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I. RNA A-TO-Z TRANSITIONS AND DNA B-TO-Z TRANSITIONS. II. CIRCULAR INTENSITY DIFFERENTIAL SCATTERING OF CHOLESTERIC LIQUID CRYSTALS

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II. CIRCULAR INTENSITY DIFFERENTIAL SCATTERING OF
CHOLESTERIC LIQUID CRYSTALS

K.B. Hall
(Ph.D. Thesis)

December 1984

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I. RNA A-to-Z Transitions and DNA B-to-Z Transitions

II. Circular Intensity Differential Scattering of Cholesteric Liquid Crystals

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December 1984

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I. RNA A-to-Z Transitions and DNA B-to-Z Transitions

II. Circular Intensity Differential Scattering of Cholesteric Liquid Crystals

Kathleen B. Hall

Abstract

The thesis is composed of two parts: Part I describes the conformational transitions of DNA and RNA from right-handed helices to left-handed helices. These transitions, referred to as B-to-Z for DNA and A-to-Z for RNA, are effected by specific solvents and temperatures. Various spectroscopic methods show the details of the transition, with near ultraviolet circular dichroism (CD) indicating the differences in the transition moments between the DNA and RNA, and nuclear magnetic resonance (NMR) and vacuum ultraviolet CD indicating that the structures are very similar. Thermodynamic parameters for the DNA polymer transition are calculated, indicating that the transition is not isoenthalpic as previously postulated. A tetramer of RNA, rCGCG, is used for obtaining thermodynamic data on the RNA A-to-Z transition. This short sequence also appears to adopt a left-handed structure, but thermodynamic data indicates that the A-form is more favorable.

Part II describes the circular differential scattering (CIDS) of cholesteric liquid crystals. These compounds have a natural helical order, with enormous scattering power. The scattering patterns obtained from the liquid crystals are influenced by the helical parameters such as pitch and handedness, as predicted by the theory. It is hoped that these model compounds will provide information for the interpretation of the CIDS spectra obtained for biological samples, such as gels of DNA or oriented viruses.
This thesis is dedicated to all those friends who with their patience and support helped me through to the finish.
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Au revoir, Berkeley.
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Introduction

My thesis is composed of two quite distinct parts, one of which describes the right- to left-handed transition of DNA and RNA; the other describes the scattering properties of cholesteric liquid crystals. If there is a common thread that runs between the two, it is helicity. The parameters of helices, their pitch, radius, and handedness, determine the properties of the helix. For DNA and RNA these properties are reflected in the number of base pairs per turn of the helix, the degree of base stacking present, the geometry of the sugar-phosphate backbone, and of course whether the helix itself is right- or left-handed. Spectroscopic techniques - absorption, circular dichroism, and NMR - are used to probe the structural peculiarities of the nucleic acids. For cholesteric liquid crystals, the parameters of pitch and handedness influence the circular intensity differential scattering patterns.

Pohl and Jovin (1) described an R to L transition of poly[d(G-C)] in 1972. The identification by X-ray crystallography (Rich et al., 1979) of the L form as a left-handed helix resulted in a veritable flood of investigations of the transition, now identified as a right-to-left-hand transition. Raman spectra of the crystal and of the left-handed solution form showed that the poly[d(G-C)] structure was the same in both conditions (3), providing further impetus for solution studies. The L form had several distinctive features beyond its left-handedness: the repeat unit was a dinucleotide rather than a mononucleotide, the guanosine was syn rather than anti, and the phosphate backbone, as a result, was not smooth but zigzagged down the length of the helix. This feature gave rise to the designation Z-form, and the transition is now called B-to-Z. The original Z-form was produced in 4.5 M NaCl. Later work (4,5,6) showed that other solvents, such as ethanol, CsCl, NaClO₄, and MgCl₂, also facilitate the transition in greater or lesser molari-
ties than 4.5. Modification of the bases by methylation (7) or bromination (8) also enhanced the transition. Other sequences undergo the transition, but not as readily. The requirement for alternating purine-pyrimidine is not absolute, either, as indicated by Jovin and by Hartmann (8,9) who showed that short interruptions in the pattern did not prevent the ability to form the Z conformation.

There have been many physical chemical investigations of the transition, but these cannot directly address what is perhaps the most fundamental question arising from the phenomenon: What is the biological role? One nagging suspicion is that the Z-form is a laboratory-constructed red herring in the quest for structure-function relationships. This fear is somewhat allayed by reports of specific Z-DNA binding proteins (10) and of reports of Z-DNA antibodies recognizing regions in intact chromatin (11,12) although no binding proteins have been isolated and the antibody story is not over yet. Theories of regulatory activity by stretches of Z-DNA have been proposed but are so far only theories; by far the most enthusiastic promoters of the theories are those who spend the majority of their time and energy working on the mechanics of the transition.

And now to further confound the theorists of biological utility, there is evidence that RNA can undergo an A-to-Z transition. Again, the regulatory aspects of Z-form can apply; where DNA:histone or DNA:polymerase interactions are now replaced by RNA:ribosome or RNA:synthetase schemes. There will be an examination of known RNA sequences to identify G-C rich regions; examples that come immediately to mind are viroids and the acceptor stem of some tRNAs. The idea of conformationally inflexible RNA is gone, to be replaced by a question: What are the biological roles of nucleic acid structural flexibility?

As a biophysicist, the structure:function role of DNA is the raison d'etre in my study of nucleic acids. The topics covered in this thesis are concerned exclusively with structure, however, leaving function to speculation.
In Chapter 1, the temperature dependence of the B-to-Z transition of poly[d(G-C)] is studied, including a calculation of a van't Hoff enthalpy for the transition. The Z' form is identified as arising by a noncooperative transition from Z-form as a function of ethanol concentration. All three forms, B,Z, and Z', are shown to be solution forms, with no evidence of condensation or precipitation, as their circular dichroism spectra lack any scattering contributions.

In Chapter 2, the physical characteristics of Z-DNA are presented, as well as some discussion of the techniques used to measure them. This is followed by the details of the enzymatic synthesis of poly[r(G-C)], which in turn is followed by a description of the results of the spectroscopic measurements on the RNA, which indicate that it, too, can form Z-form. Also in this chapter is a list of solvents that we used in attempts to induce the Z-RNA conformation, with a suggestion as to which ones to try next. There is also an attempt to calculate chemical shifts here, which was not a notable success.

Chapter 3 describes the spectroscopic properties of the tetramer rCGCG, which also has an A and a Z form. Thermodynamic values for the helix to coil and A to Z transition are given, as well as a phase diagram for the molecule.

Chapter 4 presents the data on the scattering of cholesteric liquid crystals. These molecules have a natural helical order, and scatter enormous amounts of light. The apparatus for measuring circular intensity differential scattering (CIDS) was in an initial state of development, and could only be used for measuring the scattering of objects only when the CIDS was greater than $10^3$. Liquid crystals were ideal, and had the additional feature that their helices could be oriented with respect to the incident light. The description here is phenomenological rather than theoretical.

Clearly, the studies described here are aimed at determining structure rather than at elucidating the biological function of those structures.
References


Chapter 1. The B-to-Z transition of Poly[d(G-C)]

Pohl and Jovin’s report in 1972(1) that poly(dCdG)-poly(dGdC) could undergo an “R-to-L” conformational transition remained an interesting observation until the identification of the L form as a left-handed structure by Rich et al. in 1979 (2). Since that discovery, many investigations have been performed to determine the solution conditions that actuate the transition (3,4), its sequence dependence (5,6,7,8,9), and the possible biological utility of left-handed, or Z, DNA. (10) A short review of the research on the B-to-Z transition as it is now known, is given by Jovin et al. (11) An earlier collection of work is found in the Cold Spring Harbour Symposium of 1982. (12) The volume of work on the transition is enormous, but a qualitative review of some of the spectroscopic work, primarily circular dichroism, is given here.

A. Spectroscopic Investigation

A. Introduction

Pohl and Jovin’s original observation of the transition was made using circular dichroism (CD), a technique that is extremely sensitive to molecular structure. For a molecule to exhibit circular dichroism, it must contain a chiral or asymmetric center, which gives rise to the differential absorption of right- and left-circularly polarized light. In a chiral molecule, each transition has both a magnetic and electric dipole transition moment, which interact to give a nonzero product ($\mu \cdot m \neq 0$). For a DNA base, with no sugar attached, there is no CD. Attaching the sugar breaks the symmetry, producing a CD absorption. In the DNA helix, the nucleotides interact with each other, cause exciton splitting and give the result that the CD of a polymer is not equal to the sum of the CD of the monomers. The base sequence of a DNA strand will therefore affect the CD through the various combinations of interactions present. In Tinoco’s approach
(13) to the calculation of CD of a polynucleotide, a sum of interactions is used to give the total CD.

The base interaction is described in part by the base stacking, and the subsequent degree of overlap of bases by their neighbors. The degree of overlap is important to the electronic interactions between the planar base pairs, such that when the base stacking is disrupted in the melted molecule, the absorbance increases (hyperchromicity) and the CD spectrum of both DNA and RNA becomes "conservative" (i.e. equal positive and negative bands, centered about the absorption maximum.)

Because the environment will affect the transition moment of the base, changing that environment will be reflected by changes in the CD signal. In this way, it is clear that CD can be a sensitive indicator of DNA structure. See Johnson et al. (14) for a discussion of geometric parameters and their influence on the CD spectra of polynucleotides.

There are a few problems in the interpretation of CD spectra, however. One is that the transition moments of the bases are not well known. Attempts to match theoretical CD spectra with experimental spectra have met with indifferent success, where the polymers containing alternating purine-pyrimidine sequences have been the most difficult to fit (15). With the interest in the left-to-right-handed transition of poly(dCdG)-poly(dGdC), it was natural that attempts should be made to model its CD spectra. Early efforts to calculate the CD of the B-form helix were unsuccessful (15), but the later efforts to calculate the Z-form helix were perhaps even more so, since their effect was to arrive at a spectrum for Z-form that resembled the experimental B-form spectrum (16). Using a slightly different method of calculating transition moments, Williams and Moore (17) came to the conclusion that the experimental Z-form CD spectrum really arose from a right-handed helix, and the experimental B-form CD spec-
trum from a left-handed helix. The question was, who was right?

There are several solvents that produced the Z-form of poly(dCdG)-poly(dGdC). Pohl and Jovin had used 3.5 M NaCl; Pohl (18) had used ethanol as well. He noted that in 50% ethanol, 10 mM sodium phosphate, the CD spectrum of the polymer was nearly identical to that observed in >2.5 M NaCl. However, in 80% ethanol, a third CD spectrum was observed, unlike either the B or Z spectra. High concentrations of ethanol are used to precipitate DNA, and there was later evidence that Z-DNA can condense or aggregate (3, 4, 19, 20). The aggregation or condensation of DNA can give rise to anomalous CD signals, due to differential scattering of the incident circularly polarized light. (21) The apparent CD spectrum arising from such a sample can in some cases have bands of opposite sign from that of the true CD band, a situation that can be ascertained only by doing the appropriate corrections for scattering. Because the solvents for producing Z-DNA were similar to those for producing aggregates, we wanted to apply the scattering corrections to the CD spectra to determine if in fact the spectra were true solution spectra. If there were significant scattering contributions, it might explain the theoretical difficulty in constructing a reasonable Z-form spectrum. We measured the scattering correction to each of the three forms of poly(dCdG)-poly(dGdC) (the high salt form, low salt form, and the concentrated ethanol form).

Experimental

The polymer poly(dCdG)-poly(dGdC) was purchased from P/L Biochemicals in two batches. Lot number 676-7, with an S value of 8.2, was used without further preparation, but Lot 692-63 was solubilized at 5 A256/ml in distilled water, and dialyzed once against 6X volume 5×10⁻⁴ M NaCl. Conductivity measurements of the samples using a Yellow Springs Instruments conductivity meter and comparing to a standard curve gave [NaCl] between 1-4×10⁻⁴ M. The species and
The concentration of counterions in the sample is critical to its reversible B to Z transition. At a counterion concentration greater than $5 \times 10^{-4}$ M, the polymer precipitated rather than changed conformation. Each batch varies in the amount of condensed counterion associated, so sample preparation is extremely critical. The pH of the samples was from 7.1 to 7.3. We used an extinction coefficient at 256 nm of 7100 (1) for concentration determination. For most experiments, polymer concentration was $7 \pm 5 \times 10^{-5}$ M phosphate. The polymer was solubilized in $5 \times 10^{-4}$ M NaCl or in distilled water at room temperature for at least 24 hours.

The absorbance spectra were measured on a Cary 15 spectrophotometer and compared to the published spectra of Pohl and Jovin (1) to ensure that the poly(dCdG)-poly(dGdC) was double-stranded. Circular dichroism spectra were measured with a Cary 60 spectropolarimeter (03 CD modification). The data collection and analysis system have been described previously (21). The fluorscat and FDCD techniques for determining the scattering contributions to the CD are described in Dorman and Maestre (21) and in Reich et al (22). Briefly, fluorescent detected circular dichroism (FDCD) is a technique that will correct for differential scattering of a sample over $4\pi$ radians, using a fluorescent reporter molecule as a detector of the transmitted and scattered light. It is especially useful for measuring the CD of optically active particles that scatter differentially. The fluorscat technique also allows for correction of any differential scattering that may occur in a sample, but it cannot measure any contribution that may arise from backscattering. Again, a fluorescent molecule is acting as a detector of transmitted and scattered light from the sample.

Ethanol was added by drops to the samples as they were stirred rapidly. Ethanol concentration was incremented from 10% to 85% v/v. The absorbance spectra were taken after ethanol addition and the measured values compared to
the calculated values as a function of dilution. These two values were within experimental error, indicating that there was no strand separation. Also, none of the absorbance spectra showed any apparent absorption at wavelengths outside of the absorbance bands. Such apparent absorption would have indicated that the polymer was scattering the incident light, as would happen if the polynucleotide were condensing or aggregating.

When the polymer precipitated, as it did in $2 \times 10^{-3}$ M NaCl and 70% ethanol, the precipitate was readily visible by eye, and stuck to the walls of the test tube. When there were no added counterions present (i.e., the solvent was distilled water or distilled water/ethanol) there was no evidence of precipitation as monitored by absorbance spectra. The absorbance spectrum of a precipitated sample was very broad and fluctuated with time, making its identification simple.

