through the column in the void volume while the remaining RNA contaminants are slowed by the gel and are separated away from the DNA peak. DNA containing fractions were then pooled and the DNA precipitated by adding 4 volumes of ethanol and storing at -20°C for 24 hours.

The final DNA pellet was rehydrated with the appropriate amount of 1M NaCl, 0.01 M Na_2EDTA, pH 8.0 buffer, so that its concentration would be 1 mg/ml, and was stored at 4°C. The DNA at this point contains roughly 80% covalently closed monomers, 10% linear monomers, and 10% covalently closed dimers as assayed by analytical ultracentrifugation in alkaline CsCl. This material was used directly in CD experiments as CCC DNA since it contained 90% covalently closed molecules.

For the experiments requiring linear DNA this material was further cleaved with the restriction enzyme Eco RI, which was obtained from Sigma Chemical Company. For this cleavage 2.5 mls of a solution containing 0.5 mg of DNA was first dialyzed extensively against a reaction buffer containing 0.1 M Tris, 0.05 M NaCl, 1mM Na_2EDTA at pH 7.5. The reaction was started when 0.1 ml of a 10,000 unit/ml Eco RI solution and 0.05 ml of a 0.5 M $MgCl_2$, 5mg/ml bovine serum albumin solution were added to the DNA solution and

the resulting reaction mixtures was incubated at 38°C for 2.5 hours. The cleavage was stopped by placing the reaction mixture on ice and adding 0.25 ml of 0.2 M Na_2EDTA (pH 8.0). The DNA solution was then extracted with phenol to remove the proteins, and further extracted with ether to remove the phenol. The solution was next dialyzed extensively against 0.1 M sodium acetate, 20 mM Na_2EDTA, pH 8.0, and the DNA precipitated by the addition of 4 volumes of ethanol and storing at -20°C. This linear material was then also rehydrated and stored in 1M NaCl 0.01 M Na_2EDTA at a concentration of 1.0 mg/ml. When analyzed with analytical ultracentrifugation this DNA was almost entirely linear, containing 5% CCC contaminants, and so was used directly in the CD experiments.

Determination of the 10.2 to P-form transition in pBR322

The temperature at which the pBR322 plasmid undergoes the 10.2 to P-form transition in the 95-5-0 solvent was found in a straightforward manner. The appropriate DNA stock was first dialyzed against the same 0.1 x SSC buffer used in Section II, and then diluted 20-fold with spectrophotometric grade methanol at 5°C. The solution was then transferred to a small volume 1.0 cm pathlength cuvette and its CD measured as a function of temperature. The time interval allowed between runs for temperature equilibration was 1.5 hours. After the run was completed the DNA solution was mixed with the proper solvent so it could be reversed to either 50-50-0 or 10-90-0 solvent. Since the DNA always reversed to a native form and not a denatured form, this step proved that a

P-form transition was actually being observed.

Analytical ultracentrifugation of the stock solutions used in these experiments showed that they had not significantly degraded. In the worst case the linear material contained 80% full length linear monomers and 20% slightly shorter material, while in the CCC DNA 80% of the molecules were still covalently constrained. This level of purity is not perfect, but it is acceptable for these experiments.

In Figure 9 we see a comparison of the temperature-induced P-form transitions in linear and CCC pBR322. One immediately recognizes that the transition occurs at a higher temperature (35.5°C) in CCC DNA than it does in linear DNA (25.5°C). As mentioned earlier, this elevation of the transition temperature shows us that the P-form transition involves a change in the DNA's secondary structure. This is because the covalent closure of the pBR322 molecule,which only restricts the ability of the DNA to undergo secondary structural change, has affected its ability to undergo a P-form transition. This may only happen if the P-form transition itself involves a secondary structural change.

While the transition is delayed, it still can occur, and is not totally blocked as I had anticipated it should be for the models of secondary structural change I had proposed. As one remembers both these models, one involving the nearly complete untwisting of the DNA to a ladder-like form, and the other involving a transformation to nearly equal amounts of left- and right- handed

Fig. 9 CD spectra of Linear and CCC pBR322 in 95-5-0 as a function of temperature.

(a) Linear DNA (——)8.2°, (—— ·)21.0°, (—— –)24.5°, (—— – –)25.4°, (- - - -)26.9°,(····)35°, and

(b) CCC DNA (——)8.0°, (—— ·)20.4°, (—— –)29.0°, (—— – –)34.3°, (- - - -)36.5°, (····)38°and 45°.

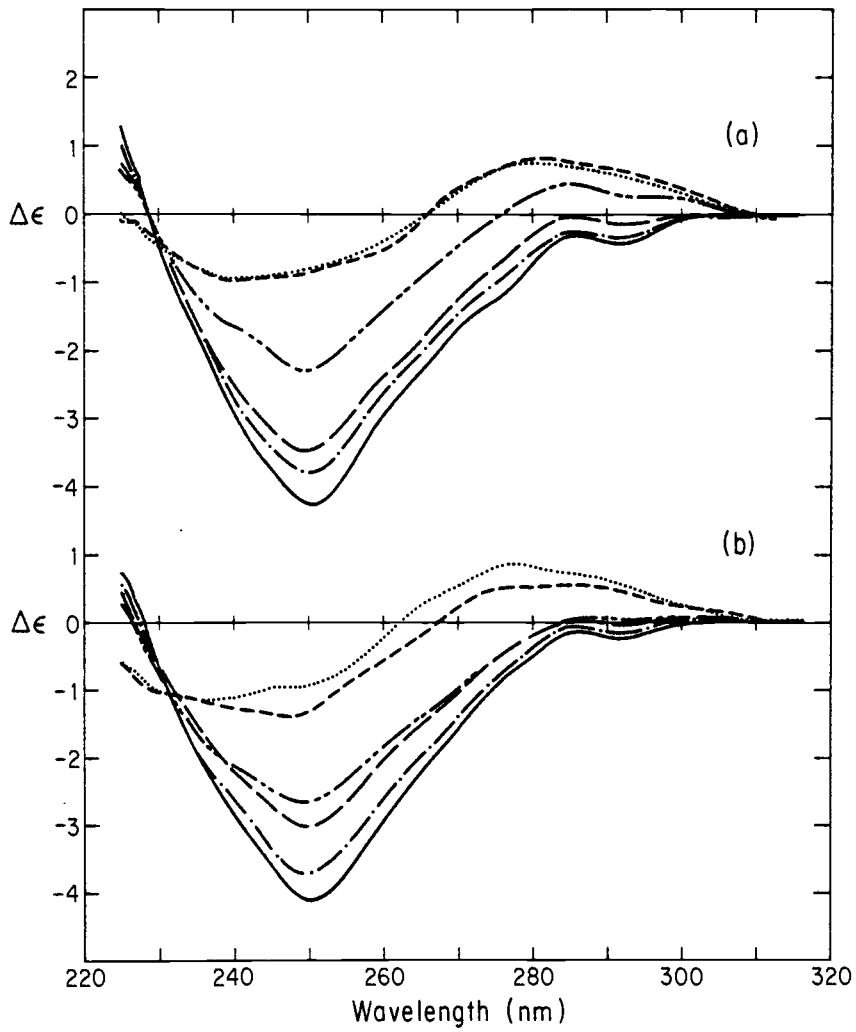

Fig. 9

helical segments, involve changing the secondary twist of the molecule to near zero. In CCC DNA any change in the number of secondary structural turns will not occur unless an equal amount of superhelical turns of the opposite sense are introduced into the tertiary structure. This change, where I hypothesize that all of the secondary structural turns would have to be transformed into the tertiary structural turns, would create a superhelical turn density so impossibly high that the transition itself should not normally occur.

The only way that these highly underwound structures could occur in CCC DNA is if the strands of the molecules became broken and were no longer truly covalently constrained. It is conceivable that this could happen here. One could postulate that because the molecule may not unwind as much as it should, a torsional strain is set up in the molecule. When this strain becomes great enough, one or both of the strands break, and the barrier to secondary structural change is released so that the P-transition may now take place. This possibility was eliminated when CCC DNA molecules which had undergone a P-form transition were isolated from their alcohol solutions, placed into aqueous solutions, and analyzed for covalent closure by using alkaline CsCl analytical ultracentrifugation. From this it was proved that no significant P-form induced breakage took place in these molecules, and that this mechanism involving strand breakage did not occur.

In summary the results of this experiment show us that while

the P-form transition does involve a secondary structural change, it is not either of the secondary structural changes postulated in our models. In Section IV I will show that the P-form is actually a combination of secondary structural denaturation and tertiary structural condensation, so I will now demonstrate that this structure does fit with this data.

The covalent closure of the molecule will only marginally affect the ability of the DNA to collapse, so this part of the P-form transition will not be radically affected by the closure of the molecule. Denaturation, on the other hand, involves at least some change in twist, and should be affected by covalent closure.

In denaturation the Watson and Crick strands of the DNA must first separate far enough that the hydrogen bonds between these strands are broken. For this to happen the strands must locally untwist, and this of course, would be opposed by the covalent closure of the molecule. However, in contrast to the previously discussed models which could not occur because they involved a uniform untwisting of the DNA molecule, denaturation may occur because a denatured molecule may have a highly irregular structure.

The irregular structure of denatured DNA allows it to kink and bend as necessary to accommodate torsional strain. This strain can also be removed from the molecule by having the opposing strands twist back on each other in a non-hydrogen bonded interaction. The denatured region therefore becomes quite flexible, and will not only be able to regain the secondary structural twist that originally re-

sided in that region, but will also be able to accept twist from neighboring regions as they also denature. The final result is that denaturation in CCC molecules occurs with precisely the behavior we see here: the transition is at first delayed by the covalent closure of the molecule, but, once it has started, the entire molecule quickly denatures, and intermediate states occur in a much narrower range of temperatures. We see then that these date are entirely consistent with the denatured-condensed structure.

VISCOSITY OF THE P-FORM

Having pretty much obtained all the information on the P-form that it was possible to get by using CD, it was now time to explore other physical-chemical methods as probes for the P-form structure. After examining several techniques, it was decided that viscosity held the best possibility for gaining further structural insight into the P-form, since this measurement could be used to tell whether or not the P-form involved a tertiary condensation as well as the secondary structural unwinding just demonstrated.

Generally the intrinsic viscosity of a molecule, in itself, is not a terribly enlightening number. This is because while this number is proportional to the cube of the radius of gyration of the molecule, it is usually impossible to obtain the proper proportionality constant without extensive outside information. In this case, however, we are looking for evidence of a collapsed tertiary state which would involve a large reduction of the molecule's radius of gyration. Thus, we are not necessarily interested in de-

termining exact numbers, but only in seeing if the numbers undergo large changes. If the P-form is a condensed state, then its values of radius of gyration and intrinsic viscosity should be greatly lower than the values corresponding to these measurements in other DNA forms, and we should be able to detect this difference.