Scattering Measurements

The scattering contribution to the CD of the three forms of poly(dCdG)·poly(dGdC) was measured by either fluoroscat or fluorescent detected circular dichroism. Z-form was produced by both high salt (3.5 M NaCl) and ethanol (50% v/v) and each preparation was examined for scattering contributions. Z'-form was described by Pohl (18) and is produced at very high (80%) ethanol concentrations. The results shown in Figure 1. indicate that in neither Z-form nor Z'-form is there any scattering contribution to the CD. This is particularly interesting in the case of the Z'-form, as it exists in high alcohol concentrations. It has been shown previously that A-form DNA in high ethanolic concentrations can be precipitated or aggregated with concomitant presence of CD scattering contributions (21,22,23). The lack of such contributions to the CD spectrum of the Z'-form shows that the measured CD is a true spectrum with no scattering contributions.
FIGURE 1. a) FDCD and CD of 4.5 M NaCl form of poly(dCdG)·poly(dCdG). b) Fluorscat and CD of 85% ethanol form of poly(dCdG)·poly(dCdG). c) Fluorscat and CD of 88% methanol form of poly(dCdG)·poly(dCdG).
The Z to Z' transition in ethanol was reported by Pohl (18). Using the techniques for scattering correction to the CD, the steps of the transition were examined to determine the solution behavior. Again, there was at no time, from 50% ethanol (Z-form) to 85% ethanol (Z'-form) any evidence of aggregation or precipitation as monitored by absorbance or of CD scattering contributions. The ethanol was added in successive aliquots, diluting the polynucleotide. For the reverse Z' to Z transition, the ethanol was diluted with 5×10⁻⁴ M NaCl.

DNA in 80% ethanol adopts an A-form, as noted above. In similar concentrations of methanol, however, DNA adopts a C-form. Therefore, it was interesting to see that methanol also produced the Z-form of poly(dCdG)-poly(dGdC) at low concentrations, and produced an A-like form (shown by CD) at high concentrations. Although the final forms in 85% ethanol and 88% methanol are not identical, neither show any signs of scattering contributions. (See Figure 1.)

Z to Z' Transition

As indicated above, Pohl identified the Z to Z' transition in high ethanol and high CsCl solvents. He described the transition as a more gradual change than was seen for the B-to-Z transition. From the data for the scattering experiments, it can be shown that the percent Z (or Z') form is proportional to the percent ethanol added. In other words, the Z to Z' transition is noncooperative. The transition occurs in increments that are established rapidly, and the Z' to Z transition, which occurs upon dilution of the ethanol, is also rapid. The Z' form is stable over several weeks at 4°C.

The Z to Z' transition shows an isosbestic point at 268 nm (Figure 2), suggesting a two-state transition. To investigate this possibility, a series of CD spectra were generated by computer that consisted of the sum of different amounts of Z and Z' form. The resulting spectra match almost perfectly the experimental data (see Figure 3) indicating that there are two forms simultaneously in
FIGURE 2. Experimental CD spectra of the ethanolic $Z$ to $Z'$ transition, showing the isosbestic point at 268 nm.
solution which contribute to the CD. Because the transition is noncooperative, meaning that sites are noninteracting, it is probable that $Z$ and $Z'$ form coexist on the same polynucleotide strand. Their relative distribution ($Z-Z'-Z$ or $Z-Z'$) cannot be determined by CD.

The $Z$ to $Z'$ transition is very similar to the B to high salt B form (C-form, or alternatively, $B'$) in that both are noncooperative. Each transition is achieved through increments that are stable at a given salt concentration (B-to-C) or percent ethanol ($Z$-to-$Z'$). The B to C transition is thought to be an increase in the winding angle of the polynucleotide (24) while maintaining the B-form backbone geometry. Since the alteration of B to C has been shown to involve very small changes in the secondary structure of DNA, we propose by analogy that similar alterations must be occurring in the $Z$ to $Z'$ transition.

Methanol also induces a $Z'$ conformation of poly(dCdG)-poly(dGdC) (Figure 4). The CD band at 280 nm is diminished in magnitude in methanol relative to the band in ethanol. The transition is again noncooperative. There is no isosbestic point in the spectra of the consecutive methanol additions, and the experimental spectra of successive methanol additions do not match the computer-generated sums of $Z$ and $Z'$ spectra, providing additional evidence that there are more than two solution forms for the methanol $Z$ to $Z'$ transition. The experimental spectra are similar to the computer-generated spectra in that there is a progressive red-shift of the maximum with increasing methanol concentrations. However, the computer-generated spectra show an isosbestic point at 273 nm, while the experimental spectra do not. The kinetics of each spectral change are as rapid in methanol as they are in ethanol, and the spectral forms at each ratio are stable. The methanol $Z'$ form appears similar, but not identical, to the ethanol $Z'$ form.

There are two possible conformations for the $Z'$-form that cannot be dis-
FIGURE 3. Calculated CD spectra of the Z to Z' transition, using sums of Z and Z' spectra.
SUM OF CONTRIBUTIONS OF ETHANOL Z AND Z' FORMS

WAVELENGTH (NANOMETERS)

EPSILON LEFT - EPSILON RIGHT

75 Z + 25 Z'
67 Z + 33 Z'
50 Z + 50 Z'
28 Z + 72 Z'

XBL 8312-4327
FIGURE 4. Experimental CD spectra of the methanolic Z to Z' transition.
POLY(DG)POLY(DG) IN METHANOL/WATER

WAVELENGTH (NANOMETERS)

POLY(DG)POLY(DG) IN H₂O
- IN 40 0/-0 MEOH
- IN 67 0/-0 MEOH
- IN 83 0/-0 MEOH
- IN 88 0/-0 MEOH

XBL 838-10965
tinguished by circular dichroism. The first is that the $Z'$-form is left-handed, since the $Z$ to $Z'$ transition is rapid and noncooperative like to the B to B' transition. Inverting the $Z'$-form CD spectrum makes it appear much like the CD spectrum of the B'-form. However, the second possibility is that the $Z'$-form may have an A-form geometry, since the CD spectrum of the $Z'$ form closely resembles the right-handed A-form CD spectrum. If $Z'$ were A-form, the polymer would be right-handed, so that the $Z$ to $Z'$ transition would be from left-handed to right-handed. Given the rapidity of the transition ($Z$ to $Z'$ and $Z'$ to $Z$) and its noncooperative nature, it seems unlikely.

B. Temperature Dependence of the B to Z Transition

Kinetics and Intermediates

The B to Z transition in ethanol is cooperative, as shown by the rate of the transition, the narrow window of temperature that produces it, and the non-linearity of the temperature dependence. The transition can be followed in ethanol/water mixtures by raising (B to Z) or lowering (Z to B) the temperature. At ethanol concentrations of 10%, 15%, and 20%, the transition exhibited temperature dependence; at 25% ethanol, the polymer was stable in Z-form until about 4°C, where, after about 48 hours, it changed to an intermediate form. The rate of change of the transitions is slow, and it is possible to step the temperature at intervals of about 1°C to observe the transition intermediates (see Figure 5). After stepping the temperature, equilibrium is reached slowly, from 20 minutes to 1 hour later. After equilibrium is reached, the intermediates are stable, at least for about an hour (the time necessary to take a CD spectrum from 350-200 nm twice.)

The CD spectra of the intermediate forms do not match the computed sums of B and Z spectra (see Figure 6) indicating that the intermediate forms are dis-
FIGURE 5. Experimental CD spectra of two intermediates in the B to Z transition. a) B-form; b) Intermediate close to B-form; c) Intermediate close to Z-form; d) Z-form.
tinct structures. The CD spectra of the intermediate forms reflect a decrease in the transition dipole-dipole couplings, perhaps as a result of a change in the stacking interactions. The intermediate forms seem to have a degree of unwinding and unstacking that may or may not involve strand separation. However, the lack of change in the absorption of the polymer shows that there is little or no hydrogen bond breakage or strand separation. The interpretation of these optical data leads to a model for the intermediary forms in which the DNA consists of a double-stranded polymer unwound with reduced stacking interactions. One possible physical model for the transition consists of a ladder of hydrogen-bonded base pairs which is straight or only slightly twisted, to account for the decrease in the magnitude (ellipticity) of the CD signal seen in the intermediates. The distance between base pairs may or may not be greater than in normal double-stranded polymer.

Thermodynamics

By doing a series of temperature shifts, the ellipticity at 290 nm was measured as a function of temperature for the transition. Figure 7 gives the data, plotted as fraction of B-form vs. temperature. Where the fraction equals 0.5, we can define a transition temperature \( (T_t) \) for each ethanol concentration. Although it is clear that the B to Z transition has many intermediate forms, it is possible to use a simple two-state model as an approximation, from which we can calculate thermodynamic parameters for the transition. From the model, an equilibrium constant is defined, \( K = (\Theta_f - \Theta)/(\Theta - \Theta_i) \) where \( \Theta_i \) is the initial ellipticity, \( \Theta_f \) is the final ellipticity, and \( \Theta \) the ellipticity at an intermediate. This equilibrium constant is used in the standard thermodynamic relationship \( K = \exp(-\Delta G^o/RT) \). To calculate the van't Hoff enthalpy, which is the enthalpy per mole of cooperative unit, the free energy is replaced by \( \Delta G^o = \Delta H^o - T \Delta S^o \), giving \( R \ln K = (\Delta H^o)/T - \Delta S^o \). Plotting \( \ln K \) vs \( 1/T \) gives the slope of \( \Delta H/R \). This allows the
FIGURE 6. Calculated CD spectra of the B to Z transition, using sums of B and Z spectra.
SUM OF CONTRIBUTIONS OF B AND Z FORM SPECTRA

WAVELENGTH (NANOMETERS)
FIGURE 7. The path of the B to Z transition, as a function of temperature and B-form content, for the three ethanol concentrations. The arrows indicate the direction of the temperature shift. a) 20\% ethanol; b) 15\% ethanol; c) 10\% ethanol.
calculation of $\Delta H$/mole for the transition, where the mole is mole of cooperative unit. For 20% ethanol, $\Delta H$ for the B to Z transition is 140 kcal/mole at $T_t$ of 34.5 ± .5°C; for 15% ethanol, $\Delta H$= 170 kcal/mole for B to Z, and -160 kcal/mole for Z to B at $T_t$ of 37 ± .5°C; for 10% ethanol, $\Delta H$= ±(-)200 kcal/mole for both transitions, at $T_t$ approximately 47± 1°C. (The error for this sample was greater than for the other two due to uncertainties in the temperature of the intermediates.)

Keeping in mind that the values obtained for the enthalpies of transition were derived using a model that is only an approximation to the actual experimental results, the calculations provide some insight into the transition mechanism. The enthalpy values obtained are expressed in terms of cooperative units. The decrease in $\Delta H$ with increasing ethanol concentration may reflect a decrease in the size of the cooperative unit, or, alternatively, a decrease in the energy needed to initiate the transition. Ivanov and Minyat (25) estimate the size of the cooperative unit in ethanol to be 25 base pairs. The $\Delta H$ per base pair for the B to Z transition, assuming the size of the cooperative unit remains constant, would then vary from about 10 kcal in 10% ethanol to about 5 kcal in 20% ethanol. It is also possible that the increase in $\Delta H$ with decreasing ethanol is due to the increase in energy required to form a nucleation site for the transition. This change in enthalpy for the transition can be compared to the change in enthalpy for the helix-coil transition of (dCdG)$_n$ oligomers of -11 kcal/base pair (26). While the length of the polynucleotide will have some influence on the $\Delta H$, the two values can be used for a rough comparison. The similarity in the enthalpies of the two transitions indicates that the values for the B to Z transition are not unreasonable.

Dickerson (27) concludes on the basis of the temperature independence in sodium chloride that the B to Z transition is isoenthalpic. Our data in ethanol/water mixtures indicates that in fact, it is not. One way to achieve the
transition from B to Z in salts is to first raise the temperature of the solution to 50°-60°C then let it cool. This also implies a high activation barrier to transition. The driving force for the transition may be solvent dependent, since clearly the behavior of the transition is strongly solvent dependent.
References


Chapter 2. Characterization of Z-RNA

A. Z-DNA Structure

In the previous chapter, the thermodynamics of the B-to-Z transition of poly[d(G-C)]·poly[d(G-C)] were discussed. Other aspects of the transition, such as the solvent conditions that actuate it, modifications of bases that facilitate it, and the physical details of the Z structure have been examined by other investigators. Some of those results are summarized here.

The original observation that Z-form could be found in 3.5 M NaCl (1) or in 50% ethanol (2) seemed to indicate that the mechanism involved a decrease in the water activity or a dehydration. The list of solvents that facilitate the transition has increased, and the great variety argues against such a simplistic explanation. The Z-form crystallography was done using crystals from a low salt solution (3) where it might be argued that the high concentration of dCGCGCG forced out the water or alternatively, concentrated the available salt. Crystallography done from high salt solutions (4) of the tetramer dCGCG produces a Z' form that is similar, but not identical to the Z-form crystal (3). The search for more physiological concentrations of ions has yielded cobalt hexamine (5) which causes the transition at micromolar amounts, and MgCl₂ at 0.7 M (1). These data suggest the possibility of specific ion replacement accompanied by water displacement.

Various modifications of the bases also facilitate the B-to-Z transition. Poly[d(G-5mC)] much more readily adopts the Z-form, at concentrations of 0.7 M NaCl, 0.6 mM MgCl₂, or 50 μM spermidine (5). Bromination of the cytosine is more effective than methylation. Addition of substituents to the 8 position of guanine have resulted in structures that, while called Z-form, have not been conclusively demonstrated to be Z (6).

The physical peculiarities of Z-form are first that there is a dinucleotide
repeat unit for the helix; second, that the guanosine is syn (purines can be either syn or anti, but in DNA they are usually anti); third, that there is a zig-zag pattern to the backbone, creating two distinct (magnetic) environments for the phosphates; fourth, that the sugar puck is 3'-endo in guanosine and 2'-endo in cytosine; fifth, that the helical axis does not pass through the bases but rather the bases move around the outside of it, forming a helix with a hole in it; and finally, the helix is left-handed.

The consequence of these physical characteristics is that they have unique spectroscopic features that can be used as a means of identification. One of the most powerful methods of structural determination is NMR, and in fact the NMR properties of the Z-form have been used extensively as a means of identifying it. The most readily visualized feature of Z-form is the two phosphorus resonances. $^{31}$P NMR shows two bands, split by about 1.3-1.5 ppm (7,8) for Z-form, where there is only one resonance for the B form of poly[d(G-C)]·poly[d(G-C)]. (There are also two resonances for the B form of poly[d(G-5mC)].) These two bands arise from the two different magnetic environments of the phosphorus atoms in the DNA backbone.

Using proton NMR ($^1$H NMR) and the nuclear Overhauser effect (NOE) the conformation of the sugar in guanosine can be determined. The GH8 proton (on the base) and the GH1' proton (on the sugar) are 3.9 Å apart (9) when the guanosine is anti. When it is syn, these two protons are 2.2 Å apart (3). As a consequence, one proton can transfer magnetization to the other. This transfer of magnetization is called NOE, and has a distance dependence of $1/r^6$, where $r$ is the distance between nuclei. When the two protons are less than 3.5 Å apart, spin transfer can occur, and the exchange measured by NMR. This is done by saturating one proton and observing the transfer of magnetization to the second, and vice versa. In the Z form, an NOE is observed between the GH8 and the GH1', indi-
cating that the sugar is syn (8).

NMR can also be used for sugar pucker determination, using J-coupling. This is an NMR technique that measures the scalar coupling (J) between nuclei. Altona (10) showed that the coupling constant $J_{1'2'}$ is dependent on the sugar pucker, having a value of 1 Hz for the 2'-endo (B DNA) and 8 Hz for 3'-endo (Z DNA). This experiment must be done on oligomers, not polymers, where the line width is narrow enough to measure an 8 Hz splitting. Polymer bands are roughly 40-50 Hz wide, making it impossible to see the $J_{1'2'}$ coupling.

While X-ray crystallography is the only way to determine helical parameters and orientation of the bases along the helical axis, there is some indication that circular dichroism (CD) can be used to determine helical handedness. Sutherland (11) and Prof. Curt Johnson (personal communication) have measured the CD in the vacuum uv (to 160 nm) of poly[d(G-C)]·poly[d(G-C)] in the B and Z form, and find that the bands are ten times larger than the near uv bands, and that in going from B to Z form, the bands invert in sign. Sutherland (12) has applied this criteria to poly[d(I-C)] which has an odd near uv CD spectrum resembling the poly[d(G-C)]·poly[d(G-C)] Z-form, and concluded on the basis of the signs of the bands that the polynucleotide was right-handed. If this technique proves reliable and can eventually be explained theoretically, it will provide another method (other than X-ray crystallography) of helix-handedness determination.

B. The search for Z-RNA

All the above studies have used poly[d(G-C)]·poly[d(G-C)] or its derivatives. The obvious question was whether the RNA, poly[r(G-C)], could also undergo an A to Z transition. While Rich (3) could see no reason why it shouldn't, Dickerson (13) was more pessimistic; he doubted that RNA could be made to adopt a Z-form.
due to both steric effects and the stability of the A-form geometry.

Dr. Tom Jovin and Dr. Johan van de Sande had observed a large change in the CD spectrum of poly[r(G-C)] in changing from 4.8 M NaClO₄ to 20% ethanol/4.8 M NaClO₄ (personal communication). However, they noted that a significant amount of precipitation occurred following the addition of ethanol, so that while intriguing, the interpretation was somewhat difficult, as condensed states do have odd CD spectra (14). The observation seemed worth pursuing, however. The difficulty was in obtaining enough sample to do NMR spectroscopy, since the structural identification by NMR was crucial to the determination of Z-form. For NMR, at least 100 ODU were necessary. Since it wasn't commercially available, it had to be synthesized, and for that we had the excellent assistance of Prof. Michael Chamberlin and the members of the Hearst lab. The synthesis conditions are described below.

Preparation of poly[r(G-C)]

Poly[d(1-C)]-poly[d(1-C)], rCTP, and rGTP, were purchased from P/L Biochemicals, and used without further purification. α³²P-CTP was purchased from ICN. RNA polymerase was purified as previously reported.(15) Both the purified holoenzyme and the A5m fractions were used; the latter fraction was used for the large scale preparation. It contains both holoenzyme and core polymerase. Polymerase activity was assayed using the single-point method as described in Chamberlin et al.(16). For this assay, the total volume of solution is 0.1 mL, where components include 20 µL of 81 solution (0.38 ml distilled H₂O, 0.2 ml 1 M Tris-HCl, pH 8, 0.3 ml 2 M KCl, 0.02 ml 1 M MgCl₂, 0.05 ml 1 M 2-mercaptoethanol, and 0.05 ml 400 mM spermidine) which is made fresh before use, 10 µl T7 DNA (2 mM in nucleotides, with no nicks on the strand - nicks greatly reduce the calculated polymerase activity as the enzyme binds but will not transcribe), 10 µl NTP mix (27 mM ATP, 11 mM GTP, and 14 mM UTP), 10 µl CTP (α³²P).
and as much deionized distilled water as necessary to bring the solution to 0.1 ml when the enzyme is added. The reaction mix is kept on ice (0°C) prior to the addition of enzyme. The enzyme is stored in 50% glycerol, which means that the reaction mix must be vortexed after enzyme addition, or the enzyme will fall to the bottom of the reaction tube with the glycerol and not diffuse into the mix. If the enzyme is free in solution at 37°C, unattached to substrate, it will die. Also, addition of excess enzyme is to be avoided, since the excess that cannot bind to the DNA will die. Normally, stock solutions of enzyme are diluted 1/100 into cold 81 solution, then that dilution diluted 1/10 or 1/20 into the reaction mix.

After addition of enzyme to the mixture on ice, the solution is vortexed briefly and incubated at 30°C for 3.5 minutes. At 3.5 minutes, 100 μl of ice cold carrier (50 mM sodium pyrophosphate, 50 mM EDTA, with 0.5 mg/ml yeast tRNA) is added, the reaction mix is put on ice, and 2.5 ml of ice cold TCA is added to precipitate the RNA and DNA. This is kept on ice for 10 minutes, then filtered onto Whatman GF/C filters (by pulling with a vacuum) then washed with 35 ml cold 1 M HCl/0.1 M sodium pyrophosphate, followed by 10 ml cold 100% ethanol to dry the filter. The filter is dried under a heat lamp, and put in a scintillation vial with standard scintillation fluid (such as Omnifluor plus toluene) to be counted. Enzyme activity is calculated by the scheme:

\[
(x \text{ cpm/0.1 ml reaction}) \times (1 \text{ mmole CTP/}x \text{ cpm}) = (\text{mmoles CTP/ml reaction mix})
\]

\[
(\text{mmoles CTP/ml reaction}) \times (1 \text{ ml reaction/}x \text{ ml enzyme}) \times (\text{enzyme dilution}) \times (1/3 \text{minutes}) \times (10^8 \text{ nmoles/m mole})
\]

1 mUnit is 1 n mole of CMP incorporated per minute, so n moles CTP/min = mU, so the last value calculated is mU/ml enzyme. This value was used to determine the amount of enzyme to be added to the synthesis reactions. The final activity in reactions was adjusted to between 8-20 mU/ml. Bovine pancreatic DNase I was
purchased from Worthington (RNase free, code DPFF). Calf thymus alkaline phosphatase (RNase free) was purchased from Boeringer-Mannheim. All other reagents were reagent-grade.

One milliliter trial reactions for determining the appropriate reaction conditions were carried out for twenty-four hours at 37°C. Samples of each reaction were removed at intervals of one to four hours, depending on the experiment. The aliquots were spotted on Whatman GF/C filter paper, and were slowly washed with 10% ice-cold trichloroacetic acid (TCA), followed by a rinse with 35 ml of ice-cold 1 M HCl in 0.1 M sodium pyrophosphate, and another rinse of 10 ml of 95% ice-cold ethanol. This method of acid precipitation was not efficient, but for the purposes of the trial reaction, it was sufficient. To monitor the final extensive synthesis, 10 μl aliquots of the reaction were added to 10 μl of 0.5 mg/ml yeast tRNA, 50 mM EDTA, 50 mM sodium pyrophosphate, to which 2.5 ml of 10% TCA was then added. These mixtures were kept at 4°C for at least 10 minutes before being poured on a GF/C filter paper and pulled through under vacuum. Washes were then as described above. The samples on the filter paper were then dried under a heat lamp, solubilized with Permafluor, and counted in a scintillation counter.

The reaction conditions were varied to maximize the yield of poly[r(G-C)]. In Table I, the amount of product as a function of divalent cation (Mg or Mn), ionic strength, and spermidine concentration is shown. The optimum reaction conditions were 0.5 mM MnCl₂, 0.5 mM spermidine. Several components of the reaction mix were not varied in the experiments: all reactions contained 40 mM Tris, pH 8.0 and 10 mM β-mercaptoethanol.

In another attempt to increase the yield of poly[r(G-C)], the amount of template and substrate was increased to 120 nM poly[d(I-C)], 10 mM GTP, 10 mM CTP. The salt conditions for this experiment are given in Table I. These conditions did
Table I. Optimization of Conditions

<table>
<thead>
<tr>
<th>poly[d(I-C)] nmoles</th>
<th>[C, GTP] mM</th>
<th>[Mn] mM</th>
<th>[Mg] mM</th>
<th>[KCl] mM</th>
<th>[spermidine] mM</th>
<th>CTP incorp. nm/ml</th>
<th>% incorp.</th>
<th>RNA/DNA</th>
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<td>0.5</td>
<td>4.0</td>
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<td>2.0</td>
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<td>4.0</td>
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<td>0.0</td>
<td>7.0</td>
<td>&lt;1.0</td>
<td>6.0</td>
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<tr>
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<td>0.5</td>
<td>4.0</td>
<td>120.0</td>
<td>0.0</td>
<td>70.0</td>
<td>8.0</td>
<td>12.0</td>
</tr>
<tr>
<td>12.0</td>
<td>1.0</td>
<td>0.5</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>50.0</td>
<td>5.0</td>
<td>8.0</td>
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<td>12.0</td>
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<td>0.5</td>
<td>0.0</td>
<td>0.0</td>
<td>0.5</td>
<td>95.0</td>
<td>8.6</td>
<td>16.0</td>
</tr>
<tr>
<td>12.0</td>
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<td>0.5</td>
<td>0.0</td>
<td>50.0</td>
<td>0.0</td>
<td>70.0</td>
<td>5.6</td>
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<tr>
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<td>0.5</td>
<td>0.0</td>
<td>50.0</td>
<td>0.5</td>
<td>90.0</td>
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</tr>
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<td>0.5</td>
<td>0.0</td>
<td>0.0</td>
<td>0.5</td>
<td>110.0</td>
<td>1.3</td>
<td>1.2</td>
</tr>
</tbody>
</table>

*Final rx conditions

CTP incorporation is in nmoles/ml of reaction mix after 24 hours.
% incorporation is acid precipitable counts/total counts x 100.
RNA/DNA is nmoles RNA(G + C)/nmoles poly[d(I-C)] per ml.
not result in an increase in yield.

To investigate the limiting steps in the synthesis, additional polymerase, substrate, or template was added in separate experiments, once every 4 hours. The results of these experiments are shown in Figure 1 and Table II. It is clear that the addition of template results in increased production of poly[r(G–C)]. This suggests that the template may be binding to the product, forming a complex that is inefficiently used by the polymerase.

To determine the size distribution of the products, aliquots of the reaction mixtures were run on a denaturing 12% polyacrylamide gel in 7 M urea, 50 mM Tris-borate, 1 mM EDTA. The samples were added to an equal volume of 8 M urea, 20 mM Tris, pH 7.4, 1 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol. This mixture was heated to 90°C for 5 minutes, then quick-cooled. Hinf I-restricted SV40 DNA was used as size markers, and those were denatured as above. The gels were then autoradiographed. A representative gel is shown in Figure 2. Densitometer tracing of the gel showed that 51% of the poly[r(G–C)] is from 100-550 nucleotides long, with 21% less than 100, and 27% greater than 550 nucleotides. The phenomenon of the apparent doublets in the lower half of the gel is unexplained, although it is possible that they arise from the different mobility of fragments ending in G or C, or are due to the presence and absence of a terminal phosphate.

The final optimized reaction condition was then 40 mM Tris, pH 8.0, 0.5 mM MnCl₂, 0.5 mM spermidine, 10 mM β-mercaptoethanol, 12 nM (nucleotide) poly[d(I–C)], 1 mM rCTP, and 1 mM rGTP. Poly[d(I–C)] was added every 4 hours to increase the final concentration by 12 nM.

The final large extensive synthesis preparation used 60 one-milliliter reactions, to avoid the possible problems of scaling up to large volumes. Each tube contained 12 mU/ml polymerase. Four of the 60 tubes contained labelled α³²P-
Figure 1. Kinetics of poly[r(G-C)] synthesis with repeated additions of RNA reactants. Optimized reaction conditions were used (see Table I).

a) substrate. CTP and GTP were added to increase their final concentration by 1 mM in each. $\alpha^{32}$P-CTP was included in the mix so as to keep the specific activity of the CTP constant. The volume change was taken into account in the calculation of yield.

b) template. Poly[d(I-C)]-poly[d(I-C)] was added to increase the final concentration by 12 nM.

c) enzyme. 11 milliunits of RNA polymerase (in its storage buffer which included 50% glycerol) was added to one milliliter of reaction. The reaction mix was then vortexed briefly.
CTP, GTP added

Template added

Enzyme added

nmoles CTP Incorporated/ml reaction

Hours
Table II. Yield with Repeated Additions of Reactants

<table>
<thead>
<tr>
<th>repeated additions</th>
<th>nmoles CTP incorp./ml</th>
<th>% incorp.</th>
<th>RNA/DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>polymerase&lt;sup&gt;a&lt;/sup&gt;</td>
<td>95.0</td>
<td>8.6</td>
<td>16.0</td>
</tr>
<tr>
<td>GTP, CTP&lt;sup&gt;b&lt;/sup&gt;</td>
<td>45.0</td>
<td>1.2</td>
<td>5.0</td>
</tr>
<tr>
<td>poly[d(I-C)]&lt;sup&gt;b&lt;/sup&gt;</td>
<td>108.0</td>
<td>12.0</td>
<td>5.0</td>
</tr>
</tbody>
</table>

All reactions contained 40 mM Tris, pH 8.0, 10 mM β-mercaptoethanol, 0.5 mM spermidine, 0.5 mM MnCl₂.

<sup>a</sup> rx carried out for 24 hours.

<sup>b</sup> rx carried out for 16 hours.
Figure 2. Poly[r(G-C)] size distribution. 12% polyacrylamide gel, 7 M urea, 50 mM Tris borate, 1 mM EDTA. The gel was run above room temperature. Size markers, shown on the side, are Hinf I fragments of SV40 DNA, heat denatured before loading on the gel as described in the text; numbers refer to the number of nucleotides in each fragment. XC is xylene cyanol which runs as an 80 nucleotide fragment in these conditions.
CTP, to allow the reaction to be monitored as before. The course of the reaction can be seen in Figure 3. Final yields were calculated to be $466 \pm 40$ nmoles poly[r(G-C)])/ml reaction mix or 10 mg total, as measured by acid precipitable radioactivity.

The four labelled samples were run on a 12% denaturing gel as described above. Again, the predominant species were from 150-500 nucleotides in length.

The 60 samples were pooled and the RNA polymerase was extracted with an equal volume of redistilled phenol. The sample was dialyzed against 1 M NaCl, 0.1 M EDTA (four changes of 4 liters) to remove the free NTP's. Their removal was followed by counting an aliquot of the dialysis buffer in a scintillation counter. When 99.999% of the free NTP's were removed, the sample was lyophilized to reduce the volume, then dialyzed against 0.1 mM EDTA.

The poly[r(G-C)] in 0.1 mM EDTA was heated to 80°C for 5 minutes to melt the RNA/DNA hybrid duplexes. These hybrid duplexes have a lower melting point than the RNA/RNA duplexes. Heating to 80°C should melt the hybrid strands, leaving the RNA/RNA and DNA/DNA duplexes. After cooling, the sample was adjusted to 0.1 M NaCl, 10 mM Tris, pH 8.0, 10 mM MgCl$_2$. DNase I was added (5000 Units) with alkaline phosphatase (10 Units), and that digestion continued at 37°C for 24 hours. The alkaline phosphatase was added to remove the charged phosphate groups from any CMP or GMP, to facilitate dialysis.