The pBR322 used in the previous experiment is actually a very small DNA molecule, and so it does not have a very large intrinsic viscosity. For this experiment it will be advantageous to use a large DNA with a large intrinsic viscosity to magnify the differences in this property between the 10.2 and P-form states. The DNA molecule from the T7 bateriophage of E coli was selected for this experiment both because it was large, and because its intrinsic viscosity in aqueous solution had already been extensively studied.[22] An already purified stock solution of the DNA was graciously provided by Dr. Walter Baase. This material was used without further purification after analysis by analytical ultracentrifugation showed that it was in good shape and was homogeneous in molecular weight.

A Zimm type viscometer[23] was borrowed from Professor J. Schellman's laboratory (University of Oregon) and was used after a few missing pieces were obtained. This device was interfaced with a Super Kim microprocessor by Paul Staskus so that the motion of the rotor could be monitored automatically. To do this a laser was first placed so that its light would enter the viscometer, pass through the upper transparent portion of the rotor, then exit from the device and fall upon a light sensitive diode. Opaque stripes

were then painted on the clear portion of the rotors so they would block the laser light twice during every revolution. If the light-sensitive diode was now placed in series with a current source, the voltage across the diode could be used to tell when the rotor had moved far enough that its paint stripe had rotated into, or out of, the laser beam. The computer was simply programmed to measure the time it took for the laser light beam to be interrupted a set number of times by the moving rotor, and these numbers were used to calculate the rotor's rotation rate.

This particular apparatus was designed specifically for work with volumes of less than 1 ml. While this feature had the advantage that it did not waste valuable sample, it also had its disadvantages. One disadvantage that could not be overcome was that the combination of this small volume apparatus, and the use of mixed solvents of low viscosity, made the data obtained in these experiments less precise than the equivalent data obtained under more routine conditions. Thus the data I gathered in these experiments has a bit more scatter than is usually reported using this technique.

A second problem which arose from the use of this apparatus, but one that could be corrected for, is the problem of the rotors' nonideal behavior. If a rotor performs within certain specifications then, an assumption may be made which makes the analysis of the data obtained with this rotor easier to calculate. My rotors did not meet these specifications, so I was forced to determine a

correction coefficient for use with this rotor, and to change the calculations accordingly.

Since the construction of the rotor, and the correction of the data for the nonideality of the rotor are significant portions of this experiment, I will first outline these features before discussing the work itself.

Rotor Construction

The rotor was made from the closed end of a 5 mm OD NMR tube. The source of the tube was not significant, but better results were obtained if the glass used in the construction of the tubes had not been treated so that water would bead up on it. This is because the inks used to mark the tubes would also bead up on this surface, making it impossible to mark the glass.

The NMR tubes were initially selected so that only those tubes with uniformly curved bottoms were used. The bottom 3 cm of these tubes were carefully cut off, padded with tape, and gently mounted in the chuck of a drill press. The cut end of the NMR tube bottom was then pressed down and ground against 240 grit sand paper until its surface was flat and its edges devoid of chips. The tube was next successively ground against 400 and 600 grit paper, and its end finally polished against jeweler's rouge until it had a nearly glossy surface.

The tube was next carefully cleaned inside and out, and an aluminum drive ring was placed inside. The drive rings were hollow aluminum cylinders which ranged in size from 2 to 10 mm along their

axis. The outer diameter of the cylinders was just small enough to allow the ring to fit inside the NMR tube, and the inner diameter was determined so that the total weight of the rotor and drive ring would not sink the assembled rotor when it was floated in the 95% alcohol solvent. It was found experimentally that the rotors made with the 10 mm long drive rings exhibited unacceptably high shear rates, so only rotors with 2 to 3 mm drive rings were used in gathering data.

After the drive ring was in place the cut end of the tube was lightly fire polished. Only enough heat was applied to the tubes to reanneal the glass to remove the strain placed in it during the grinding process. If the tube was heated too much, the flat surface of the cut end would distort, and the entire top of the rotor would have to be reground.

Two lines of approximately 2 mm width were next painted inside the rotor. These lines, located directly across from each other, were made with an opaque glass marking ink, and were designed to entirely block the laser light when it shown upon them.

Since the ideal rotor floats with no distortion of the liquid surface around its top, the amount of ballast necessary to achieve this result had to be determined and added to the rotor. This mass was found by first weighing the rotor, then floating it in the 95% alcohol solvent, and adding small amounts of water until it floated correctly. The rotor was then re-weighed, so the difference between its two weights determined the mass that had to be added to the

rotor. The difference in density between the 47.5-5-47.5 solvent and the 95-5-0 solvent did not significantly change this weight, so rotors could be used interchangeably between these two solvents.

Once the proper amount of ballast had been determined, GE RTV 8111 silicon rubber compound was prepared and placed in the bottom of the rotors as this ballast. As the polymer cured the rotor was spun about its axis, with this axis placed at a 45°angle to the horizon so that the rubber compound would be uniformly distributed around the bottom of the tube.

The following day, when the rubber had hardened, the rotor was tested for precession by spinning it in the viscometer. If precession was found, small amounts of the rubber compound were either removed or added until the precession had reached a barely preceptible level. At this point the rotor was ready for use.

Corrections for Rotor Nonidealities

As mentioned earlier, the small size of the rotor made it impossible to create an 'ideal' rotor for which certain assumptions may be made in the data analysis. I will now show where this problem arises and how it may be corrected for.

The experimental equation relating the speed of rotation of an external magnetic field to the rotation of this type of rotor in a viscous solution is:[24]

$$C(\omega_m - \omega_r) + D = \eta\omega_r \qquad \text{Equation 1.}$$

Where ω_m is the speed at which the external magnetic field rotates, ω_r is the speed at which the rotor rotates in the sample and η is

the absolute viscosity of the sample. C represents the force driving the motion of the rotor which arises from eddy currents set up in the aluminum drive ring by the rotating magnetic field. D may be related to the ferromagnetic impurities within the rotor that try to keep it directly aligned with the magnetic poles of the external field. In other rotors of this design, built for measurements in larger volumes of aqueous solutions, the C term is usually 100 times greater than the D term.[24] In calculating the relative viscosity then, the D term may be ignored, the C term canceled out, and the relative viscosity determined without knowledge of these coefficients, as is seen in the following derivation.

$$\eta_{rel} = \frac{\eta}{\eta_o} = \frac{\dfrac{C(\omega_m - \omega_r)}{\omega_r}}{\dfrac{C(\omega_{m_o} - \omega_{r_o})}{\omega_{r_o}}}$$

$$= \frac{\omega_{r_o}}{\omega_r} \cdot \frac{(\omega_m - \omega_r)}{(\omega_{m_o} - \omega_{r_o})}$$

Here the zero subscript denotes a measurement on a sample containing only solvent.

In the rotors which I constructed the C term was at best only 10 times the D term, making this approximation clearly inapplicable. At first it would seem that this problem could be solved very simply by using Equation 1 to determine the C and D coefficients of the rotor when it was placed in a solvent of known absolute viscosity. The problem with this approach is that my rotors will only operate in mixed solvents which do not have well defined

absolute viscosities, and therefore the C and D terms may not be directly determined.

While the absolute values of the C and D coefficients may not be easily obtained, it is possible to find the ratio of C to D. If one examines Equation 1, one sees that if $\omega - \omega_o$ is plotted versus ω, the slope of the line is C/η and the intercept of the line is D/η. Thus, if one takes the slope and divides it by the intercept, one can determine the C/D ratio without knowing absolute viscosity of the solution. This ratio may now be used to derive the proper η_{rel} for this data, as seen in the following set of equations:

$$\eta_{rel} = \frac{\eta}{\eta_o} = \frac{\dfrac{C(\omega_m - \omega_o) + D}{\omega}}{\dfrac{C(\omega_m - \omega_{mo}) + D}{\omega_{mo}}}$$

$$= \frac{\omega_{mo}}{\omega_o} \cdot \frac{C(\omega_m - \omega_o) + D}{C(\omega_m - \omega_{mo}) + D}$$

$$= \frac{\omega_{mo}}{\omega_o} \cdot \frac{D}{D} \cdot \frac{C(\omega_m - \omega_o) + D}{C(\omega_m - \omega_{mo}) + D}$$

$$= \frac{\omega_{mo}}{\omega_o} \cdot \frac{C(\omega_m - \omega_o + D)}{D} \cdot \frac{D}{C(\omega_m - \omega_{mo}) - D}$$

$$= \frac{\omega}{\omega} \cdot \frac{C/D\ (\omega_m - \omega_o) + 1}{C/D\ (\omega_m - \omega_{mo}) + 1} \qquad \text{Equation 2}$$

For data analysis the C/D ratio of a rotor was determined from the data obtained on that day and then this ratio was used to calculate η_{rel} via Equation 2.

Determination of Viscosity

Again for these experiments the buffer used in the aqueous

DNA stock solution was 0.1 x SSC.

In determining the viscosity of materials in mixed solvents one encounters yet one more problem not normally seen in the aqueous situation. This problem arises from the fact that the solvent is composed of a mixture of two or more solvents of greatly different viscosities. Thus, if even a small error is made in measuring the amount of one of these solvents that goes into either the sample or solvent solutions, the error introduced into the relative viscosities between these solutions is of the same order of magnitude as the change in viscosity introduced by the solute itself. To remedy this the DNA sample was always extensively dialyzed against the solvent so that the concentrations of alcohols and water would always be equilibrated between these solutions.

The temperature of the viscometer was regulated to ± 0.01°C with a Lauda temperature bath filled with a 50-50 methanol-water circulating solvent. This solution was used instead of the usual ethylene glycol-water liquid because it was less viscous, and would carry fewer bubbles into the viscometer where they could block the laser beam enough to falsely trigger the computer interface. When runs at 8°C were done on humid days, it was also necessary to use air jets to keep water vapor from condensing on the surfaces of the viscometer where the laser beam entered and exited.

Just before use both the rotor and the stator were extensively cleaned in ethanolic KOH and profusely rinsed, first with distilled water, and then with methanol. After both rotor and stator were

dry, a solvent sample was placed in the viscometer, and the rotation rate of the rotor measured at four different speeds of external magnetic field rotation. At each speed four separate measurements of roughly four minutes each were taken to determine the rotor's rotation rate. The rotor and solvent were then removed and both rotor and stator rinsed with methanol and dried to prepare for the next sample.

A 1 ml sample containing roughly 50 μg/ml DNA was next placed in the viscometer. Since roughly 0.6 ml of this sample had to be removed from the viscometer to lower the rotor to its optimum position, this liquid was saved so its optical density could be determined to find its exact concentration, and its CD could be measured to confirm that the solution contained the proper DNA form. After this aliquot was removed, the same measurements performed on the solvent were taken on the sample. When these measurements were completed, 0.6 ml of solvent was added to the viscometer to raise the rotor to a place where it could be removed, cleaned, and dried. The diluted DNA remaining in the stator was then thoroughly mixed in situ so it could be used directly as the next DNA sample of lower concentration. Thus, after the rotor was dry, it was simply replaced in the viscometer, 0.6 ml of the solution was again removed to place the rotor at its optimum height, and the measurements continued on a more dilute sample. Again the liquid removed from the viscometer was saved so that its exact concentration could be determined from its UV absorption. After this sample was

measured the entire procedure was repeated one more time, so that the relative viscosity was determined for yet a third even more dilute DNA concentration. When the DNA measurements were finished, the rotor and stator were cleaned and rinsed with methanol, and the solvent measurements were repeated a second time to finish the experiment.

The C/D ratio for that rotor on that day was then determined from the data, and the η_{rel} calculated as outline above. A plot of η_{rel} -1 divided by concentration was then made and extrapolated to zero concentration to determine [η] (intrinsic viscosity).

The data from these experiments are summarized in Table 1. As mentioned earlier there is some scatter in these data, and the absolute uncertainty is roughly ± 10dl/g.

The somewhat surprisingly low viscosity of the 10.2 form when compared to the 10.4 form may be interpreted in two ways. The high viscosity of the aqueous DNA is largely due to the fact that after all of the dilutions of buffer are complete, the solvent contains only about 1mM Na^+. Because there is little sodium in the solution to neutralize the charge of phosphates on the DNA backbone, the charge repulsions of these groups serve to extend and stiffen the chain, thus creating a structure with high viscosity. At first glance it would seem that this mechanism should make the DNA in the alcohol solvents even more stiff and extended since the lower dielectric constant of the medium would serve to increase the charge repulsion between the phosphates of the DNA backbone. The Manning

Table 1 Intrinsic Viscosity of T7 DNA in Various Conformational States

Solvent	95-5-0				47.5-5-47.5	0-100-0
Temperature (°C)	8.0	25.0	30.0	30.0 → 8.0	8.0	25.0
Form	10.2	10.2	P	P	P	10.4
[η] (dl/g)	180	180	70	70	10	370

polyelectrolyte theory, however, shows that this lower dielectric constant also increases the attraction of the phosphates for the Na^+ in the medium.[25] Thus, more of the phosphate charges are directly neutralized by ionic interaction with this cation, and the DNA in the alcohol solvent may therefore become less rigid than in an aqueous solvent. This mechanism does not seem unreasonable when one finds that the viscosity of the 10.2 form DNA is actually a realistic number. The 10.2 form viscosity is almost exactly comparable to the viscosity of aqueous DNA in 10 mM Na^+.[22] Thus, one interpretation of the low viscosity of the 10.2 form is that the DNA here is in an extended state whose flexibility is equivalent to that of aqueous DNA in 10 mM Na^+.

An alternative mechanism to explain the low viscosity of the 10.2 form is that the DNA has already started to undergo a limited condensation, thus reducing its radius of gyration, and therefore its viscosity. Based on the data presented here it is not possible to distinguish between these mechanisms.

I will prove in the next section that the P-form involves a denaturation, so it is pertinent at this point to ask what effect denaturation would have on the DNA's viscosity. If we first examine the relationship between native and denatured DNA viscosities in aqueous solution to see if this will tell us what to expect in nonaqueous solvents, we get confused because the viscosity of the denatured form may be higher or lower than the viscosity of the native form. In low salt, <0.002 M Na^+, the viscosity of denatured

aqueous DNA can be much greater than the viscosity of its native counterpart. In high salt, >0.01 M Na, quite the opposite is true, and denatured DNA can have a much lower viscosity than the native form. It is therefore not immediately obvious which relationship should hold in the nonaqueous solvent.

It is possible to find a handle on this problem if we return to the idea that the low viscosity of the 10.2 form is due to its being in an extended but flexible form. Since the viscosity of this native extended form is equivalent to the viscosity of aqueous DNA in 10 mM Na^+,[22] it is logical to use the viscosity of aqueous denatured DNA at this salt level as an estimate of the viscosity of denatured DNA in the 95-5-0 solvent.

In 10 mM Na^+ aqueous denatured DNA has a viscosity of 100 dl/g. While our 95-5-0 P-form viscosity of 70 dl/g is lower than this, it it not so low that we can say with certainty that the DNA is fully collapsed. It is far more likely that our viscosity data are telling us that in this solvent and buffer the DNA does not undergo a complete collapse, but is instead in an intermediate state. While this is a bit disappointing, we see that this is not entirely unreasonable when we are reminded of earlier data. We saw in Section II that the P-form in this solvent does not have the same heat stability that the 47.5-5-47.5 P-form does, and shows partial denaturation even at temperatures as low as 70°C. This could easily happen if the P-form in the 95-5-0 state was not as fully collapsed, and therefore not as well protected from denaturation as the 47.5-

5-47.5 P-form. Thus this interpretation of the viscosity data, which says that the 95-5-0 P-form is not a fully collapsed state, is consistent with earlier work.

The above argument was based upon the assumption that the low viscosity of the 10.2 form is due to an extended but flexible conformation. If one uses the alternate explanation that the low viscosity of this DNA form occurs because the DNA has already started to collapse, then all bets are off, and we have no handle on which to predict the effect of denaturation on viscosity. One would expect, though, that if the DNA were already collapsing, then the denaturation would have no great effect on the viscosity because the viscosity would be due primarily to the tertiary structure of the molecule instead of its secondary structure. Thus, the even lower viscosity of the final P state would only indicate a more complete collapse. This reasoning, though, does not predict the lower stability of the 95-5-0 P state, and so it does not seem likely that this is the correct explanation.

While the results with the 95-5-0 solvent may be interpreted to mean that this P-form is either a slightly condensed intermediate state, or a more fully collapsed state, the results in the 47.5-5-47.5 solvent can be interpreted only in terms of a full, major collapse. The reduction of the viscosity by more than an order of magnitude from the 10.2 form to the 47.5-5-47.5 P-form cannot be explained in any other manner. Thus, we have our first independent proof that the P-form is a condensed state of DNA tertiary confor-

mation.

The experiments seen here have shown us that the 10.2 to P-form transition involves both a change in the DNA's secondary structure and a change in its tertiary structure. The experiments, however, have not shown us the exact nature of these changes. In the next section we will use two other techniques, infrared spectroscopy and electron microscopy, to demonstrate that the secondary change is in fact a denaturation, and to visualize the true tertiary form of the P-state, thus finally determining the nature of the P-form structure.

SECTION IV: THE CONFORMATION OF P-FORM DNA

SYNOPSIS

The P-form of DNA has been studied by use of infrared (IR) spectroscopy and electron microscopy (EM). The IR data show that the P-form has little or no hydrogen bonding, while the data from the EM show that the P-form has a condensed tertiary structure. In earlier work we demonstrated that the P-form is devoid of base-stacking. When that information is combined with the new IR data, we conclude that the P-form is denatured because it lacks any of the interactions associated with a normal secondary structure. This is in apparent contradiction to earlier work which showed that the P-form may be easily transformed back to a native state by adding water. However, the lack of secondary structure can be overcome by the presence of a collapsed tertiary state which does not allow non-hydrogen bonded strands to separate. Thus, the complementary strands can renature quickly upon the addition of water. The collapse to a condensed tertiary structure occurs when roughly 90% of the charge on the DNA molecule is neutralized by counterion condensation, as calculated by the Manning polyelectrolyte theory, and is consistent with other collapsed DNA states in this respect. This structure explains all physical properties of the P-form that have been observed.

INTRODUCTION

The P-form of DNA refers to a structure found when this mol-

ecule is in a solvent mixture of high alcohol content (methanol or a methanol-ethanol mixture) and low water content.[6,9] When the P-form was first studied both the hyperchromism of its absorbance and the low intensity of its circular dichroism (CD) demonstrated a lack of base stacking. This property, when combined with the nonreversible character of the transition in solvents of low water content, argued that this state was a simple denaturation.[6] Later study, however, showed that P-form DNA could be quickly transformed back to a fully native structure by simply adding water to the solvent.[7] This fast, complete recovery of the native form with large complex DNAs argued that the P-form was not a denaturated state, but was perhaps some other new and unknown structure.

There are four models that can account for the properties of P-form DNA observed to date. These are (1) a condensation into a tertiary structure,[8,9] (2) a highly underwound helix with strong hydrogen bonds between the base pairs but no base stacking,[9] (3) a helix composed of sections of both right- and left- handed twist,[9] and (4) a structure in which the complementary DNA strands have almost completely separated, but are held together by some short regions that are exceptionally stable.[9] Since the available data were consistent with all of these models there has, up until now, been no proof which clearly favors one of these models over the others.

In this paper we present experiments which confirm that the P-form is a condensed structure and which eliminate the other models. We find though that the condensed P-form structure is not a

simple collapsed state of native DNA. It is, instead, an unusual combination of tertiary structural collapse and secondary structural denaturation.

To prove that this structure is correct we first use infrared (IR) spectroscopy in the 1550 to 1750 cm^{-1} region to show that the P-form has little or no inter-base hydrogen bonding and therefore demonstrate that the P-form's secondary structure is truly denatured. We then use the electron microscope to show the tertiary structure of the P-form state by directly visualizing the collapsed molecule.

We therefore believe the the P-form DNA molecule is a molecule which is denatured in the sense that there is no base stacking or hydrogen bonding, but it is also a molecule which may be readily transformed back into a native state because an overall tertiary structure keeps non-hydrogen bonded strands in close proximity and allows them to quickly renature when water is added back into the solvent system. This condensed-denatured explanation takes away all the mystery associated with the P-form because it can easily account for all the properties that have been observed.

MATERIALS AND METHODS

When referring to solvent mixtures we will continue to use the convention originally proposed by Johnson and Girod.[6] A three number code will refer to the percent, by volume, of solvent in the order methanol - buffer - ethanol. Thus 85-5-10 is a solvent system composed to 85% methanol, 5% buffer, and 10% ethanol by initial

volume. The change in volume that occurs when the solvent is mixed, or when the sample is made up at different temperatures is not significant.

Infrared Spectroscopy

Highly polymerized calf thymus DNA was obtained from Worthington Biochemical Corporation and used without further purification. Since the wavelength region we are looking at in the IR is normally obscured by O-H vibrations, both D_2O and alcohols deuterated in the hydroxyl position had to be used. The source of the D_2O was Bio-Rad, while the alcohols were obtained from Sigma.

For this work a D_2O buffer stock solution was made to contain 15 mM NaCl and 1.5 mM Na citrate. No attempt was made to precisely adjust the pH, although the apparent pH was measured and found to be close to 7.5, the value used in previous papers. When this buffer was diluted by a factor of twenty with the deuterated alcohols the final salt concentration was .75mM NaCl -.075mM citrate, the same as in previous papers.

Since the solubility of DNA is limited in solvents composed of mostly alcohol, the following procedure was adopted for sample preparation. First a weighed amount of DNA was placed under vacuum overnight to remove any residual moisture. After this treatment the DNA was transferred to a dry screw-top vial, and both the entire volume of buffer and either 1/2 of the total methanol volume (in the 47.5-5-47.5 solvent case) or 1/4 of the total methanol volume (95-5-0 solvent) was added. The vial was then sealed and gently

agitated at room temperature overnight. The next day the vial was transferred to a cold room (~ 5°C) and another aliquot of the methanol was added. The tube was then kept continuously but gently agitated, and either the remaining methanol was added in two equal aliquots, or the remaining ethanol was added in four equal aliquots with 8-12 hours between each addition. Using this technique we could make solutions of up to 2mg/ml DNA in the 95-5-0 solvent, and between 1-1.5 mg/ml in the 47.5-5-47.5 solvent.