Following a phenol extraction to remove the protein, the sample was dialyzed against 10 mM sodium phosphate, pH 7.0 (3 changes of 4 liters). An aliquot was removed for optical studies at this stage, to allow calculation of yield.

The final yield of poly[r(G-C)] from this reaction was 400 ODU, or about 20 mg, as calculated from optical measurements. The melting temperature ($T_m$), measured in a Gilford 250 spectrophotometer with an Apple processor, in 0.1 mM EDTA, was 75°C. After allowing the poly[r(G-C)] to reanneal, the $T_m$ was remeas-
Figure 3. Kinetics of the final preparative synthesis reaction. Four of the sixty reaction volumes were monitored by acid precipitable counts; the two dots at each time point indicate the minimum and maximum yield for the four samples.
Hours

Poly [d(I-C)] added

Nmoles CTP incorporated/ml reaction

0 2 4 6 8 10 12 14 16 18 20 22 24
ured. The melting profile of the reannealed polymer was identical to the initial melt. This indicates that the poly[r(G-C)] exists as hairpins, or duplexes that exist within a single strand of polymer. Whether this is true at higher ionic strengths is not known, since the T_m is too high to measure.

Discussion

The use of an RNA polymerase preparation that contained both holoenzyme and core polymerase probably increased the initiation of transcription, since the lack of a promoter required for specific holoenzyme binding might have decreased the efficiency of transcription. However, both species will initiate efficiently at nicks. Since the poly[d(I-C)] was assumed to contain nicks, this was an advantage, since it offered more initiation sites. The poly[r(G-C)] was to be used primarily for NMR studies, so the possibility that this type of initiation would produce shorter fragments rather than long ones was not a disadvantage. In fact, the predominant size class produced in the synthesis was ideal for NMR.

The components in the final reaction mix merit some comment. Manganese seems to allow E. coli RNA polymerase to use a broader range of templates(17,18). Whether it is an effect on the polymerase itself or on the conformation of the template is not clear.

Spermidine binds to the RNA, inhibiting the binding of polymerase. Since poly[r(G-C)], with its high T_m, is a poor substrate for RNA polymerase, it is important that the spermidine be present to prevent the complex from forming.

The lack of counterions (no KCl) in the reaction may either lower the T_m of the hybrid duplexes or affect their structure. Since RNA:DNA duplexes are certainly forming,(19) the lower ionic strength may allow them to continue to function as templates for polymerase.

One concern in choosing optimum reaction conditions was the ratio of RNA
produced to DNA template added. Because the RNA:DNA duplex had to be disrupted and the DNA removed, the ratio needed to be as high as possible. In the event that removal proved difficult, the percentage of such duplexes would then be small. For this reason, in the large preparation, there was no further template addition past 16 hours. In fact, the optical melt of the purified poly[r(G-C)] showed no shoulder at 55°C, the approximate $T_m$ of the DNA duplex, indicating that removal was complete.

These conditions are in some respect peculiar to the template used and the product produced. Poly[d(I-C)] is one of the poorest templates for RNA polymerase, since it lacks a promoter, and includes a nonstandard base. It may also have an unusual conformation, as judged by its circular dichroism spectrum. The product, poly[r(G-C)], is also among the most difficult of polynucleotides to produce, with its high $T_m$ and uncertain conformation. Given the combined problems associated with this synthesis, it seems likely that other synthetic polynucleotides may allow more efficient transcription under similar conditions.

C. Double-stranded RNA Can Adopt a Z-form Conformation

After the synthesis was complete, it was necessary to repeat Jovin's experiments to observe the CD change that he had seen. In my initial experiment, the conditions were 6 M NaClO$_4$, 0.1 mM EDTA, pH 6. At room temperature, the poly[r(G-C)]-poly[r(G-C)] was A-form. Diluting with ethanol to give 20% ethanol/4.8 M NaClO$_4$ did not produce any drastic change in the CD spectrum. However, when 10 mM sodium phosphate was added, a large change in the CD spectrum was observed, which later proved to be similar to that observed by Jovin. This apparent pH dependence was not expected by a comparison with the B-to-Z transition of poly[d(G-C)], since the deoxy polymer has no such dependence.
The requirement for ethanol complicated the NMR experiments, since fully deuterated ethanol is not commercially available. The large excess of ethanol protons, resonating near the peaks of interest for the polymer, made the NMR experiments very difficult. However, like poly[d(G-C)], poly[r(G-C)]-poly[r(G-C)] has a temperature dependence. At room temperature in 6 M NaClO₄, the CD spectrum was A-form, but at 45°C, the CD spectrum changed. This property allowed NMR experiments to be done at 45°C in 6 M NaClO₄, and at 45°C in 3 M NaClO₄ where the CD spectrum was A-form, to compare the two spectra.

Poly[r(G-C)]-poly[r(G-C)] has been shown by a variety of spectroscopic methods (NMR, circular dichroism, and absorbance) to undergo a conformational change that we believe is an A-form to Z-form transition. There is a distinct change in both the circular dichroism and absorption spectra of the polymer in 6 M NaClO₄ above 35°C; the transition also occurs in other dehydrating conditions as a function of temperature. Proton (¹H) and ³¹P NMR show that in the 6 M form, at 45°C, the guanine is syn, and there are two widely separated phosphorus resonances. This is the signature of left-handed Z-form DNA.(8) In the low salt form, the guanine is anti, as found in B-form DNA and A-form RNA, and the separation between phosphorus resonances is very much reduced. From this evidence, we propose that poly[r(G-C)]-poly[r(G-C)] undergoes a conformational transition from right-handed A-form to left-handed Z-form. This is the first major conformational change reported for a double-stranded RNA.

Phosphorus NMR spectra of three forms of poly[r(G-C)]-poly[r(G-C)] are shown in Figure 4. There are two lower salt forms that are identified by ³¹P NMR; one in 10 mM NaPO₄ where the resonances are separated by 0.40 ppm, and the other in 3 M NaClO₄ at 45°C with two phosphorus resonances, separated by 0.50 ppm. The similar chemical shifts of the two peaks imply similar phosphodiester torsion angles in the GpC and CpG residues. In contrast, the spectrum of
Figure 4. Phosphorus NMR spectra of poly(dCdG)-poly(dCdG) in three ionic strengths at 45°. a) poly(dCdG)-poly(dCdG) with no added NaClO$_4$, b) poly(dCdG)-poly(dCdG) in 3 M NaClO$_4$, c) poly(dCdG)-poly(dCdG) in 6 M NaClO$_4$. Spectra were measured on the UCB 200 NMR, 81.75 MHz (200 MHz proton). Each sample contained approximately 30 mM (nucleotides) in polymer, 10 mM HEPES, pH=7.0 (N-2-Hydroxyethylpiperazine-N'2-ethanesulfonic acid), 0.1 mM EDTA. Chemical shifts were referenced to the internal reference, trimethyl phosphate.
(a) Poly \([r(G-C)]\) in 10mM HEPES, pH 7.0, 45°C

(b) Poly \([r(G-C)]\) in 3M NaClO4, 10mM HEPES, pH 7.0, 45°C

(c) Poly \([r(G-C)]\) in 6M NaClO4, 10mM HEPES, pH 7.0, 45°C
poly[r(G-C)]-poly[r(G-C)] in 6 M NaClO₄ at 45°C shows a separation of 1.34 ppm. This pattern is similar to that seen by Patel et al. (8) for poly[d(G-5mC)] and poly[d(G-C)] double strands.

The large separation of the phosphorus resonances is characteristic of Z-form-DNA, with its dinucleotide repeat unit which has greatly different phosphodiester torsion angles in CpG as compared to CpC residues. This produces a Zig-zag form with two distinct chemical and magnetic environments for the phosphorus. These data suggest that the poly[r(G-C)]-poly[r(G-C)] has adopted a Z-form; this conformation will be referred to as Z-RNA.

We have used transient proton nuclear Overhauser effect (NOE) measurements to investigate the conformation about the glycosidic bond. NOE arises from through-space dipolar interactions between spins, and can be observed as a change in intensity of one peak upon irradiating another. These spins must be spatially proximal, since the magnitude of the NOE falls off as 1/r⁶. In Z-DNA structure as determined by X-ray crystallography (3) the guanosine residues adopt a syn conformation about the glycosidic bond, and in this form, the guanosine H8 (GHB) and GH1' protons are only 2.2 A apart. The guanosine residues adopt an anti conformation in A- and B-form, however, and the GHB to GH1' distance increases to 3.75 A. Patel et al. showed that in low salt, the only NOE from an aromatic proton in poly[d(G-C)]-poly[d(G-C)] was from the CH6 to its neighboring CH5; these protons are separated by only 2.2 A. When the polymer was placed in 4 M salt, in addition to the CH6 to CH5 NOE, an NOE was observed between the GHB and GH1', indicating a transformation to syn.

The proton NMR spectrum of poly[r(G-C)]-poly[r(G-C)] in 6 M NaClO₄, 10 mM NaP0₄, pH=7 at 45°C is shown in Figure 5a. Only resonances due to the GHB and CH6 protons appear in the region between 7 and 8 ppm, and the peak at 7.82 ppm has been assigned to the GHB using deuterium exchange at high temperature. As
FIGURE 5. Proton spectra at 500 MHz of poly(dCdG)-poly(dCdG) (≈30 mM in nucleotides) at 45°C in 6 M NaClO₄, 10 mM phosphate buffer, pH 7.0, and ≈ 0.1 mM EDTA. Chemical shifts are referenced to the internal standard trimethylsilyl propionate (TSP). Spectra were measured on a JEOL 500 MHz instrument at Stanford University. a) Spectrum in D₂O. The large narrow resonances below 5 ppm correspond to residual solvent protons, EDTA, and reference TSP. b) Transient NOE difference spectrum obtained by irradiating the GHB proton (indicated by the arrow) with a 50 ms saturation pulse before obtaining the spectrum. The difference spectrum represents the spectrum with an on-resonance presaturation pulse subtracted from the spectrum with the presaturation pulse 10 kHz off resonance. A large NOE to the GH₁' is observed. The reverse experiment (not shown) results in an NOE to the GHB proton upon irradiation to the GH₁'. c) Transient NOE difference spectrum obtained by irradiating the CH₆ proton (indicated by an arrow) as described above. A large NOE can be seen to the CH₅ proton. Irradiation of the CH₅ proton results in a large E to the CH₆ (not shown).
shown in Figure 5b, the GH8 shows a large NOE to a resonance at 5.87 ppm, which is assigned to the GH1'. The presence of this NOE shows that the guanosine residues adopt the syn conformation. These NOE results, together with the phosphorus NMR data, provide the basis for设计ing this high salt form of poly[r(G-C)]-poly[r(G-C)] as 'Z-RNA'. The CH6 proton also shows a large NOE to a peak at 5.17 ppm, which has been assigned to the neighboring CH5.

The proton NMR spectrum of poly[r(G-C)]-poly[r(G-C)] in 3 M NaClO₄, 10 mM NaPO₄, pH=7, at 45°C is shown in Figure 6a. Deuterium exchange at high temperature was used to assign the resonance at 7.51 ppm to the GH8 proton. In A-form, exchange is extremely slow, so the poly[r(G-C)]-poly[r(G-C)] was heated to 80°C for 7 hours in 1 mM NaPO₄, 0.1 mM EDTA, where it is expected to be single-stranded, to effect exchange. In contrast, the Z-form showed exchange after 2 hours.

Panels 6b and 6c show the NOE difference spectra in 3 M NaClO₄ that result upon irradiation of the peaks at 7.63 and 7.51 ppm, respectively. Saturating the CH6 peak at 7.63 ppm results in a large NOE to a peak at 5.11 ppm, and this peak has been assigned to CH5. When the GH8 peak at 7.51 ppm was irradiated, only several small NOE's were observed, indicating that guanosine is in the anti conformation. It is seen from the NOE spectra that there is a significant amount of spin diffusion among the various protons. This is seen on polymer spectra, and can to some extent be diminished by the duration of the presaturation pulse. It was not entirely eliminated in these experiments, however, and appears as small NOE's in all the spectra.

The absorption and the circular dichroism (CD) spectra of the two forms of RNA (Figure 7) corroborate the NMR conclusions. The Z-RNA absorption spectrum (6 M NaClO₄, 45°C) has a shoulder at 295 nm; this is very similar to the spectrum of Z-DNA. (1) Two types of CD spectra are seen for
FIGURE 6. Proton spectra of poly(dCdG)-poly(dCdG) in 3 M NaClO₄. All other conditions are as described in the legend to Figure 5. a) Spectrum at 500 MHz in D₂O. b) Transient NOE difference spectrum obtained by irradiating the CH₆ proton (indicated by an arrow) as explained in Figure 2 legend. A large NOE to the CH₅ is observed. c) Transient NOE difference spectrum obtained by irradiating the GH₈ proton (indicated by an arrow). Only small NOEs due to spin diffusion are observed.
(a) Poly [r(G-C)] : Poly [r(G-C)]

3M NaClO4
45°C

(b) CH6

(c) GH8
FIGURE 7. Absorption (top) and CD (bottom) of two forms of poly(dCdG)·poly(dCdG). Absorption spectra were measured with a Gilford model 250 spectrophotometer; CD spectra with a JASCO J500C spectropolarimeter. Both instruments have thermoelectric cell blocks. The absorbance and CD were measured in 10 mM phosphate, pH 7, 6 M NaClO₄ at 22°C (solid line) and 45°C (dotted line). An extinction coefficient of 6560 at 260 was used for the poly(dCdG)·poly(dCdG) at 22°C (20). The CD spectrum at 22°C is similar, but not identical to, the spectrum seen in buffer alone. It is identical to that for the polymer in 3 M NaClO₄. At 45°C, 6 M NaClO₄ the CD spectrum is identical to that seen in conditions of lower perchlorate and higher temperatures, and is similar to that in 20% (v/v) ethanol in 4.8 M NaClO₄. Concentration is 1.5×10⁻⁴ M (nucleotides).
poly[r(G–C)]-poly[r(G–C)]. The first is represented by the spectrum at 6 M NaClO₄, 22°C. It is identical to the CD spectrum in 3 M NaClO₄ and is similar to a CD spectrum measured in 10 mM sodium phosphate (not shown) which matches the CD spectrum of Gray et al. in 1 mM sodium phosphate, 0.1 mM EDTA, pH 7.8. These CD spectra are all presumed to be from an A-type conformation of the polymer (24). Because the CD spectrum of the polymer in 6 M NaClO₄, 22°C and 3 M NaClO₄ are identical, we used the latter solvent at 45°C for NMR measurements; at 22°C the NMR linewidths are too broad for analysis. The CD spectra in dilute phosphate differ from those in NaClO₄ by a small change in the band at 285 nm. This band is particularly sensitive to solvent, and diminishes at high ionic strength. This CD band may be correlated to the small differences in phosphorus resonances between the RNA in 10 mM HEPES and in 3 M NaClO₄.

The CD spectrum in 6 M NaClO₄, 45°C is very different from the others, and is identified as that of Z-RNA. The CD remains positive in the region above 215 nm. The negative bands above 280 nm and below 240 nm in A-RNA change sign in Z-RNA; the positive band near 260 remains positive.

The transition temperature is a function of the solvent conditions. In 6 M NaClO₄, the transition temperature is approximately 35°C; in 4.8 M, it is about 45°C; in 4 M, about 80°C; in 3 M, it is above 80°C and unmeasurable in our instrument. The addition of ethanol to the perchlorate solution decreases the transition temperature, so that in 4.8 M NaClO₄/20% ethanol, it is Z-RNA-form at room temperature. This temperature-dependence of the transition to Z-RNA-form is analogous to that of poly[d(G–C)]-poly[d(G–C)]. For that polynucleotide, Z-form is favored at higher temperatures in ethanol/water solutions.(8,21,22) However, we have found that solvents which cause a B to Z transition in poly[d(G–C)], such as 4 M NaCl or 1 M MgCl₂, do not cause an A to Z transition in poly[r(G–C)]-poly[r(G–C)]. This is consistent with the work of Westerink et al.
(23) and Uesugi et al. (25) who found no Z transition in these salt solutions for r(C-G) oligonucleotides.

The kinetics of the transition also vary with the solvent and the temperature. In 6 M NaClO₄, a temperature shift from 21°C to 35°C results in an A to Z-RNA transition that takes about an hour. The reverse transition takes place over 5 hours. A temperature shift from 23°C to 45°C, (or addition of 20% ethanol (v/v) and concomitant dilution to 4.8 M NaClO₄), however, produces the Z-RNA form in about 10 minutes, although again the reverse transition takes several hours.

The possibility that the transition could result from a melting of the poly[r(G-C)]-poly[r(G-C)] was investigated using absorbance where melting would show a hyperchromicity. Solutions of poly[r(G-C)]-poly[r(G-C)] were melted at both CD concentrations and NMR concentrations. The results indicate that there is in fact a hypochromicity in the transition from A to Z-RNA form, measuring absorbance at 260 nm. Poly[d(G-C)]-poly[d(G-C)] also shows a hypochromicity in the B to Z transition. (1)

This is the first major conformational change reported for a double-stranded RNA; it shows that RNA has conformational flexibility, contrary to current belief. The solution conditions for obtaining Z-form seem extreme; further study should find more physiological conditions. This was in fact the case for DNA Z-form. Other sequences of double-stranded RNA will no doubt also be shown to have conformational flexibility, prompting many questions about the function of conformational diversity in vivo.

D. Do RNA and DNA adopt the same Z-form?

The question of whether the RNA Z-form and the DNA Z-form are the same structure is an interesting one, since the RNA begins from an A-form which is radically different than the DNA B-form. Was the final state the same, given the
different starting states?

To address this question, we compared the chemical shifts of the two polymers in the Z-form. These results are shown in Table III. The chemical shift of a proton is dependent on its environment, both the solvent structure around it and the chemical composition of the molecule it is a part of. The chemical shifts of the GH8, CH5, and CH6 protons on the two polymers in the Z-form are very similar, indicating that the two structures are also very similar. The different values for the CH1' and GH1' are to be expected, since the sugar rings are very different.

Beyond the comparison of the experimental values of chemical shifts, there are ways to calculate what the expected values should be. Mitra et al. (26) and Mitra et al. (27) have calculated the theoretical $\Delta \delta$, or the change in chemical shift in going from the monomer to the dimer for the two Z forms (high and low salt crystal forms), A-form, and B-form, among others. The major contribution to the $\Delta \delta$ comes from ring current shifts arising from the local magnetic field of the neighboring bases.

Briefly, because the bases are planar and have electron clouds ($\pi$ bonds) diffused around the plane, the base will act approximately as a current loop in the presence of an applied magnetic field. The induced current will in turn produce a magnetic field, just as with an electron circulating about a wire. The induced magnetic field will then either shield or deshield a magnetic nucleus in its vicinity from the applied magnetic field, relative to its orientation. In the monomer, there is no field other than the applied field, so that the chemical shifts of the nuclei (protons) on the base are due to solvent, monomer structure (sugar pucker, syn or anti sugar) and base composition. In the polymer, effects from ring currents (as well as other effects such as the magnetic anisotropy of the phosphorus) add to these contributions, so that the chemical shifts change, giving rise to a $\Delta \delta$. These calculations are not trivial, as Mitra et al. and
Table III

<table>
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<th>PROTON</th>
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<td>GH1'</td>
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<td>CH1'</td>
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Giessner-Prettre et al. (28) show. The geometry of the polymer, as in the distance between base pairs, the base pair overlap, the angle of the phosphorus backbone, and the orientation of the sugar must be known to perform an accurate calculation. Even so, there are ad hoc terms applied, especially in measuring the contribution from the phosphate backbone to the base protons. While we were not prepared to apply the entire treatment to Z-RNA, and in fact would be unable to do so, given the lack of X-ray data on which the calculations are based, we did want to know what a simple ring current calculation could tell us about Z-RNA structure, and for this we used a graphical approach.

The ring current contours for the DNA bases have been described by Giessner-Prettre and Pullman (29), assuming a distance of 3.4A between the base pairs. The contours are illustrated in their paper, and were used for our calculation with no changes. For a model of Z-form, we used Wang's low salt Z-form illustrated in the 1979 paper. The idea was to calculate the Δδ's for one C·G base pair sandwiched between two others (nearest neighbor contributions only.) The figure from Wang's paper was enlarged by Xerox until the bases in the illustration were the same size as those in Pullman's paper. Then the bases with the ring current contours were placed over the bases from the X-ray structure to create a base pair in the correct orientation. This base pair with contours was then used as the "bread" for the "sandwich". To avoid end effects (since the crystal was a hexamer) the "bread slices" were base pairs <3 10> and <5 8>, and the Δδ's were calculated for base pair <4 9>. This is illustrated in Figure B. The position of the CHδ, CHδ, and GHδ protons from <4 9> were noted on the base pairs with contour lines <3 10> and <5 8> separately, using the helical structure from X-ray studies. The angle between the base pairs in the Z-form is designated as 0°. This angle was changed by small increments to increase the calculated Δδ when we found that the values at 0° were too small relative to the experimental values. This is also illustrated in Figure B, and the results from the calculations for the three
protons are given in Table IV.

The purpose of changing the angle was to determine how much the geometry needed to be changed to produce calculated ∆δ's that matched the experimental values. The first thing to notice in the calculations is that the values for the ∆δ of the GH8 proton are not even close to the measured values, indicating that either the conformation used is completely wrong, or that ring current contributions do not appreciably affect that proton. To decide which of those explanations is the case, it is necessary to look at the other data. If, when comparing calculated to experimental values, a difference of ±0.15 ppm is considered acceptable (28), then clearly there are a number of winding angles that will give a good fit. For each proton, CH5 and CH6, those values were considered that fell within this criterion. Then the intersection of those sets was selected as meeting the acceptable error for both protons. In that smaller set, there was only one combination of winding angles that produced a best fit to the experimental values: <3 10> < <4 9> = 10° and <4 9> < <5 8> = 2.5°.

The fact that one combination gives a best fit for the two cytosine protons points out the sensitivity of the ∆δ's to ring current shifts and ultimately to base stacking. Because there is such a correspondence and yet the GH8 proton remains outside ring current effects suggests that another parameter exerts more influence on the GH8 magnetic environment. In fact, Mitra et al. add a factor due to the magnetic anisotropy of the phosphate backbone to "calculate" the chemical shift of the GH8 proton. Because the diamagnetic and paramagnetic anisotropies of the phosphate group are not at all well understood, this factor is more or less a fudge factor, and its use is empirical.

There are really two points to be learned from this exercise. The first concerns the incredible sensitivity of the protons to the positions of the bases above and below them. As can be seen in Table IV, very small changes in the winding
Figure 8. Graphical method of calculating chemical shifts due to ring current contributions. The base pairs pictured with the contour lines around them are in the orientation of Wang’s Z-form; the positions of the protons of the base pair above or beneath the base pair with contour lines are shown as dots, and were determined from the positions shown for the base pairs <3 10>, <4 9>, and <5 8> in Wang’s paper (see text). The base pair <4 9> was rotated by the angle indicated in the figure to increase the calculated chemical shift of the protons. The helix axis was fixed with respect to the bases, and acted as the rotation axis for the base pair. The position of 0 angle was the original orientation given by the Z crystal structure, and the rotation angles are relative to this angle (positive in one case, negative in the other, resulting in a tightening of the helix or an increase in the base stacking.)
Table IV

Ring Current Shifts of CH5 Proton

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Ring Current Shifts of CH6 Proton

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Δδ (δ_{duplex} - δ_{monomer})

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<th>Experimental (A)</th>
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*Best Fit
angle can produce large changes in the $\Delta \delta$. The second point is that the graphical method of calculation is clearly not refined enough for quantitative use. To tighten the helix by the degrees indicated to achieve a best fit would mean that the Z-RNA structure was not the same as Z-DNA structure, and in fact there is no reason to suppose that it should be. However, this calculation was very simplistic when it is compared to the treatment of Mitra et al., where next- and next-next-nearest neighbor base pairs are included, as well as other factors already mentioned. In the detailed breakdown of the contribution to the calculated $\Delta \delta$ from ring current, anisotropy factors, etc, it is reassuring to note that the values we calculated for the Z-form match closely the nearest-neighbor contributions of Sarma, indicating that graphical methods and computational methods give very similar values. However, whereas we had to twist the helix to match experimental with calculated values, Sarma's additional factors allow the match of all protons to the Z-form with no such helical deformations. They then proceed to do the same calculations for other DNA structures, and were able to fit experimental data remarkably well, including demonstrating that there is a significant difference between the low salt Z-form and the high salt Z-form (ZI and ZII, respectively) predicted by their method.

This last observation brings up an interesting point. Because the chemical shifts are so sensitive to conformation (environment) of the helix, the nearly identical values of the chemical shifts of the CH8, CH5 and CH6 protons of Z-DNA and Z-RNA indicate to me that these structures are extremely similar if not identical. However, Prof. Alex Rich's lab has produced a crystal of d(CGC)r(GCG) which X-ray crystallography shows adopts the ZII (high salt) form. This leads Rich to believe (personal communication) that Z-RNA has ZII structure, since DNA under these conditions is ZI. The NMR data does not support this conclusion. The calculated $\Delta \delta$'s for ZII (Sarma) are very different from those of ZI, and do not match our data. This may be a case where the solution structure is not identical.
to the crystal structure.

**E. Solvent Effects on the A-to-Z Transition**

Using NMR criteria, it appears that Z-RNA has the same structure as Z-DNA. Z-RNA cannot be induced by the solvents that induce Z-DNA, however. Table V gives a summary of the salts and solvents used to try to induce the Z-RNA conformation, indicating which successfully induce Z-DNA. The combination of perchlorate and ethanol does not produce the same CD spectrum of the Z-form as does the perchlorate/methanol combination; the difference is in the relative peak heights of the first two CD bands. As mentioned in Chapter 3, the Z-form CD spectrum of the tetramer, rCGCG more closely resembles that of the polymer spectrum in perchlorate/methanol. Clearly the conditions for producing Z-RNA are more stringent and restricted than for Z-DNA. Also, the RNA has a pH dependence, unlike DNA. This has been described earlier. Another example of this occurs with 500 μM spermidine, where at lower pH (approx 6) the RNA forms fluffy, nearly transparent precipitates that are easily broken up by shaking or by addition of sodium phosphate to 10 mM, pH 6.8.

**Methods**

Because the pH was important, all solutions were brought up to near pH 7, either by addition of 1 M sodium phosphate, pH 6.8 to give 10 mM sodium phosphate, or by addition of HCl or NaOH. For CsCl and LiCl, addition of the phosphate caused immediate precipitation of the salts. CsF at 8 M had a pH=12, which was never satisfactorily explained, since calculations with the pKₐ of HF indicated that the pH should have been nearer 9. Using concentrated HCl, a 4 M CsF solution was brought to pH 7; a more concentrated CsF solution required so much HCl that it was significantly diluted. At pH 7, however, the amount of HF generated is considerable, and the solution irreparably etched the quartz cuvettes containing
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<th>Conformation</th>
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<th>DNA</th>
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Table V continued

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<td>A</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>7</td>
<td>25°C</td>
<td>A</td>
<td></td>
<td>C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80°C</td>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>4</td>
<td>25°C</td>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>25°C</td>
<td>A</td>
<td></td>
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<td></td>
<td>80°C</td>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>25°C</td>
<td>A</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>7</td>
<td>25°C</td>
<td>A</td>
<td></td>
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<td></td>
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<td>A³</td>
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<td></td>
<td></td>
<td>37°C</td>
<td>A</td>
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<td>A⁴</td>
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<td>7</td>
<td>25°C</td>
<td>A</td>
<td></td>
<td>Z</td>
</tr>
<tr>
<td>Sat'd. CsCl / 5 mM MgCl₂</td>
<td>7</td>
<td>25°C</td>
<td>A⁴</td>
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</tr>
</tbody>
</table>

¹The RNA formed an aggregate that did not precipitate, but that scattered differentially enormous amounts of light. The sample was used in CIDS experiments.

²The CD spectrum resembled the CD spectrum of DNA in the Z-form. More experiments are being done to determine the structure of this form.

³More than 0.5 mM MnCl₂ caused the RNA to precipitate.

⁴More than 5 mM MgCl₂ caused the RNA to precipitate.
Table V, continued

<table>
<thead>
<tr>
<th>Solvent</th>
<th>pH</th>
<th>Temperature</th>
<th>Conformation RNA</th>
<th>Conformation DNA</th>
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<tr>
<td>4 M CsF</td>
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<td>A</td>
<td>Z&lt;sup&gt;5&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td>80°C</td>
<td></td>
<td></td>
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<td>32 mM spermidine</td>
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<td>25°C</td>
<td>A</td>
<td>1 mM aggregates&lt;sup&gt;6&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td>80°C</td>
<td>A</td>
<td>B-form GC</td>
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<tr>
<td>Co(NH&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;6&lt;/sub&gt;Cl&lt;sub&gt;3&lt;/sub&gt;</td>
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<td>A</td>
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<td></td>
<td></td>
<td>60°C</td>
<td>A</td>
<td></td>
</tr>
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<td></td>
<td>500 M</td>
<td>6</td>
<td>25°-50°C</td>
<td>forms flocculent ppt&lt;sup&gt;7&lt;/sup&gt;</td>
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<td>7</td>
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<tr>
<td></td>
<td></td>
<td>50°C</td>
<td>A</td>
<td></td>
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</table>

<sup>5</sup>Poly[d(G-C)] B-to-Z transition takes 200 hours. This experiment was terminated after 2 days, due to the destruction of the cuvettes by the HF generated in the neutralization of the CsF.

<sup>6</sup>50. μM causes poly[d(5-Me-C-G)] to adopt a Z conformation

<sup>7</sup>The precipitate is redissolved by addition of sodium phosphate to 10 mM, pH 6.8, or the clumps can be broken up by shaking.