The IR data were taken on a Beckman IR-7 spectrometer operated in the double beam mode with a spectral slit width of 3.25 cm^{-1}. Demountable variable pathlength cells were used with teflon spacers so the nominal pathlength between the CaF_2 windows was 200μm. A cell containing a solvent blank with no temperature control was run as a reference while the sample cell was temperature controlled. This arrangement gave a relatively flat baseline in the 1750-1650 cm^{-1} region, but there was some curvature in the 1600-1550 cm^{-1} region, probably due to a slight mismatch in cell pathlengths.

The IR sample cell holder was designed so that it would also fit into both a Cary 14 spectrometer and a Jasco J-41 CD machine. This allowed us to directly measure the CD and OD of any sample in the UV region to find the concentration of the DNA and to confirm that the DNA was in the expected secondary form. These additional measurements were actually fairly easy to obtain because the strength of the electronic interactions in the UV are about an order of magnitude stronger than the interactions in the IR, so our

samples with IR absorbances of about 0.1 had absorbances in the 260 nm region of about 1.0.

While a CD measurement can tell when a transition from a 10.2 bp/turn B-form has occurred, it cannot tell if the new state is a denatured state or a P-form because the CDs of these molecules are virtually identical in the alcohol solvents. These forms can only be distinguished when water is added to the solution to see whether or not the DNA reverses to a native form.[9] We therefore routinely checked putative P-form samples used for the IR measurements by reversing them to a solvent of 50% water. This reversal was done by simply flushing the cell, which held a volume of about 0.1 ml, with 40 mls of a solvent containing 50% water. The CD of this rinse solution could then be taken to confirm the presence of either a native or a denatured state by directly placing it in a 10 cm pathlength cell.

Electron Microscopy

The DNA used for electron microscopy was T7, which was graciously provided by Dr. Walter Baase. The alcohols used were filtered through Millipore GVWP filters to eliminate any large particulate matter.

Since the standard NaCl citrate buffer used in the P-form transition is nonvolatile and would increase in concentration as the solvent evaporated from the grid, we switched to the volatile salt ammonium acetate for the bulk of the EM work. We choose to work at a final diluted concentration of 1mM ammonium acetate, since the

NH_4^+ concentration here would be close to the Na^+ concentration that exists when the sodium chloride - citrate type buffer is used. This change in salt has only positive effects the 10.2 bp/turn B-form to P-form transition, a point which will be dealt with in the Results section.

Since one must always worry about introducing artifacts in the different steps of EM grid preparation, we tried to create a simple straightforward procedure. In this method the grids were simply immersed in the solvent of choice, rinsed in an identical solution containing no DNA, then dried under vacuum to quickly remove both solvent and salt. No attempt was made to stain the DNA, or to spread it, or to make it adhere to the grid by making the grid more hydrophilic.

Grids were prepared by evaporating carbon onto a freshly cleaved piece of mica, then floating the carbon film off the mica surface and onto untreated copper grids. The grids were then stored desiccated at room temperature until use.

The actual application of DNA to the grid was done in a cold room at 5° C. To do this an open 6 cm Petri dish containing several milliliters of solvent was placed inside a closed 9cm Petri dish. After a few minutes, when the vapor inside the dish had equilibrated, a few drops of the DNA solution were placed into the cap of a 1.5 ml Eppendorf type centrifuge tube, and this was also placed inside the large Petri dish. The grid was placed carbon side up in this DNA solution for five minutes, and after that time removed and gently

rinsed for 10 seconds in a solution of identical solvent composition containing no DNA. The grid was then placed carbon side up on a piece of Parafilm and put immediately under vacuum to remove both solvent and salt. The rinse step was included here to allow DNA which was not attached to the grid to be rinsed away and could also be used to apply latex spheres of known diameter to the grid to be used as internal size standards when measuring the DNA.

The DNA solution used to prepare the grid and the original solution were recombined and the CD of this material was taken to confirm that the DNA was in the expected form. If the solution contained P-form DNA, the sample was further reversed to a solvent of 50% water and the CD taken to be certain that the reversibility characteristic of the P-form was present.

For contrast enhancement, grids were linearly shadowed with platinum at angles between 5:1 and 10:1. The actual microscopy was performed on a Phillips EM 300 operated at either 40KV or 60KV. Strand lengths were measured on enlarged prints by using a Hewlett-Packard 9864 digitizer coupled with a 9821A calculator. Widths of fibers were calculated by measuring shadow lengths of both DNA and spheres directly on the negative using a Nikon Shadowgraph.

RESULTS AND DISCUSSION

Of the four models that were previously proposed as possible P-form structures, both the highly underwound helix[9] and the mixture of right- and left- handed helical segments[9] would involve base

pairing. To test for this hydrogen bonding we chose to measure the infrared absorbance in the 1550 to 1750 cm^{-1} range because this region of the IR contains the C=0 in plane vibrations as well as in plane NH and NH_2 deformations, and is therefore sensitive to the hydrogen bonding of the molecule.[26]

Shown in Figure 10 are the IR spectra of both the B-form (with 10.2 base pairs per turn instead of the usual 10.4 base pairs per turn) and the P-form of DNA in the 95-5-0 solvent system. The spectrum for the 10.2 form is typical of native DNA, while the spectrum for the P-form is typical of strand-separated DNA.

In this experiment the 10.2 form measurements (IR, CD, and UV-OD) were done first by holding a sample of DNA at 10-15°C. The cell was then heated above 30°C to push the DNA into the P-form and all measurements repeated. Finally the cell was cleaned and filled with a solvent blank and the appropriate baselines were taken. Confirmation that the low temperature IR corresponds to a 10.2 form was found in that solution's CD spectrum. Proof that the high temperature IR data corresponded to a P-form state was found first in that solution's CD, and second in the ability of that DNA to regain a native structure when the solvent was transformed into a 50% water mixture.

Y. Kyogoku et al.[27] and H. Fritzche[28] have shown that denaturation may be followed in this IR region by examining the ratio of an absorbance corresponding to a peak in the native spectrum to the absorbance corresponding to a peak in the denatured spectrum. When

Fig. 10 IR spectra of calf thymus DNA in the deuterated 95-5-0 solvent (——) 10-15°C and (····) above 30°C.

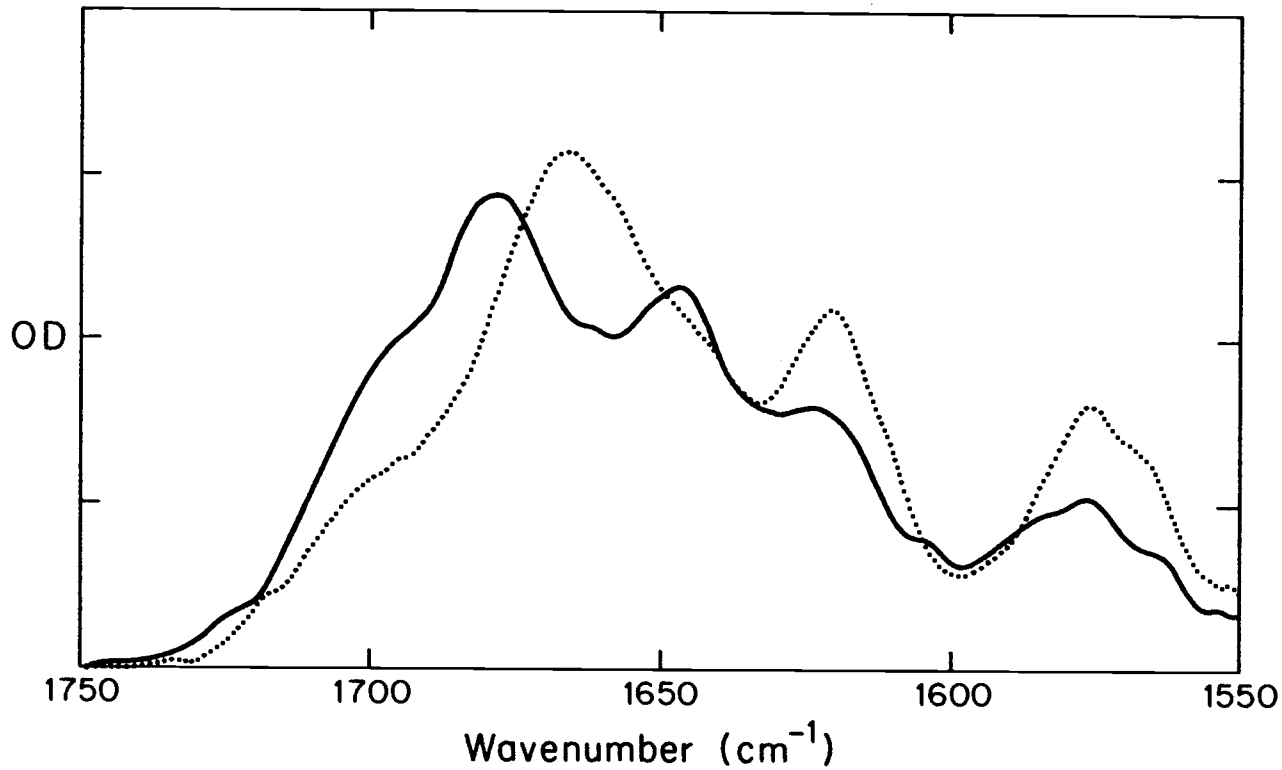

Fig. 10

this ratio is plotted against temperature one can create melting curves very similar to those obtained when using the hyperchromicity at 260 nm to monitor denaturation. In this procedure one first locates the absorbance peaks located near 1685 cm^{-1} in the native spectrum and near 1665 cm^{-1} in the denatured spectrum. One then measures the absorbances at these wavenumbers and calculates the ratio of the 1665 cm^{-1} to the 1685 cm^{-1} peak. Their results for native calf thymus DNA were generally quite close to 0.9, while the denatured values ranged from 1.2 to 1.6.[27,28] This last number is inherently unstable because the absorbance near 1685 cm^{-1} in the denatured spectrum lies on a steeply sloped portion of the IR curve, so small errors in the placement of this point will create large errors in the final ratio.

For our experiments in the 95-5-0 solvent this ratio is 0.8 for the 10.2 form and 1.4 for the P-form. Thus, our 10.2 form data fit quite well as a native form and our 95-5-0 P-form is clearly denatured. We have also obtained the IR spectrum of the 47.5-5-47.5 P-form, but have not displayed these data because they are similar to the 95-5-0 P-form data. The absorbance ratio here was also 1.4, again also showing complete denaturation.

Before leaving the IR data we would like to point out that the IR of the 10.2 form of DNA in the 95-5-0 solvent system is slightly different from the IR of the 10.4 form in D_2O alone. Both in the literature and in our own experiments (not shown) there seems to be a difference between the spectra at 1695 cm^{-1}. In the 95-5-0

solvent this band appears as a shoulder on the 1680 cm^{-1} peak. In D_2O this shoulder has a greater intensity and may be equal in height to the 1680 cm peak. It is not clear whether this difference is a real difference between the structures of 10.2 and 10.4 B-forms of DNA, or whether it reflects either a different solvent-DNA interaction or the different sample concentrations (the D_2O work was done with the DNA in films or gels with at least an order of magnitude higher DNA concentration).