Most samples contained 10 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7, except those that had to be brought to pH 7 by addition of acid or base, due to precipitation of the salt by the phosphate.
the sample in 24 hours, signaling the end of the experiment. (The high pH of CsF makes it impossible to use with RNA and DNA as hydrolysis will occur. There are reports in the literature where CsF has been used to effect the B-to-Z transition (30) and for comparing its effects on DNA with those of other salts. No mention was made of buffering it, which raises doubts about the validity of the observation.)

MgCl₂ at 4 M has a pH of 4. Addition of NaOH produces a precipitate, which presumably is Mg(OH)₂ which is very insoluble. Addition of sodium phosphate had no effect on the pH.

The Co(NH₃)₆Cl₃ was made by Dr. P. Cruz using the method described in (31). The absorption spectrum was checked to ensure its identity, but the more significant affirmation was its ability to facilitate the B-to-Z transition, which it did in about 36 µM. The stock solution of 5 M did not totally dissolve although the solubility limit was not exceeded, so dilutions were made from a 50 mM solution that was completely dissolved.

Because the kinetics of the transition were not known, the solutions were kept for some time (from 2 weeks to 2 months) and occasionally remeasured. (The sample of poly[r(G-C)]-poly[r(G-C)] in 5 M NaCl, after heating to 80°C and then cooling, retained an A-form CD spectrum. When it was remeasured 2 months later, having been kept at room temperature in an Eppendorf tube, the CD spectrum showed enormous scattering contributions as far out into the visible as 500 nm. When the scattering corrections to the CD were made using the Fluorscat cell, the remaining CD signal was still greatly disproportionate to the absorbance of the sample, an indication that the RNA was in a ψ-form (37). The sample was given to Dr. Skip Shimer for CIDS measurements.)

Why Perchlorate?
The list of solvents/salts that facilitate the DNA B-to-Z transition is lengthy, and it is difficult to find a common denominator among them. Hypotheses such as specific ion effects and lowering water activity have been raised, but neither effect is uniform among the many solvents. With RNA, there is one salt, either alone or in combination with temperature or alcohol, which facilitates the A-to-Z transition. This raises the question of what property of sodium perchlorate allows the RNA transition?

One possible explanation may be found in the effect of perchlorate on the melting temperature of DNA and RNA. Figure 9 shows the melting curves of calf thymus DNA in 1 and 6 M NaClO₄, where the Tm of calf thymus DNA is lowered by 36°C when the salt concentration is increased. The same response was seen for poly[r(A-U)], which exhibited a decrease in melting temperature (but no change in the CD spectrum) with increased perchlorate concentration (data not shown). Presumably poly[r(G-C)] would respond similarly, even though the Tm's are too high to measure. Analogous experiments with 1 and 5 M NaCl showed no such decrease (although the Tm's for the polymers were nearly identical in the two NaCl concentrations) indicating that the effect is mediated by the anion.

In fact, this lowering of the melting temperature of DNA was observed in 1962 by Hamaguchi and Geidushek(32). They identified a number of solvents that exerted this effect on DNA, calling them "chaotropic" agents (chaotropic meaning tending to disorder). In order of effectiveness, the series is CCl₃COO⁻→CN⁻>CF₃COO⁻>ClO₄⁻>CH₃COO⁻>Br⁻,Cl⁻,CHO₂ where all species are measured at 4 M, pH 7. The explanation advanced for this phenomenon was that the effect was mediated through modification of water structure, although they noted that water activity could not be correlated with the chaotropic ability of the anion. The mechanism was not postulated, but thought to be related to the inability of the anion to salt out nonelectrolytes. This seemed quite unsatisfac-
Figure 9. The melting curves of calf thymus DNA in 1 and 6 M NaClO₄, the solvents used for the A- and Z-form, respectively, of poly(r(G-C)). The transition is broader in the 6 M NaClO₄ for both RNA and DNA. Melting was measured in a Gilford 250 spectrophotometer at 260 nm, using a 1 cm pathlength. Concentration of calf thymus DNA was $1.5 \times 10^{-4}$ M.
tory as an explanation.

Robinson and Grant (33) investigated the chaotropic solvents with respect to their ability to salt in the nucleic acid bases. They found that the strongly chaotropic anions were also those that increased the solubility (or the molar activity coefficient, \( S_i/S^0 \), where \( S_i \) is the solubility in either salt or water) of the free bases. The hydration of the bases was not increased, however, suggesting that the increased solubility was due to specific base:anion interactions. If the free base is assumed to behave like a base in a random coil, then the effect of the anion would be to stabilize the structure of the random coil over the helix. In thermodynamic terms, \( \Delta G_{\text{helix-coil}} \) would be expected to be lower in chaotropic solvents.

This last conclusion was suggested by Tunis and Hearst (34) to explain the results of KCF\(_3\)COO (KTFA) on nucleic acid structure and hydration. Their experiments on hydration of double- and single-stranded DNA showed that the double strand is highly hydrated in KTFA, but not in ClO\(_4^−\), with lower hydration of the bases in the denatured form. Hearst (35) showed that there was no correlation between helix stability and net hydration, but that there could be a correlation between helix stability and hydration in the single strands.

If this effect of lowering the \( \Delta G_{\text{h-co}} \) is true for all chaotropic solvents, then perhaps it is the mechanism that allows perchlorate to facilitate the A-to-Z transition. A-form is a very stable state, and in fact it has been thought to be the only state of RNA. If this conformation can be destabilized, enough to allow flexibility without denaturation, then the conformation is free to change, as it does in poly[r(G-C)] from A to Z. This hypothesis is easily testable, simply by determining whether any of the other chaotropic solvents can facilitate the transition.

These experiments are being done. The concentration of anion needed for the transition will probably differ from salt to salt. In 4 M CCl\(_3\)COO, the T\(_m\) of DNA
that is 37% CG is lowered to less than 25°C, suggesting that in 6 M (if CCl₃COO is that soluble) the strands may fall apart at 10°C. This may mean that CCl₃COO, for example, may be effective in promoting the A-to-Z transition in millimolar amounts. Obviously, the thing to do is to try the experiments, using both different anion concentrations and different temperatures. If these solvents are effective, then a mechanism should be developed.

The construction of a phase diagram for the transition might be possible with one of the chaotropic solvents. Perchlorate does not decrease the melting temperature of poly[r(G-C)] enough to see the A→SS or Z→SS transition, but as indicated previously, perchlorate is not the most effective of the chaotropic solvents. Construction of a phase diagram would map the condition for transition precisely. An example of such a phase diagram is shown in Chapter 4 for the tetramer rCGCG. It also provides thermodynamic parameters, such as the ΔHₜₐₖ as a function of salt, where the values for A-to-coil can be compared to those for Z-to-coil. (I expect the ΔHₐ₋ₚₚ to be higher than ΔHₜ₋ₜₚ). The ΔHₐ₋ₚ can then be calculated. Presumably an analogous diagram could be constructed using poly[d(G-C)], so that the conditions for transitions and the enthalpies of transition can be compared for deoxy and ribo.

A-form variations with solvent

While the other solvents tried do not produce Z-RNA, they do have some effect on the A-RNA CD spectrum. These results are summarized in Table VI and two examples are shown in Figure 10. The most prominent feature in the CD spectrum that is sensitive to salt is the band at 285 nm. This band is present in 10 mM sodium phosphate, pH 6.8, and there has a magnitude nearly equal to the main band at 293 nm. In some salts, however, it can virtually disappear (LiCl, MgCl₂) whereas in CsCl, it increases in magnitude.
Table VI

Variations in A-form CD Spectra with Solvent

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$\Delta \varepsilon_{295}/\Delta \varepsilon_{283}$</th>
<th>$\Delta \varepsilon_{265}/\Delta \varepsilon_{295}$</th>
</tr>
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<tbody>
<tr>
<td>10 mM NaH$_{3/2}$PO$_4$</td>
<td>1.27</td>
<td>1.95</td>
</tr>
<tr>
<td>4.8 M NaClO$_4$</td>
<td>1.78</td>
<td>2.44</td>
</tr>
<tr>
<td>Sat'd CsCl</td>
<td>0.92</td>
<td>1.79</td>
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<tr>
<td>9 M LiCl</td>
<td>1.43</td>
<td>2.1</td>
</tr>
<tr>
<td>0.9 M MgCl$_2$</td>
<td>1.64</td>
<td>1.87</td>
</tr>
<tr>
<td>500 μM Co(NH$_3$)$_6$</td>
<td>4.5</td>
<td>2.22</td>
</tr>
<tr>
<td>32 mM spermidine</td>
<td>1.75</td>
<td>2.64</td>
</tr>
</tbody>
</table>
Figure 10. While other solvents do not produce Z-RNA, they do alter the shape of the A-form CD spectrum. This alteration is probably an effect of the interaction of one of the bases with a particular solvent ion. CD spectra of several representative A-form structures: a) poly[r(G-C)] in 7 M LiCl, pH 7. b) in saturated CsCl.
The movement of this 285 band can arise either from structural changes in the polynucleotide, or from interactions of the base in the polymer with solvent. I am inclined to favor the latter explanation. With only two bases (G and C) to consider, it should be possible to discover which base is identified with that transition and so decide where the solvent is interacting. The N7 position on guanine is quite reactive, and may possibly be interacting with solvent, but it would be interesting to really identify the base. Perhaps NMR of A-forms in the solvents of interest could help in detecting which base is interacting, on the basis of changes in the chemical shifts of the GH8, CH6 or imino resonances as a function of salt.

\[ \text{MgCl}_2 \]

Quite by accident, we discovered that there is a third form of poly[r(G-C)] that is present in 4 M MgCl2. As mentioned earlier, 4 M MgCl2 has a low pH that is resistant to change. Those pH measurements were made using pH paper, where the indicator showed pH 4. Later pH measurements by Ms. Edie Leonhardt, using the pH meter, showed a pH of 6. In any case, addition of NaOH causes precipitation of, presumably, Mg(OH)2, so experiments were done without buffering. In attempting to reproduce a peculiar result I had obtained with poly[r(G-C)] in 3.6 M MgCl2, Ms. Leonhardt found that there is a third form of poly[r(G-C)], present in 4 M MgCl2, the CD of which looks very similar to that of Z-DNA. This form is stable with time, and is a function of MgCl2 concentration and temperature. The CD and absorbance of the Mg-RNA is shown in Figure 11. Experiments are continuing to investigate the kinetics of the transition as a function of salt concentration and temperature. To determine whether the form is Z or an alternative form of A, the NMR must be done, since CD is not able to unambiguously determine structure.
Figure 11. The absorption (top) and CD (lower) spectra of Mg-RNA. MgCl₂ is 4 M, pH≈4. RNA is 1.5 X 10⁻⁴ M. Spectra are taken at room temperature, when the sample was several weeks old. While the Mg-RNA CD spectrum resembles the CD spectrum of the DNA Z-form, the magnitude of the RNA signal is reduced from that of the DNA, in contrast to the more usual case, where the molar ellipticity of the RNA exceeds that of the DNA.
Poly[r(G-C)]
4M MgCl₂
23°C

Poly[r(G-C)]
4M MgCl₂
25°C
The appearance of an RNA CD spectrum that is virtually identical to a DNA CD spectrum is exciting, since it poses many questions about the calculation of CD and its relation to structure. If in fact the Mg-RNA is A-form, it may give some support to Williams and Moore's calculations (36) which produced a Z-DNA CD spectrum arising from a right-handed structure. One interesting feature of the RNA spectrum is the magnitude of the molar extinction relative to that of the DNA. Usually, RNA has a greater extinction; here that is not true.

F. Vacuum UV CD

Near uv CD cannot be used for structural determination, but, as discussed previously, it is possible that vacuum uv CD (160-200 nm) may eventually be used to distinguish helical handedness. The CD bands in the VUV are 10-12 times larger than those in the near UV, and seem to be much less sensitive to solvent effects. Johnson and Baase have measured the VUV CD spectrum of B and Z poly[d(G-C)], and those results are shown in Figure 12. While the origin of those bands is not clear, their response to change in helical handedness is dramatic.

The near UV CD spectra of poly[d(G-C)] and poly[r(G-C)] are very different, both in the right- and left-handed forms. If the VUV CD is in fact an indicator of handedness (whatever the origin of the transitions) then it might be expected that the RNA spectra should resemble the DNA spectra, both for right- and left-handed forms. Fortunately, the solvent for RNA, sodium perchlorate, is the perfect solvent for VUV work, as it is transparent in the region of interest. The experiments were done in Prof. Curt Johnson's lab in Oregon, using an instrument designed and built specifically for VUV work.

The results of the experiments on poly[r(G-C)] are shown in Figure 13. The positions of the bands in A-RNA are red-shifted from those in B-DNA, but otherwise the spectra are similar. In Z-RNA, the bands reverse sign from those in A-
Figure 12. The vacuum ultra violet (VUV) CD spectra of poly[d(G-C)] measured by Prof. Curt Johnson and Dr. Walt Baase on the VUV CD instrument in Prof. Johnson's lab in Oregon. Pathlength was 10 microns. Concentration was $1 \times 10^{-3}$. 
poly d(G-C)·poly d(G-C) 22°C

- B-form, 10mM Phosphate
- A-form, 0.67mM Phosphate
- 80% hexafluoroisopropanol
- Z-form, 10mM Phosphate
- 2M NaClO₄
Figure 13. VUV CD spectra of poly[r(G-C)], again measured in Prof. Johnson's laboratory. Note that the sign and positions of the bands in the vuv are almost identical to those of the Z-DNA bands. Also to note is that the bands of the A-DNA are nearly identical to those of the A-RNA. The relatively small molar extinction of the Z-form may be due to the presence of a small amount of A-form in the solution; this is also suggested by the small negative band near 300 nm. The mixture of states would also result in the broadening of the CD band centered around 190 nm.
poly r(G-C)·poly r(G-C)

---

A-form 22°C
Z-form 46°C

6M NaClO₄
10mM Phosphate
0.1mM EDTA

\[\lambda \text{ (nm)}\]
RNA, and are now slightly red-shifted from those in Z-DNA. This provides further evidence that Z-RNA is left-handed. It also supports the idea that those bands in the VUV are sensitive to helix structure rather than base structure, as is the case for the near-uv CD bands. If there were more empirical evidence, or alternatively, a theoretical explanation for the origin and behavior of the bands, VUV CD could be a most useful way of determining helical handedness.