While we see that the IR data have ruled out the two models which involve strong base pairing, this method alone does not have the sensitivity to rule out the model in which there is strand separation for all but some short region that is exceptionally stable. However, the residual hydrogen bonding involved in this model does not seem reasonable when one remembers the heat stability of the P-form state shown previously.[9] If the hydrogen bonds are almost entirely absent when the P-form transition occurs at 30°C, then further heating of the solution to 100°C should easily destroy the few remaining hydrogen bonds so the structure should not be heat stable. Since the 47.5-5-47.5 P-form is heat stable, and may be heated to 100°C without effect, this model may also be eliminated. We are left then with only the structural model of tertiary condensation to explain the P-form properties.

EM has already been used to detect tertiary structures of DNA in both aqueous[29] and nonaqueous solvents,[30-32] and seemed like an ideal tool to use to test for the presence of a tertiary condensa-

tion in the P-form. In the EM studies already done on DNA structures in alcohol solvents, the usual method of preparing an EM grid was to place a drop of aqueous buffer containing the DNA on the surface of the grid and then to simply immerse the grid in a solvent of given alcohol concentration. This procedure does not appeal to us because the DNA is being subjected to an almost discontinuous environment and the exact salt and solvent conditions that exist around the DNA when it is attached to the grid are not at all clear. Thus, there is no way in which the secondary form of the DNA may be independently checked.

We therefore designed our own procedure (outlined in the Methods section) which we feel takes care of these deficiencies. The first major advantage of this method is that the DNA is never subjected to discontinuous solvent conditions. The second advantage of this method is that the CD can be measured for the solution from which the grids were made, allowing us to monitor the secondary structure of the DNA. The reliability of our EM results may be further strengthened by using an internal control. Since the transition is not reversible in this solvent, we can make sets of samples which vary only in the form of the DNA within the sample, but do not vary in solvent content or temperature. To do this a single large sample is split into two identical portions, one of which remains in the cold in the 10.2 form, while the other is heated to 30°C for an hour in a sealed vial to push it into the P-form.[9] After heating, the P-form sample may be returned to the cold where it

will retain the P-form. We now have two samples with identical solvent and solute concentrations at the same temperature so that any differences observed between them on the EM grid indicate differences between sample structures.

The final step, when the solvent is evaporated from the grid under vacuum, presents the only uncertainty. As solvents containing nonvolatile salts, like NaCl or Na-citrate, are evaporated, the salt will remain behind and concentrate in the solvent remaining on the grid. This will result, not only in salt crystals appearing on the grids as a dirty background, but may also induce the DNA to aggregate. This is indeed an artifact commonly seen when nonvolatile salts are used in this manner.[33] While we were aware that these problems could exist in preparations which included the NaCl-citrate buffer, we still attempted our first EM experiments in this salt (not shown) because, up until now, the P-form had only been demonstrated in solvents containing this salt. As expected, we observed aggregation artifacts in these preparations. When we examined grids of P-form DNA in the 47.5-5-47.5 solvent made with this salt we would sometimes see dense two-dimensional nets of aggregated DNA. However, these experiments were also encouraging because the nets would often thin out to the point where short, thick individual fibers could be seen. These fibers were later shown to be similar to P-form fibers isolated from preps using ammonium acetate as the salt. In the 95-5-0 solvent these artifacts were slightly different in form, and mostly one-dimensional linear aggregates were seen.

These fibers, though, did not resolve into the same short thick fibers seen for the 47.5-5-47.5 P-form, but instead seemed to taper into extremely thin strands resembling native DNA. Since similar aggregates were seen for both the 10.2 form and the P-form, the exact meaning of this experiment is unclear.

One explanation for these results is that the P-form of DNA in this salt and the 95-5-0 solvent is simply not as condensed as in the 47.5-5-47.5 solvent. This explanation is appealing because it is congruent with previous observations of viscosity and stability. Indeed, when one remembers that DNA will denature when methanol is either replaced with the only slightly more polar solvent ethylene glycol,[9] or when just a bit less methanol is included in the solvent, it is easy to understand that the DNA might be in an incompletely collapsed state.

We continued our EM investigation using ammonium acetate as the buffer salt, since this volatile substance is removed from the grid with the solvent under vacuum, and was expected to give much cleaner results.

First though, we must show that this change in salt does not radically change the P-form transition in these solvents. Shown in Figure 11 are the CD spectra of P-form and denatured DNA in the 47.5-5-47.5 solvent and ammonium acetate salt. When these curves are compared to the corresponding curves for NaCl-citrate buffer we see that the major difference between these spectra is the positive band above 260 nm which, has almost doubled in intensity. Since this

Fig. 11 CD spectra of calf thymus DNA in the 47.5-5-47.5 solvent with ammonium acetate salt. (——) Native DNA (P-form) and (····) heat denatured DNA.

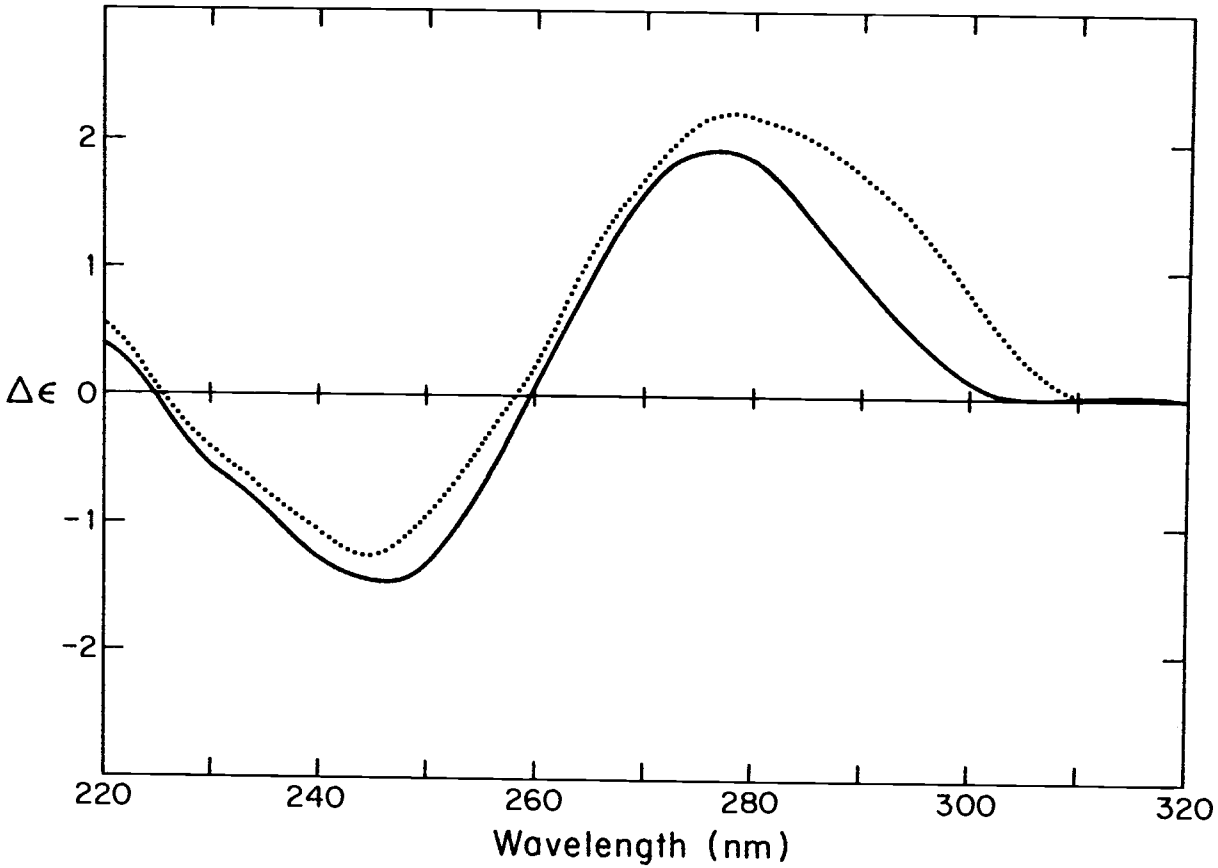

Fig. 11

change happens in the denatured spectrum as well as the P-form spectrum, we can conclude that this difference is due to a slightly changed base-base interaction in the underlying denatured state and is not due to a change in the P-form itself. Shown in Figure 12 are CD spectra obtained in this salt for DNA exposed to the 47.5-5-47.5 P-form conditions and reversed to 25-50-25 solvent and for native DNA which was placed in this solvent without undergoing P-form conditions. The spectra are identical and the reversibility proves we have the P-form with the ammonium acetate salt.

Besides changing the shape of the final CD curve, the different salt also has a slight effect on the temperature of the 10.2 to P-form transition. Shown in Figure 13 is a comparison of transition curves in the 95-5-0 solvent done in the two different salts. One can immediately see that the ammonium acetate salt lowers the transition temperature about 5°C. It should be noted here that this lower transition temperature is the reason that the EM grids were prepared in the cold. By applying the DNA to the grids at 5°C instead of room temperature we tried to eliminate the possibility that a sample could accidentally get warm enough to undergo a P-form transition prematurely. Again this final P-form state is fully reversible, but these data are not shown because they are almost exactly identical to Figure 12.

The lower transition temperature of the 95-5-0 P-form in the ammonium acetate salt probably indicates that this P-form is more stable than its NaCl-citrate counterpart. We have observed other

Fig. 12 CD spectra of calf thymus DNA in the 25-50-25 solvent with ammonium acetate salt. (——) DNA reversed from 47.5-5-47.5 (P-form) and (- - - -) Native DNA

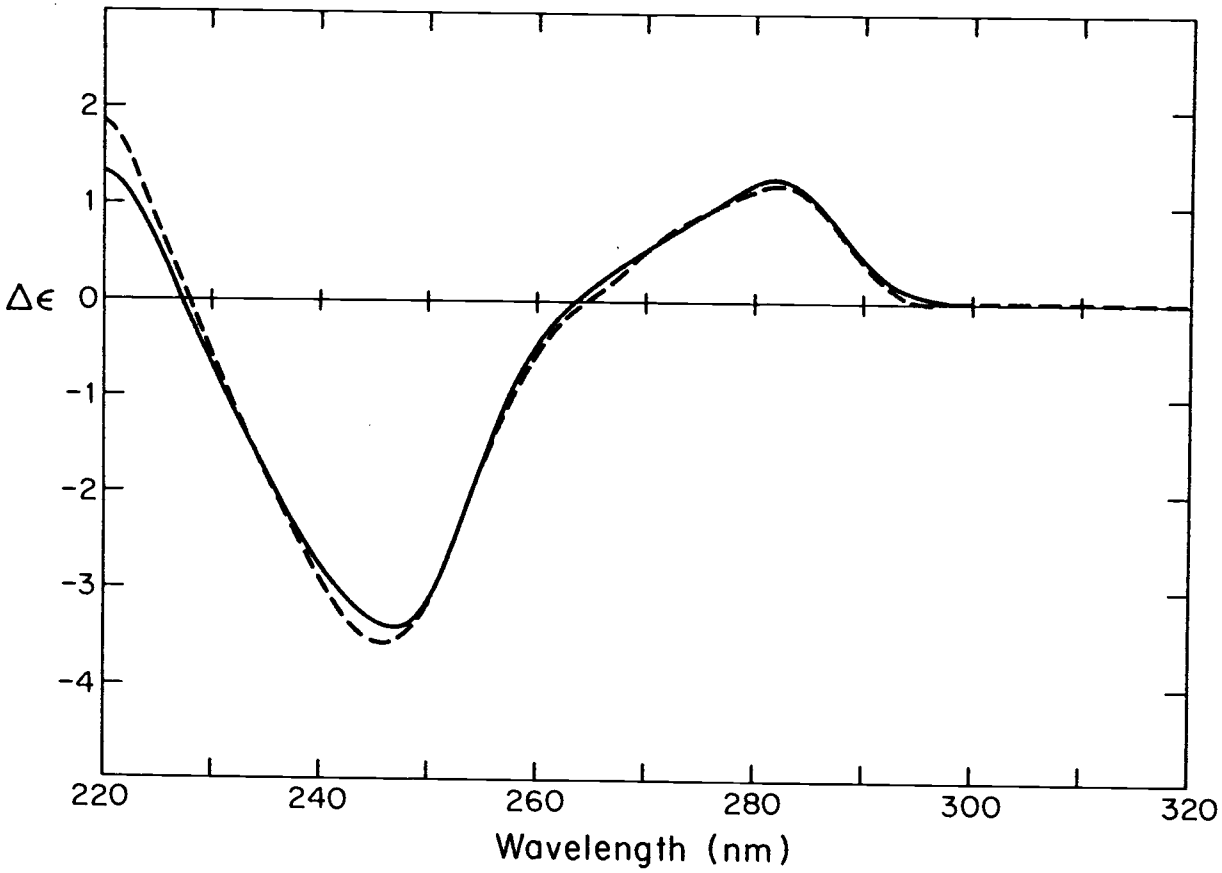

Fig. 12

Fig. 13 CD spectra of calf thymus DNA in the 95-5-0 solvent with different salts. (a) NaCl - citrate buffer: (——) 8.0°C, (—— .) 22.5°C, (— —) 25.0°C, (—— – –) 27.5°C, (- - - -) 30.8°C, (····) 32.0°C and (b) ammonium acetate salt: (——) 8.5°C, (— —) 20.0°C, (—— ·) 22.6°C, (— — —) 25.6°C, (—— – –) 28.2°C, (····) 33.5°C.

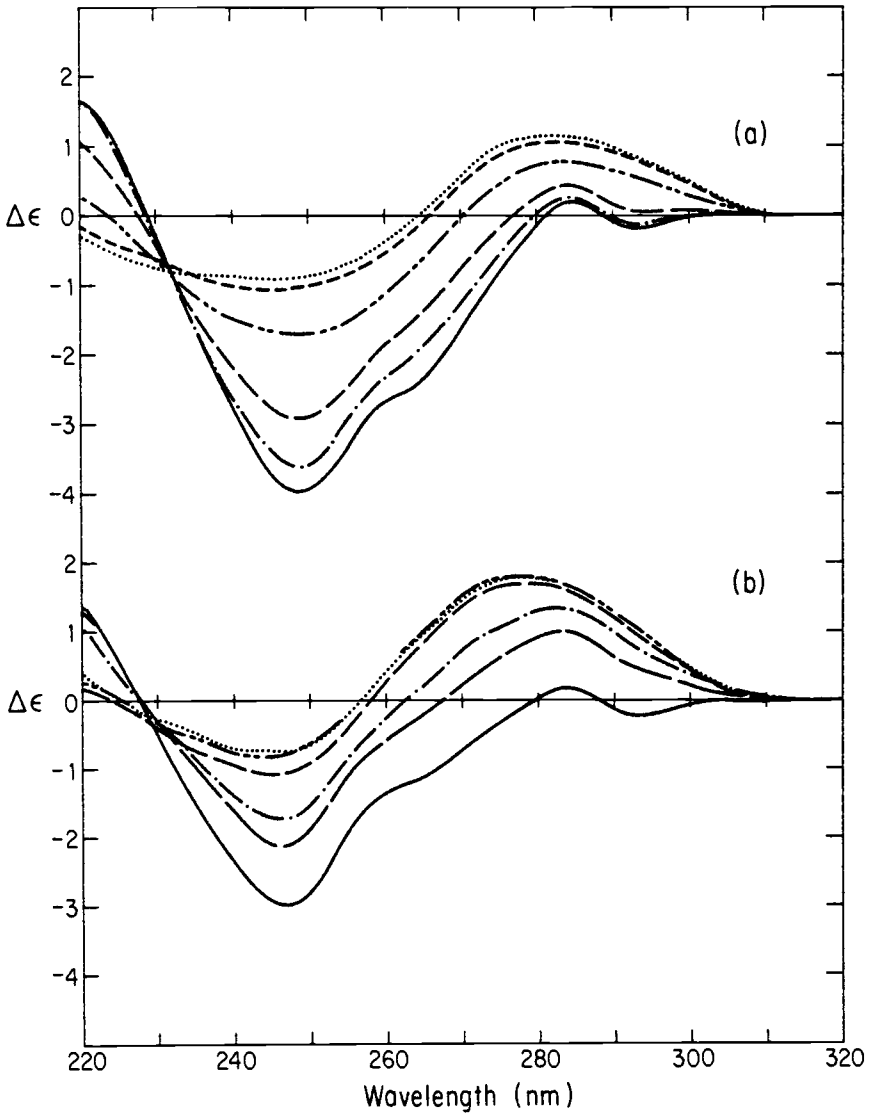

Fig. 13

indirect evidence that confirms this. In doing reversibility experiments in the NaCl-citrate buffer in this solvent we have observed that there was always a small component of the DNA which did not reverse to the native form, but denatured instead. When we started using the ammonium acetate buffer however, this nonreversible component was reduced, and we started finding much better reproducibility in our reversibility experiments.

We believe that these effects, the better stability and the lower transition temperature of the P-form transition in the 95-5-0 solvent with ammonium acetate buffer, are due primarily to the change of cation from Na^+ to NH_4^+. The effect of this change in cation should not be surprising because in many ways the NH_4^+ ion acts in a manner similar to increased alcohol concentration. At low concentration the NH_4^+ ion causes an increase in the rotation angle (decreases the number of base pairs per turn), relative to the rotation angle in Na^+.[34] At high concentration this produces a structure which has a CD similar to the CD of DNA in 95-5-0 at low temperature.[5,35] Thus the cation NH_4^+ ordinarily mimics the effects of alcohols on DNA conformations. When used in this system the NH_4^+ cation acts in the same manner as the alcohol, and has an effect similar to that of using a slightly higher amount of alcohol in the solvent. It both lowers the transition temperature and increases the stability of the P-form.

Now that we have shown that the change in cation from Na^+ to NH_4^+ has only positive effects on the 10.2 to P-form transition, let

us examine what the electron microscope tells us about the P-form state in this salt. Figure 14 parts a, b, and c shows T7 DNA in the 95-5-0 solvent with the ammonium acetate salt. Although the 10.2 form tended to aggregate, a number of long, extremely thin strands were isolated, one of which is shown in Fig. 14(a). Fig. 14(b) and (c) show P-form structures which were obtained from solutions containing DNA which had been heated to 30°C and cooled so they could be applied to the grid under conditions identical to those in which the 10.2 strand was applied to the grid. Fig. 14 (b) shows these structures at the same magnification as the 10.2 strand, so a direct size comparison may be made, while Fig. 14 (c) shows the structures at a higher magnification so more detail may be seen. The diameter of the 10.2 strands was 2-4 nm and lengths in excess of 9 μm were observed. We did not, however, find enough measurable strands of this type to do a significant analysis of length. The P-form structures were comparatively easy to observe and measure, and are about 0.23 μm long with a diameter of 20 nm.

The P-form in the 47.5-5-47.5 solvent is also in a collapsed state but appears to be somewhat different in form as can be seen in Fig. 14 (d). The magnification here is the same as in Fig. 14(c), so that the details of the two structures may be compared. The P-form here appears to be longer, thinner, and perhaps more flexible. It now measures 0.6 μm long and 10 nm wide.

It is instructive to compare these observations to others already reported in the literature. The length of the T7 molecule in

Fig. 14 Electron micrographs of T7 DNA in various alcohol solvents (a) 95-5-0 at 5°C (10.2 form) (b) and (c) 95-5-0 heated to 30°C and cooled to 5°C (P-form) and (d) 47.5-5-47.5 at 5°C (P-form)

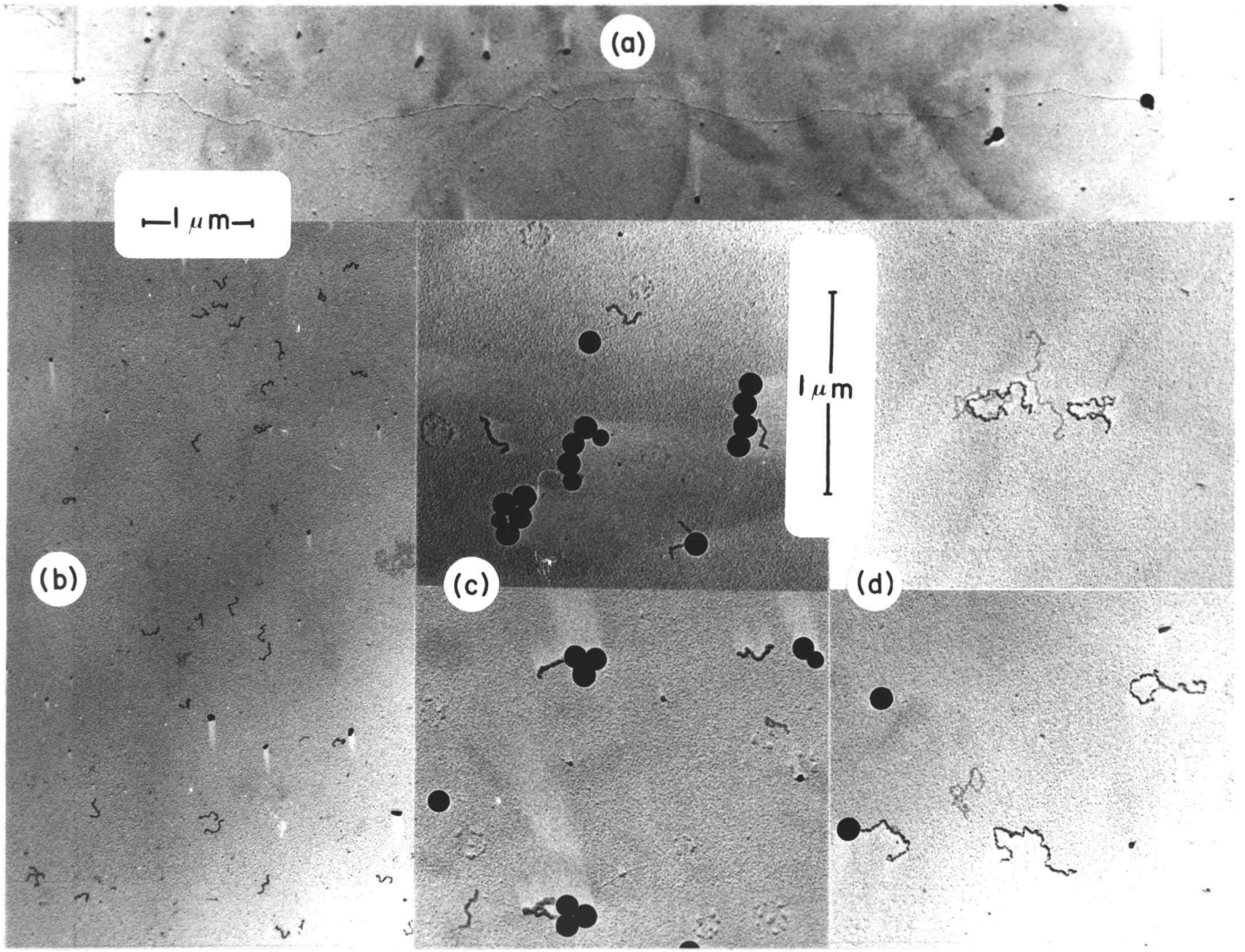

Fig. 14

aqueous solution (10.4 bp/turn) is commonly given as 12.5 μm and studies indicate that this value is reduced to 12.1 in methanolic solutions.[32]