Baase and Johnson also measured the VUV spectrum of the A-form of poly[d(G-C)] produced by high concentrations of ethanol. Their data show that in the VUV region, the CD of the A-DNA matches almost exactly that of the A-RNA. This DNA A-form is very different from the DNA Z' described in Chapter 2. A comparison of the positions of the CD bands of the two forms shows that there are significant differences: the A-form CD spectrum has a negative band around 295 nm, with a larger negative band around 210 nm; the Z'-form CD spectrum has no negative band above 250 nm, and the bands around 210 nm do not seem to differ from those of the Z- or B-form spectra. From this comparison, it appears that the identification of the high ethanol form as a Z' form is substantiated; it does not match the (right-handed) A-form spectrum. It is always possible that the differences in the CD bands are due to solvent interactions, since alcohols do red-shift absorbance bands of nucleic acids. However, the qualitative differences in the two spectra are such that this explanation is not a likely one. To settle the question of handedness, or at least to answer the question of whether the DNA in ethanol has the same VUV CD spectrum as the DNA in isofluoropropanol, the CD experiment should be repeated using the ethanol sample.
References


Chapter 3. A and Z forms of the tetramer rCGCG

We have presented evidence that the polymer of poly[r(G-C)] can undergo an A-to-Z transition. For this phenomenon to have any biological utility, the transition must occur in short lengths of C-G sequences, since long stretches of alternating G-C are not found. Therefore, it was necessary to find the shortest length of alternating G-C that would adopt a Z-form. The deoxy tetramer dCGCG assumes a Z-conformation (1-3) whereas the hexamer dGCGCGC does not (4). For this reason, we investigated the ribo tetramer rCGCG, which was a gift from Prof. Thomas Neilson.

Uesugi et al. (5) have shown that the ribo tetramer with 8-bromo- guanosine adopts a Z-like structure in low salt solutions. In that paper, they also noted that the unsubstituted tetramer was unable to adopt a Z-form. Westerink et al. (6) reached the same conclusion, using a different solvent system. As I will show here, however, this apparent inability to form a Z conformation is due to the choice of solvents, not to an inherent property of the tetramer. As shown in Chapter 2, there are many solvents that facilitate the B-to-Z transition, but very few that work for RNA. This specific solvent requirement holds for both the polymer and the tetramer.

The data presented here describe the spectroscopic characteristics of the A- and Z-forms of the tetramer. A phase diagram for the transitions from duplex to single strands is constructed as a function of salt concentration and temperature. Finally, the ΔH for the A-to-Z transition is calculated.

A. The A and Z forms

We received 20 ODU of rCGCG from Prof. Neilson. One batch of 10 ODU was dissolved in 0.5 ml of 1 M NaClO₄ with 1 mM EDTA, pH 7; the other 10 ODU was dis-
solved in 0.5 ml of 6 M NaClO₄, 1 mM EDTA, pH 7. The two salt concentrations were chosen from previous experiments with the polymer, where at < 3 M NaClO₄, it was A-form and where at 6 M NaClO₄, it was Z-form either at room temperature or at 35°C. This scheme also provided an equal molar concentration of tetramer in the two salt concentrations, so that when an intermediate salt concentration was needed, it could be achieved without dilution of the tetramer. These stock solutions were kept at 4°C. The concentration of the tetramer was calculated using the extinction coefficient of Uesugi et al. (7) who calculated it to be $8.7 \times 10^3$ at 260 nm in 0.1 M NaCl, 10 mM sodium phosphate. Using this value is probably valid for the 1 M perchlorate solutions, but is in error for 6 M perchlorate solutions, where the absorption of the tetramer is decreased. Nevertheless, it was used for all solutions.

To determine the conformation of the tetramer in the two perchlorate concentrations, absorbance and circular dichroism were used. The absorbance was measured in the Gilford 250 spectrophotometer where the temperature could be controlled. The melting point of the tetramer was expected to be low, so the absorbance spectra were measured at 0°C to get the maximum amount of single strands. Figure 1 shows the absorption spectra of the tetramer in 1 and 6 M NaClO₄, at 0°C (duplex) and 50°C (single strands). The spectra of the duplex forms are not identical; the tetramer in 6 M NaClO₄ has a large shoulder on the main absorption band. This shoulder, centered near 262 nm, is characteristic of the Z-form absorption spectra of both ribo and deoxy polymers. The effect of the shoulder is to change the ratio of $A_{295}/A_{260}$ in the A and Z forms, so that the absorption at 295 nm can be used as an indication of the presence of Z-form (8). For the tetramer, this ratio is 4.4 in 1 M NaClO₄ and 3.4 in 6 M NaClO₄.

The appearance of this shoulder in the absorbance spectrum indicates that there is a qualitative change in the transition moments of one or both of the
Figure 1. The absorbance spectra of the tetramer rCGCG in 1 and 6 M NaClO₄. The measurements were made on the Gilford 250 spectrophotometer, at 0°C, in a .2 mm pathlength cell. Concentration of the tetramer in 1 M NaClO₄ is $2.1 \times 10^{-3}$ M (NT); in 6 M NaClO₄, $1.86 \times 10^{-3}$ M, calculated using the extinction coefficient of Uesugi et al..
bases. Either a conformational change of the duplex is allowing for more or less base overlap that affects the coupling of the transition moments of the bases, or there is a new base/solvent interaction that produces a new absorbance band. A comparison of the absorption spectra of the single-stranded tetramer in the two perchlorate concentrations shows that while both spectra have maxima at 254 nm, the second maximum is blue-shifted by 3 nm in the higher salt concentration. This shift suggests either a different base/solvent interaction, or a different conformation of the single-stranded tetramer in the two salt concentrations. It is not possible to distinguish between these two cases by absorption.

While the absorption data were suggestive of a conformational change, they were not conclusive. The circular dichroism spectra of the samples are shown in Figure 2, again keeping the solution at 0°C. The CD spectrum of the tetramer in 1 M NaClO₄ is almost identical to that of poly[r(G-C)] in A conformation. The trough is blue-shifted 2 nm and the positive band is red-shifted 3 nm in the tetramer spectrum relative to the polymer spectrum; the ratio of $\Delta\varepsilon_{266}/\Delta\varepsilon_{292} = 1.21$ for the tetramer where $\Delta\varepsilon_{263}/\Delta\varepsilon_{294} = 1.85$ for the polymer. The tetramer in 1 M NaClO₄ clearly adopts an A-form duplex. The tetramer CD spectrum in 6 M NaClO₄ is not identical to the Z-form CD spectrum of the polymer in perchlorate, the differences being in the peak positions and the relative peak heights. Where the polymer has 3 bands at 283 nm, 260 nm, and 221 nm, with the 260 band having the greatest ellipticity, the tetramer has bands at 286 nm, 260 nm, and 221 nm, with the largest band at 286 nm. The 6 M perchlorate CD spectrum of the tetramer more closely resembles the CD spectrum of the polymer in 4.8 M NaClO₄/20% ethanol. These CD bands may be reflecting structural differences or again base/solvent interactions. Alternatively, the bands in the tetramer may contain contributions from end effects which are not seen in the polymer. (Perhaps it is an indication that there is a family of RNA Z-forms, analogous to the Z1 and Z11 forms of DNA.) Nevertheless, the tetramer appears to be adopting a Z-form in 6 M
Figure 2. Circular dichroism spectra of the tetramer in 1 and 6 M NaClO₄ at 0°C. Spectra were taken with a Jasco J500C, using a thermoelectric cell block taken from a Zeiss spectrophotometer. The pathlength was .2 mm, and the microcell was positioned in the thermoelectric block with a spacer. The baseline, 1 or 6 M NaClO₄ in the same cell with the same pathlength (i.e. with the spacer in the microcell) was subtracted from the spectrum of the sample. All spectra are the average of two spectra, since the signal/noise of the measurements was not good. The spectra were measured at the 2 millidegree/cm scale, then expanded for plotting by the data processor.
As in the polymer, the negative CD band at 295 nm in the A-form spectrum becomes positive and blue-shifted in the Z-form spectrum for the tetramer. This change in sign of the band is certainly due to structural changes of the molecule, not simply to solvent effects. This CD evidence supports the idea that the appearance of a band at 262 nm in the absorbance spectrum arises from conformational changes in the molecule. If this is assumed to be the case, then the question arose of whether the band, or rather the absorption at 295 nm, could be used to quantify the amount of Z-form present.

The tetramer adopted an A-form in 1 M NaClO₄, 0°C, and Z-form in 6 M NaClO₄, 0°C. Was there a temperature or a perchlorate concentration where it would exhibit an A-to-Z transition? CD was much more sensitive to conformational changes than was absorbance, so it was used for structural determination. CD spectra were taken of the tetramer in 1 M, 2 M, 3 M, 3.5 M, 4 M, 5 M, and 6 M NaClO₄ as a function of temperature. The spectra of the molecule at 0°C are shown in Figure 3. It is apparent from these spectra that at intermediate salt concentrations, the molecule is not in a pure state, but is either in an intermediate conformation (neither A nor Z) or in a mixture of states (A plus Z, either on the same duplex or as a mixture of the two conformations on different molecules). If it were a mixture of A- and Z-form duplexes, then it should be possible to match the experimental spectra to computer-generated spectra that consist of the sums of A and Z spectra.

The calculated spectra were generated by measuring the CD spectrum of the A-form tetramer in 1 M NaClO₄ and the spectrum of the Z-form tetramer in 6 M NaClO₄ and then adding the two together in various proportions. This approach suffered from a difficulty in normalizing for concentration. The absorption of the tetramer in 6 M NaClO₄ was 10% less than that of the tetramer in 1 M NaClO₄.
Figure 3. The CD spectra of the tetramer in the various concentrations of perchlorate, all at 0°C. These spectra have two isosbestic points, one at 275 nm, the other at 256 nm, suggesting that there are two states comprising the spectra. The noise in the spectra make location of the isosbestic points accurate to within 3 nm.
although the two solutions should have had 20 ODU/mL. This 10\% decrease in absorption seems to be a function of the perchlorate rather than a true concentration difference. The calculated CD spectra were not corrected for this apparent concentration difference, so that the mixtures may err on the side of too much A-form conformation. However, because there was no easy way to digitize the data, this was the only approach available.

The calculated spectra show isosbestic points at 275 nm and 254 nm. When the experimental spectra of the tetramer at 0°C in the different perchlorate concentrations were plotted together, they showed isosbestic points at 275 nm and at 256 nm, suggesting that in fact those spectra at intermediate salt concentrations are a sum of A and Z forms. The two sets of data show disagreement in the magnitudes of the bands below 250 nm, indicating that there may be other contributions to the spectra, perhaps from single strands or from various end effects that are dependent on the salt concentration.

To investigate further the number of conformations composing the CD spectra, we used a technique of spectral decomposition. As described in Cantor and Tinoco\(^{(9)}\), the method consists of constructing a matrix of \(N\) spectra by \(M\) wavelengths. This matrix is then multiplied by its transpose to give a square matrix, \(U\), which is \(N \times N\). When the matrix \(U\) is diagonalized to give its eigenvalues, the number of eigenvalues indicates the number of linearly independent bases that comprise the spectra. To find the eigenvectors, the original matrix is multiplied by the transpose of the eigenvalue matrix. Ideally, the number of eigenvalues equals the number of eigenvectors comprising the system; in practice, \(N\) eigenvalues are obtained, but only a few of them are significant. These few indicate the number of eigenvectors. As described in \((9)\), the sum of the residuals is the standard deviation, \(\sigma^2\).

As applied to the tetramer spectra, if there are three components, A, Z and
single strands, then this treatment should yield 3 significant eigenvalues. This analysis was performed for the tetramer in 3.5 M NaClO₄, where there was significant absorbance at 295 nm, but where the CD indicated also a significant amount of A-form. The CD spectrum was recorded at 11 temperatures, the baseline subtracted, and the ellipticity read from the chart paper at 49 wavelengths. This 11 X 49 matrix was used for the algebraic operations described above. Diagonalization yielded two significant eigenvalues, with a standard deviation of less than 0.02, indicating that two eigenvectors were necessary to describe the system. This result is disappointing; I expected three eigenvalues. It means that on the basis of the CD data, it is not possible to distinguish the number of species in the solution; the two eigenvectors could represent a mixture of A and single strands with Z and single strands, or any combination of the three. Thus the question of the number of components in solution cannot be determined by this method.

Because the absorbance at 295 nm was characteristic of Z-form, any hypochromicity at this wavelength seemed indicative of the presence of Z-form. Using this criterion, the tetramer in salt concentrations ≥ 3 M perchlorate adopted Z-form in measurable proportions. In Table I, the melting temperatures calculated from the change in absorbance at 256 nm are compared to the Tₘ's calculated using the absorbance at 295 nm. In all but one experiment, the Tₘ calculated from A₂₉₅ is greater than the Tₘ calculated using A₂₅₆. The error in the measurement of the absorbance at 295 nm is also greater, since the absorbance at 295 nm is so much less than the absorbance at the maximum, although as seen in the Table, the percent hypochromicity exceeds the percent hyperchromicity for the samples. The discrepancy between the Tₘ's calculated at the two wavelengths is greater at high concentrations of the tetramer in 6 M NaClO₄, for reasons that are not clear. Because of the greater error in the absorbance at 295 nm, the Tₘ's used for thermodynamic calculations and for construction of a
Table I

Melting Temperatures of rCGCG

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$T_m$</th>
<th>Wavelength</th>
<th>Percent Absorbance Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M NaClO$_4$</td>
<td>24.3°C</td>
<td>256 nm</td>
<td>+9%</td>
</tr>
<tr>
<td>2 M NaClO$_4$</td>
<td>22.5°C</td>
<td>256 nm</td>
<td>+8%</td>
</tr>
<tr>
<td>3 M NaClO$_4$</td>
<td>19.5°C</td>
<td>256 nm</td>
<td>+6%</td>
</tr>
<tr>
<td></td>
<td>22°C</td>
<td>295 nm</td>
<td>-7%</td>
</tr>
<tr>
<td>3.5 M NaClO$_4$</td>
<td>17.5°C</td>
<td>256 nm</td>
<td>+6%</td>
</tr>
<tr>
<td></td>
<td>23°C</td>
<td>295 nm</td>
<td>-8.6%</td>
</tr>
<tr>
<td>4 M NaClO$_4$</td>
<td>20°C</td>
<td>256 nm</td>
<td>+7%</td>
</tr>
<tr>
<td></td>
<td>17°C</td>
<td>295 nm</td>
<td>-10%</td>
</tr>
<tr>
<td>5 M NaClO$_4$</td>
<td>14°C</td>
<td>256 nm</td>
<td>+5%</td>
</tr>
<tr>
<td></td>
<td>13.7°C</td>
<td>295 nm</td>
<td>-23%</td>
</tr>
<tr>
<td>6 M NaClO$_4$</td>
<td>17.5°C</td>
<td>256 nm</td>
<td>+5%</td>
</tr>
<tr>
<td></td>
<td>12.8°C</td>
<td>295 nm</td>
<td>-24%</td>
</tr>
</tbody>
</table>
phase diagram were calculated using the absorbance at 256 nm. However, the change in absorbance at 295 nm was used in a qualitative sense to determine if some Z-form was present in the solution, and to some extent in a quantitative sense to judge how much Z-form was present, by the percent hypochromicity in the different perchlorate concentrations.

The construction of a phase diagram for the tetramer utilized the $T_m$ to determine a phase change from duplex to single-strands, and utilized the CD spectra to determine which conformations were present in solution. The phase diagram is shown in Figure 4. The concentration of tetramer was 20 ODU/mL for all samples; using the extinction coefficient of Uesugi et al. the concentration ranged from $2.1 \times 10^{-3}$ M (NT) in 1 M NaClO$_4$ to $1.86 \times 10^{-3}$ M (NT) in 6 M NaClO$_4$. Samples in intermediate salt concentrations were obtained by mixing the 1 and 6 M NaClO$_4$ stock solutions in the appropriate proportions.