Structures like the P-form have been observed in several studies using different concentrations of salt and alcohol. Lang observed structures similar to ours with lengths of 3.0 μm in 10% ethanol and low salt, 0.7 μm in 30-95% ethanol and low to moderate salt (0.001-0.2 M), and 0.16 μm in 95% ethanol with 0.15 to 0.2 M ammonium acetate. He called these first, second, and third order supercoils, respectively.[30] While our 47.5-5-47.5 P-form seems to be roughly equivalent to his second order supercoils, our 95-5-0 form does not seem to match any of these structures.

However, Eickbush and Moudrianakis have shown that size is a function of salt.[31] They studied fibers condensed from 95% methanol and 95% ethanol solutions as a function of salt using the longer Lambda DNA molecule. After adjusting for this length difference their data would be equivalent to finding T7 fibers with a length of 2μm in 95% ethanol and extremely low salt (0.5mM) and a length of 3 μm in 95% methanol at the same very low salt concentration. As the ionic strength of their solutions was increased the length of these fibers decreased steadily until at 150mM ammonium acetate the 95% ethanol fiber was a very short rod of slightly less than 0.2 μm length. Their findings then would indicate that fibers similar to ours could be obtained by simply varying the ionic strength of the solvent until a proper match was

found.

Since there was no independent check of the secondary structure of the molecules which were applied to the grid in these other works, it is not clear whether the presence of similar structures under different conditions indicates that these other studies were isolating P-form fibers under different solvent conditions, or whether this simply shows that other secondary forms of DNA may be condensed into similar tertiary structures.

When examined with the Manning polyelectrolyte theory,[25] the collapse to the P-form is completely consistent with other DNA condensations. Wilson and Bloomfield have shown that self-association of DNA to a collapsed state occurs when the linear charge density of DNA is reduced to 89-90% by counterion condensation.[36] Their experiments showed that this occurred in aqueous solutions with spermine and spermidine, and in solvents of 50% methanol with Mg^{+2} and putrescine. For monovalent counterions the amount that the linear charge density of a polyelectrolyte is reduced by direct counter ion condensation is given by the formula:[25]

$$1-(|N|\xi)^{-1}$$

where $\xi = \frac{Q_e^2}{\varepsilon kTb}$ and Q_e is the charge of a proton, ε is the bulk dielectric constant and b is the average linear spacing of the polyelectrolyte in the absence of associated ions.

Let us now apply this equation to P-form DNA in the 95-5-0 solvent where we know the transition temperature, to calculate the charge neutralization. Under these conditions the transition oc-

curs at roughly 30°C. For the bulk dielectric constant we use 32.6, a linear interpolation of the dielectric constants of 90% and 100% methanol at this temperature.[37] For the value of b we use 1.7Å, the value of b for B-form DNA (10.4 bp/turn) in aqueous solution. Strictly speaking we should use a slightly different value since we are working with a transition from a 10.2 bp/turn DNA form, but for a first approximation this seems a reasonable value. Using these parameters, we get 90% charge neutralization, as expected from the Wilson and Bloomfield work.[36]

If we invoke a counterion condensation as a cause for the collapse, then we can also postulate a link between denaturation and condensation. In denaturation the bulk of the DNA is attempting to unwind to a random state. If the DNA is sufficiently long it will not be able to unwind fast enough to remove all torsional strain from the molecule, and there will be some temporary local areas of overwinding to help relieve the strain. This local overwinding would in turn result in a local decrease in the DNA's linear charge density. If one examines how this factor appears in the above formula, one can see that such a change would result in increase in counterion condensation for this region. Since the molecule is already close to collapsing due to charge condensation, this local increase in the linear charge density could be enough to trigger a small region to collapse, and this nucleation could start the collapse of the entire molecule.

This mechanism would also explain why we get an intramolecular

collapse of individual molecules in the ammonium acetate buffer rather than intermolecular aggregation of all the molecules together. In this mechanism the local nucleation of the collapse is strictly an intramolecular event. The collapse of one molecule has no effect whatsoever on any other molecules, so an intermolecular aggregation would not be expected to occur.

Although at first glance it seems hard to find any biological significance for a transition which occurs in a solvent of 95% alcohol, there may indeed be a biological role in the interactions which create the P-form. DNA conformations in mixed alcohol-water solvent systems were originally studied because the CDs derived from these solutions were similar to CDs derived from certain biological systems.[38-40] It is possible that DNA conformations in these solvents of low water content could be used to model interactions of DNA with proteins, where accessibility of the DNA to water is limited, and perhaps even replaced with access to less polar protein moieties. Under these conditions of low water activity the DNA changes form the classical 10.4 bp/turn B form to structural variant of 10.2 bp/turn. If either the solvent hydrophobicity or temperature are increased further, this 10.2 bp/turn form may in turn change to the collapsed-denatured P-form. The ability of the DNA to collapse by itself without the use of polyamines or specific protein interactions could be a great aid to organisms, like viruses or spores, who wish to package the DNA into small stable containers. The ability of this collapsed state to resist permanent heat dena-

turation is an added factor which could insure that such a packaged DNA would still be viable after heat stress. We see then that if an organism wishes to package and protect its DNA, all it needs to do is to isolate it in a low water, somewhat hydrophobic environment, and the DNA will take care of itself. No additional specialized protein interactions are required, and no special cations like the polyamines need to be present.

Finally we would like to note that the P-form is not as new and unique as we once thought. A series of papers in the early 1960's by Herskovits, Singer and Geiduschek[41-43] described a phenomenon which they refer to as "reversible denaturation". After carefully studying their conditions and results, we believe that they were observing a P-form type transition.

Thus, with this paper we have answered a 10-year-old question on the nature of the P-form. Our results show that the P-form is actually a combination of two important features: it is both a secondary denaturation and a tertiary condensation. It is this unusual combination of structures that gives the P-form its many unique properties.

Acknowledgements

We would like to thank Dr. Dimitrij Lang for his helpful discussion on EM grid preparation, Dr. Joseph Nibler for the use of his IR instrument and Dr. Walter Baase for the gift of T7 DNA. We would also like to acknowledge the technical assistance of Chris Weiss and use of the Botany and Plant Pathology Electron Microscope

Facility at Oregon State University.

This work was supported by NIH Grant GM21479 from the Institute of General Medical Sciences.

SECTION V: SUMMARY

This thesis now contains the bulk of what we now know about the P-form. It is the purpose of this final section to try to summarize this information and bring it into focus. I will do this by defining exactly what I mean by the collapsed-denatured P-form state and by briefly reviewing the evidence that proves its existence. I will then intuitively examine the interactions behind the P-form transition to see if further insight can be gained in understanding both the P-form state itself and DNA structure in general.

In defining the collapsed-denatured P-form state I will start with the denatured portion because it is the easier to define. By denaturation I mean that no hydrogen bonding exists between the Watson and Crick strands of the DNA. The denaturation of the P-form state was first seen by such indirect evidence as the low intensity CD spectrum in both near and far UV, the hyperchromic absorption of the P-form in the 260 nm region, and in the weak but positive LD that was also seen in this region.[8] The delayed transition of the 10.2 to P-form transition in CCC pBR322 could also be taken as evidence that was consistent with denaturation. Since all these properties could be interpreted in other ways, the denaturation of the P-form state was not conclusively proven until this work, where the denaturation was finally and unequivocally shown in the IR data presented in Section IV. This data proved that the P-form state contained no hydrogen bonds and therefore is de-

natured under this definition.

If the hydrogen bonds between the complementary strands are broken, how is it possible that the P-form's most distinguishing feature, the reversal of a denatured form to a native form may occur?

This brings us to the second part, the condensed portion of the P-state. Let us first define condensation as the folding of the normally extended, spaghetti-like molecule, into a compact, stable structure. In this structure segments of the molecule not directly associated along its linear sequence now interact and probably even make direct contact with one another. We see then, that it is the nearness, or even the direct contacts among the folded portions of the DNA molecule, that keeps the strands from wandering away from each other. Since the opposite strands stay close to one another they do not "lose sight" of their proper complement, and, when water is added into the system, they find their neighbor quickly and renature to a native form.

It should be pointed out here that earlier in this work the statement was made that "...large complex DNAs could not renature under these conditions." This statement was made to illustrate the point that when the strands of large complex DNAs are separated from each other in solution, it is impossible for a strand to find both its complement, and its proper position along that complement in the time scale of the experiment, and thus renaturation could not occur under ordinary circumstances. With the P-form we have an ex-

traordinary circumstance, the collapse of the molecule. This changes the rules, and now allows the renaturation of large complex DNAs because the tertiary structure of the P-form molecule does not allow the opposite strands a chance to escape each other. It should be emphasized that this ability to renature will only occur in molecules which were placed into the P-form during denaturation, and that previously denatured molecules placed in P-forming solvents will not magically renature, because their strands will have already lost their registration with each other.

The intimate contact between nonadjacent segments of the DNA in the collapsed structure also restrains the molecule so that it moves within a much smaller overall volume and the DNA therefore has a greatly reduced radius of gyration. In this work the smaller radius of gyration was observed in the extremely low viscosity of the 47.5-5-47.5 P-form. If we turn to the work of Herskovits et al. we see it also in such measurements as light scattering, sedimentation velocity, and flow birefringence.[41-43] The most graphic demonstration of the condensed state is seen, of course, in the electron micrographs. Here we are able to directly see the transformation of an extremely long threadlike molecule into a short, fat, wormlike state.

With the proof and acceptance that the P-form is a collapsed state, the nonreversible character of the P-form transition in the alcohol solvents can now be explained. Once the DNA has collapsed into small condensed structure, its interaction with the solvent is

minimized. Anyone who has ever tried to dissolve DNA directly in methanol or ethanol knows that these are very poor solvents for DNA and that they will not dissolve precipitated DNA. Thus, once the P-form collapse occurs, the combination of a low DNA exposure and poor solvent conditions makes it impossible for the liquid phase to dissociate the condensed structure, and the DNA remains in the P-form.

Now that the reader is aware of the meaning of the condensed-denatured P-from state, and he has seen the overwhelming evidence proving its existence, two final questions need to be asked. Why does the P-form occur? What does its occurrence tell us about the nature of DNA itself?

In Section IV I offered a rather mechanistic and perhaps imaginative explanation of the occurrence of the P-form based on the Manning polyelectrolyte theory.[25] While this mechanism may or may not be correct, I believe great insight into the nature of the condensed-denatured P-form state, and how this state is fundamentally different from the aqueous denatured state, may be derived from the general principles that lie behind the polyelectrolyte theory.

According to this theory only 75% of the phosphate charges along the backbone of the DNA are directly neutralized in aqueous solutions containing monovalent cations. The charge repulsion that results from this incomplete neutralization is one of the major forces behind all DNA conformation. On the primary structural level it is this interaction that forces the two phosphate backbones to

be located across the helical axis from each other and is, in part, responsible for the hydrophobic inside - charged outside structure of the molecule at this level. In secondary structure it is the interaction between the phosphate groups repelling each other trying to expand the molecule and the hydrophobic forces trying to minimize solvent exposure that gives rise to the many different helical structures. Finally, on the tertiary level, one may view the entire gamut of DNA tertiary structures from small collapsed 'donuts'[29] to extremely long rigid extended conformations as the result of the interplay between this repulsive force trying to tear the DNA molecule apart, and the hydrophobic forces and hydrogen bonds trying desperately to hold it together.

At moderate temperatures and salt levels these opposing forces reach an equilibrium to create the moderately stiff, somewhat flexible helical structure known as B-form DNA. The flexibility and size of this structure may be changed by altering the ionic strength of the medium. If the ionic strength increases, the indirect shielding increases, the charge repulsion decreases, and the DNA becomes less rigid and more flexible. This effect may be taken to an extreme with divalent cations which will also reduce the direct charge neutralization and complete collapse to highly condensed structures will result.

In the other extreme, if the ionic strength on the medium is decreased excessively, the repulsion between the phosphates becomes so great the hydrophobic and hydrogen bonding forces within the DNA

can no longer hold the helix together and the molecule will rip itself apart and denature. Thus, while one usually thinks of the denaturation of aqueous DNA as a process in which one adds energy to pull the DNA strands apart, the truth of the matter is that one adds energy to destabilize the forces holding the molecule together, and that the molecule tears itself apart because there is no longer any force opposing the DNA's self-destructive phosphate charge repulsion.

With this in mind let us now examine the P-form state. The P-form transition is obtained by either raising the temperature of a methanolic solution, or by adding ethanol to this solution. Both of these changes have the same effect of decreasing the dielectric constant of the medium. This decrease in the dielectric constant, as we saw in Section IV, in turn has the effect of increasing the amount of charge condensation along the DNA helix, thus lowering the charge repulsion within the DNA structure to the point where the molecule may collapse upon itself. Thus, when the hydrogen bonds holding the strands of the DNA together are destroyed, the molecule is in a fundamentally different environment than when this occurs during aqueous denaturation. Unlike the water case, the charge repulsion along the phosphate backbone is now largely neutralized and the repulsive force driving the strands apart has been greatly reduced. The DNA is also now in a compact tertiary structure, so even if the enfeebled repulsive forces tried to weakly force the strands apart, the strands would quickly encounter other

parts of the molecule and would be unable to separate from each other.

Thus we should picture the P-form state, and the reversible character of the denaturation that occurs in this state, as being a direct consequence of the decreased dielectric constant of the medium. It is this decreased dielectric constant which increases the charge condensation along the helix and thereby reduces the charge repulsive forces that would ordinarily separate non hydogen bonded strands from each other. This understanding of the forces behind the P-form structure also helps us to explain the last untouched property of the P-form, its heat stability.

When a solution of P-form DNA is heated two forces will change to stress the P-form structure. Both of these forces, however, will tend to stabilize the structure rather than destroy it. One force which changes is the phosphate repulsion due to incomplete charge condensation. As the solution is heated the dielectric constant of the solution will further decrease,[37] and more counterions will condense on the DNA thereby further reducing the repulsive forces within the condensed structure. The second changing force exerted on the P-form structure will be the hydrophobic interaction. This force, which is caused by the tendency of hydrophobic residues to avoid a hydrophilic medium, is presumably much weaker in P-form DNA than in aqueous DNA because the moderately hydrophobic nature of the solvents involved will more easily solvate the highly hydrophobic bases. However, even if its contribution to the sta-

bility of the P-form structure at room temperature is only minimal, as the DNA is heated it will play an increasingly stronger stabilizing role. The thermodynamics of this force are typified by both positive enthalpy and entropy terms. Thus, when calculating the free energy of a transition, this positive entropy term is multiplied by -1 and the temperature, to make the hydrophobic force an increasing stronger stabilizing force as temperature increases. One can see then that this force will become more favorable with increased temperatures, and will act to further increase the stability of the P-form state. Thus both the hydrophobic forces and the increased charge condensation will be acting in favor of the collapsed state at higher temperature, and we can now see why the P-form should indeed be heat stable.

We have then the answers to the last two questions which were posed. We now have an understanding of the nature of the forces which cause the P-form to occur, and in understanding these forces we have gained greater insight into the nature of the forces behind the structure of DNA in general.

We see that this thesis has evolved from early work which simply defined the properties and occurence of an unknown state of DNA, to experiments which demonstrated the actual physical nature of the P-form, and then to an analysis of the forces behind the P-form structure which in turn has given us greater insight into the nature of DNA itself. While some questions may still remain on the exact nature of the P-form's secondary structure, and on differing solvent conditions that may induce a P-form transition, the original engima

posed by the contradictory properties of the P-form has now been solved, and this work is as complete as any scientific work ever is.

REFERENCES

1. Wang, J.C. (1978) Cold Spring Harbor Symp. Quant. Biol. 43, 29-34.
2. Wang, J.C. (1979) Proc. Natl. Acad. Sci. USA 76, 200-203
3. Baase, W.A. and Johnson, W.C., Jr. (1979) Nucleic Acids Res. 6, 797-814.
4. Girod,J.C., Johnson, W.C., Jr., Huntington, S.K., and Maestre, M.G. (1973) Biochemistry 12, 5092-5096.
5 Ivanov, V.I., Minchenkova, L.E., Schyolkina, A.K., and Poletayev, A.I. (1973) Biopolymers 12, 89-110.
6. Johnson, W.C., Jr. and Girod, J.C. (1974) Biochim. Biophys. Acta 353, 193-199.
7. Kay, E. (1976) Biochemistry 15, 5241-5246.
8. Nordén, B., Seth, S., and Tjerneld, F. (1978) Biopolymers 17, 523-525.
9. Zehfus, M.H. and Johnson, W.C., Jr. (1981) Biopolymers 20, 1589-1603.
10. Johnson, W.C., Jr. (1971) Rev. Sci. Instrum. 42, 1283-1286.
11. Chen, G.C. and Yang, J.T. (1977) Anal. Letters 10, 1195-1207.
12. Nelson, R.G. and Johnson, W.C., Jr. (1970) Biochem. Biophys. Res. Commun. 41, 211-216
13. Sprecher, C.A., Baase, W.A., and Johnson, W.C., Jr. (1979) Biopolymers 18, 1009-1019.
14. Lewis, D.G. and Johnson, W.C., Jr. (1974) J. Mol. Biol. 86, 91-96.

15. Wang, A. H.-J., Quigley, G.J., Kolpak, F.J., Crawford, J.L., van Boom, J.H., van der Marel, G., and Rich, A. (1979) Nature 282, 680-686.

16 Gray, D.M., Edmondson, S.P. Lang, D., and Vaughan, M. (1979) Nucleic Acids Res. 6, 2089-2107.

17. Nordén, B. and Seth, S. (1979) Biopolymers 18, 2323-2339.

18. Bolivar, F., Rodriguez, R.L., Greene, P.J., Betlach, M.C., Heyneker, H.L. and Boyer, H.W. (1977) Gene 2,95-113.

19. Sutcliffe, J.G., (1978) Nucleic Acids Res. 5,2721-2728

20. Sutcliffe, J.G. Personal Communication

21. Summerton, J., Bestwick, R., and Atkins, T. In Press. Analytical Biochemistry.

22. Rosenburg, A.H., and Studier, F.W. (1969) Biopolymers 7, 765-774.

23. Zimm, B.H. and Crothers, D.M. (1962) Proc. Natl. Acad. Sci. USA 48,905-911.

24. Gill, S.J. and Crothers, D.M. (1962) Proc. Natl. Acad. Sci. USA 57,562-566.

25. Manning, G.S. (1978) Q. Rev. Biophys. 11, 179-246.

26. Shimanouchi, T., Tsuboi M., and Kyogoku, Y. (1965) Advan. Chem. Phys. 7,435-498.

27. Kyogoku, Y. Tsoboi, M., Shimanouchi, T., and Watanabe, (1961) Nature 189, 120-126

28. Fritzche, H. (1971) Biochim. Biophys. Acta. 228, 344-349

29. Chattoraj, D.K., Gosule, L.C., and Schellman, J.A. (1978) J. Mol. Biol. 121, 327-337.

30. Lang, D., Taylor, T.N., Dobyan, D.C., and Gray, D.M. (1976) J. Mol. Biol. 106, 47-107.

31. Eickbush, T.H. and Moudrianakis, E.N. (1978) Cell 13, 295-306

32. Vollenweider, H.J., James, A. and Szybalski, W. (1978) Proc. Natl. Acad. Sci. USA 75, 710-714.

33. Lang, D. Personal Communication.

34. Anderson, P., and Bauer, W., (1978) Biochemistry 17, 594-601.

35. Studdert, D.S., Patroni, M., and Davis, R.C. (1972) Biopolymers 11, 761-779.

36. Wilson, R.W. and Bloomfield, V.A. (1979) Biochemistry 18, 2192-2196.

37. Akerlof, G. (1932) J. Am. Chem. Soc. 54, 4125-4139.

38. Dorman, B.P., and Maestre, M.F. (1973) Proc. Natl. Acad. Sci. USA 70, 255-259.

39. Hanlon, S., Johnson, R.S., Wolf, G., and Chan, A. (1972) Proc. Natl. Acad. Sci. USA 69, 3263-3267

40. Rill, R., and VanHolde, K.E. (1973) J.Biol. Chem. 248, 1080-1083

41. Herskovits, T.T., Singer, S.J., and Geiduschek, E.P. (1961) Arch. Biochem. Biophys. 94, 99-114.

42. Geiduschek, E.P., and Herskovits, T.T. (1961) Arch. Biochem. Biophys. 95, 114-120

43. Herskovits, T.T.(1962) Arch. Biochem. Biophys. 94, 474-484.