There are several points to note from the diagram. The first is that the $T_m$ of the duplex decreases as the salt concentration increases and as the proportion of Z-form increases. The decrease in the $T_m$ may be a result of the properties of chaotropic solvents (see Chapter 3 for a discussion) or it could be a result of the lower stability of the Z-duplex. Clearly the effect of chaotropic solvent will be significant, but whether it is the dominant effect is not clear. Second, there is a large region of the diagram that contains both A- and Z-form duplex. The CD spectra of the samples were measured as a function of temperature, to observe any A-to-Z or Z-to-A transition that might occur. With the polymer, such a transition is clearly visible, as the band at 294 nm disappears. With the tetramer, there was never any clearly definable transition; rather, the spectra always resembled a sum of the two forms which both melted with increasing temperature. As the spectral decomposition showed, it is not possible to unambiguously assign the components of the spectra; so the spectra can only be said to resem-
Figure 4. The phase diagram of the tetramer as a function of perchlorate concentration and temperature. Note that there is a large area in the middle region where there is a mixture of the A and Z duplex forms. The error in the $T_m$ values is about $\pm 1^\circ C$. 
ble the sum of the two contributions, based on similar isosbestic points and absorbance data. For this reason, the tetramer is thought to be either A or Z-form, existing as an equilibrium mixture of the two conformations in perchlorate concentrations between 3 and 5 M. Presumably, if the temperature could be lowered, a condition could be found where either the A or Z form was favored at these intermediate salt concentrations. It would be useful to know the proportions of the two duplex forms in those salt concentrations. NMR could be used to calculate the relative proportions by observing the peak areas as a function of temperature and salt. Alternatively, a hexamer or octamer would have a higher Tm that might allow the observation of an A-to-Z transition.

B. Thermodynamics

Because there was no observable A-to-Z transition in the tetramer, the thermodynamics describing that transition were calculated indirectly; first measuring the ΔG°, ΔH°, and ΔS° of the A-to-single strand and the Z-to-single strand transitions, then subtracting the two sets of values to give the A-to-Z transition. In this scheme, (A → SS) + (SS → Z) = (A → Z) with appropriate addition of thermodynamic parameters.

To calculate the ΔH° for the transitions, where the transition is defined as (2S → S2), the relation \( 1/T_m = (R/ΔH^°)\ln C + (ΔS^°/ ΔH^°) \) was used (11) where the Tm was measured at several concentrations of tetramer in either 1 or 6 M NaClO₄. A plot of 1/Tm vs ln C where C is the concentration of single strands, gives a slope of R/ΔH, where the slope and intercept are calculated using a least squares linear regression. The normalized melting curves for the tetramer in 1 M NaClO₄ as shown in Figure 5. The concentration ranged from 2.36 X 10⁻³ M (NT) to 5.86 X 10⁻⁴ M (NT). Similar melting curves were obtained for the tetramer in 6 M NaClO₄ (not shown) where the concentration ranged from 2.3 X 10⁻³ M to 1.24 X 10⁻² M.
Figure 5. The normalized melting curves for the tetramer in 1 M NaClO₄. Concentration range is described in the text. Note that in most cases, there is no clearly definable lower baseline for the melting curve. This ambiguity results in the calculation of higher apparent Tₘ values.
The tetramer A-form duplex is stable at low concentrations, while the Z-form duplex is not. This agrees with Uesugi's observation (1) about relative stabilities of dCGCG in 1 and 6 M NaCl, which suggests that the denaturing effect of perchlorate is not the dominant factor in duplex instability. While the Tm's of the A-form duplex could be measured at tetramer concentrations \( \leq 20 \text{ ODU/mL} \), that concentration was the lower limit of duplex stability for the Z-form. To obtain more concentrated samples, the tetramer was twice passed through a G-10 desalting column to remove the perchlorate, then lyophilized to concentrate the molecule. It was brought up in 6 M NaClO₄, 1 mM EDTA, 10 mM sodium phosphate, giving a concentration of 105 ODU/mL. At this concentration, the tetramer precipitated at 0°C, so the solution was heated to 60°C before taking aliquots. After heating, the tetramer seemed to stay in solution as judged by visual inspection and by the reproducibility of the melting data. Where a sample had precipitated, the melting curves were biphasic.

The melting temperatures were calculated graphically, assuming that the lower baseline of the melting curve was flat and intercepted the Y-axis at that absorbance measured at 0°C. There was no curve-fitting done, which was the method used by Petersheim and Turner for calculating melting temperatures. Placing the lower baseline as I did introduces an error into the calculation of the Tm, with the error being greater when the Tm was lower. This error would give an apparent Tm higher than the true melting temperature, thus biasing the subsequent calculations of thermodynamic parameters. Some additional error comes from the difficulty in determining the Tm in a curve that is very broad, as are the melting curves of the tetramer. Keeping these potential problems in mind, it is possible to calculate the thermodynamic parameters for the two transitions. These plots of \( 1/T_m \) vs in C are shown in Figure 6. The plots are linear, as
Figure 6. The plots of $\ln C$ vs $1/T_m$ for the A-to-ss and the Z-to-ss transition are linear, as expected for two-state systems. From a least squares fit to the data, the slope of the plot of the tetramer in 1 M NaClO$_4$ is $-4.18 \times 10^{-5}$, ±23%; the intercept is $3.08 \times 10^{-3}$, ±2%; $r=0.95$. For the tetramer in 6 M NaClO$_4$, the slope is $-2.39 \times 10^{-5}$, ±12%; the intercept is $3.36 \times 10^{-3}$, ±0.4%; $r=0.99$. Calculating the enthalpy from these data gives $\Delta H^\circ = -47$ kcal/mole (1 M NaClO$_4$) and -87 kcal/mole (6 M NaClO$_4$).
expected for a two-state transition. The thermodynamic parameters obtained from the plots are given in Table II.

The calculated values indicate that A-duplex is favored over Z-duplex at 25°C by 3 kcal/mole. This is consistent with the results of the A-to-Z transition in the polymer, where the low temperature form of the duplex is A-form, and heat must be added to produce the Z-form. The calculated enthalpy indicates that the A to Z transition is a favorable one; the unfavorable entropy gives the reaction an unfavorable ΔG. One caveat that must be mentioned with this approach to calculating the A-to-Z transition thermodynamics is that no correction has been applied for the behavior of the solvent. The assumption is that the effect on the ΔH will be small, so that errors in the measurement will exceed the separate solvent contribution. Some evidence for solvent effects can be found in the entropy term, which is larger in the 6 M perchlorate solution.

The idea in determining these parameters is to use them in comparisons with similar data for DNA to determine relative stabilities and energies of transitions, as well as using them to determine the size of the cooperative unit in the A-to-Z transition in the polymer. The error in these measurements is relatively large, due to baseline determinations, broad transitions, and low melting temperatures. The same study should be repeated using a longer oligomer where some of these problems can be eliminated. Nevertheless, these data support the previous study with the polymer transition.

Further experiments on the tetramer using NMR will provide more structural details, as well as identifying the proportions of the two duplex forms in solution. The kind of structural resolution NMR affords will be useful in comparisons between ribo and deoxy oligomers. NMR studies must also be done to confirm what the absorbance and CD studies have only indicated, which is that there is a Z-form of the tetramer. Until the guanine is shown to be syn, the
Table II

Calculated Thermodynamic Parameters

<table>
<thead>
<tr>
<th>Transition</th>
<th>$\Delta H^\circ$</th>
<th>$\Delta S^\circ$</th>
<th>$\Delta G^\circ_{298}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS A$^a$</td>
<td>-47 kcal/mole</td>
<td>-146 cal/mole°K</td>
<td>-3 kcal/mole</td>
</tr>
<tr>
<td>SS Z$^a$</td>
<td>-83 kcal/mole</td>
<td>-279 cal/mole°K</td>
<td>0</td>
</tr>
<tr>
<td>A Z$^b$</td>
<td>-36 kcal/mole</td>
<td></td>
<td>3 kcal/mole</td>
</tr>
</tbody>
</table>

$^a$calculated from plot
$^b$calculated from difference between transitions
duplex will not be unambiguously identified as Z-form.
References


Chapter 4.
Circular Intensity Differential Scattering by Cholesteric Liquid Crystals

The theory of CIDS has been developed by Bustamante et al. (1-4). There are separate theoretical treatments for the scattering of circularly polarized light by oriented systems and by randomly oriented systems. The power of the theory to allow the unambiguous determination of helical handedness immediately brought to mind visions of watching the B-to-Z transition progress. However, the development of the theory preceded the development of an instrument to measure the phenomenon. The first instrument that was built was completely contained in a black cloth-lined box to keep it light-tight. That scheme was abandoned following the realization that we needed much more room to maneuver, not to mention more distance between the sample and the detector. The second instrument occupied the better half of an optical bench. Using this instrument, we determined that the cell design was critical (square cuvettes did not work; there were too many reflections from the corners) and more important, that to measure something as small as DNA, photon counting was necessary. To utilize this second generation instrument, we needed a sample that scattered lots of light. So, while we worked on the photon-counting technology, we used the existing instrument to measure the scattering patterns from cholesteric liquid crystals. Liquid crystals have been shown to scatter tremendous amounts of light, so the sensitivity of the instrument would not be the limiting factor. Then, if we could observe the change of sign with handedness using left- and right-handed cholesteric liquid crystals, and if we could observe some change in the CIDS pattern with change of helical pitch, we could be confident that the instrument was working in the way that we thought that it was, and so the way would be paved for more biological samples.
A. CIDS and the CIDS Instrument

First to briefly review CIDS. The defining equation is \( \text{CIDS} = (I_L - I_R)/(I_L + I_R) \), where \( I \) is the intensity of right- or left-circularly polarized light. The conventions used for describing the two polarizations are shown in Figure 1. The simplest case of CIDS is that of elastic scattering, outside of the absorption band of the sample. When the circularly polarized light is incident upon an object that is in itself chiral, one sense of the light is scattered to a greater or lesser extent than the other, depending on the geometric parameters of the chiral object. The problem then becomes first to describe the interaction between the incident light and the object, and second, to describe the scattered light. When there is no intrinsic optical activity of the sample, then the scattering arises from the geometric placement of elements on the helix, an example of "form CIDS". In this treatment, there are four main assumptions. First, that the wavelength of the light is much larger than the dimensions of the scatterer. This allows the interaction to be described as the induction of a point dipole \( \vec{\mu}_{\text{ind}} = \vec{\alpha} \cdot \vec{E}_0 \), where the element (or scatterer) is described by a polarizability tensor \( \vec{\alpha} \). Second, that this is elastic scattering, where there is no energy change. Third, that the scattering is observed at a distance much greater than the wavelength. Classically, this enables the use of the far field approximation of the electric field of an oscillating dipole, ignoring the nonradiative near field. Fourth, that the first Born approximation is used, where the electric field felt by a dipole is only the incident field, with no contributions from nearby induced dipoles. (In fact, to accurately describe the CIDS patterns of liquid crystals, the second Born or higher approximations must be used.)

The resulting scattered electric field is thus the superposition of the electric field of each scatterer, and has the following features: a) it is a spherical wave; b) it is a transverse wave; c) it falls off as \( 1/r \); d) the polarization of the
Figure 1. A graphical depiction of right- and left-circularly polarized light, using the convention for describing the polarization used in the papers of Bustamante et al. The electric vector describes a helix as a function of time, shown in the lower figure.
\[ \hat{e}_L = \frac{1}{\sqrt{2}} (\hat{x} + \hat{y}) \]

\[ \hat{e}_L = \frac{1}{\sqrt{2}} (\hat{x} - \hat{y}) \]
scattered light is described by the interaction of the polarizability tensor with the incident polarized light \((\mathbf{a}, \mathbf{e}_o)\). The immediate result is that if the scatterer is isotropic, then \(a = a_1\), and \(\mathbf{e}_o\) is left unchanged. Consequently, \(I_L = I_R\), so the CIDS is zero. CIDS is an interference phenomenon arising from phase differences in the scattered electric field originating from different scatterer positions.

For calculation of CIDS, \(\mathbf{e}_o\) is replaced by \(\mathbf{e}_L\) and \(\mathbf{e}_R\), and the expression for \(E\), the electric field, is squared to give \(I = (c/8\pi)|E|^2\). The expression contains a term that selects for a helical orientation of scatterers, such that if the angle between two elements is 0° or 90°, the CIDS will be zero. Also, at \(k = k_o\), there is no phase shift, so again the CIDS is zero. It should be noted here that for the second Born approximation, it is possible to have a CIDS signal at 0°, and to measure CIDS when the noninteracting scattering groups are isotropic.

The instrument for measuring CIDS is illustrated in Figure 2. It consists of a laser light source, here a HeCd laser with a 442 nm line, a telescope to expand the laser beam, the Pockels cell that produces left- and right-circularly polarized light, the slit system to control the acceptance angle of the photomultiplier (PMT) detector, and the Cary 6003. The Cary acts as a source of modulating voltage for the Pockels cell, voltage for the PMT, lock-in amplifiers for signal processing, and a chart recorder for signal measurement. The gain circuit of the Cary was modified to measure the enormous \((10^{-2})\) CIDS of the liquid crystals. The telescope is necessary to provide an area that is the effective average over the microdomains of the liquid crystal. Using the 1.2 mm diameter beam of the laser, the CIDS pattern was not independent of the position of the laser on the sample. By increasing the beam diameter to approximately 1 cm, this problem was solved. The photomultiplier is mounted on an arm that rotates in the scattering plane. It is able to rotate 360°, but measurement is possible only from
Figure 2. A schematic diagram of the CIDS instrument, including the cell mount used for liquid crystal measurements. The photomultiplier tube moves around the sample in the scattering plane, mounted on an arm that is controlled by a goniometer.
Oriented Liquid Crystal

Cary 6003

SLit System

Telescope

Laser

He Cd Laser
442 nm
50 mW (cw)
0° to 174° and from 186° to 360° as the beam is clipped by the PMT housing from 174° to 186°. The rotation is controlled by a goniometer that is able to move in arbitrary intervals. For the liquid crystal measurements, the usual interval was 2.5° to 5°. The angular resolution is controlled by the slit system, which can be fixed by the use of apertures and by varying the distances from the apertures to the sample. For the liquid crystals, the angular resolution was 2°.

The alignment of the optical elements in the optical path is critical. It is clear from subsequent work on alignment of the Pockels cell by Dr. Skip Shimer that the system used in the experiments described here was not optimized. However, the magnitude of the liquid crystal CIDS signals provides some confidence that the linear anisotropy contributions arising from stray linear dichroism from the Pockels cell would be small. Also, the use of the beam expander gave concern about the integrity of the polarization of the light after it passed through the lenses. The expanded beam, after passing through the Pockels cell, did show an expanded version of the maltese cross that was used for Pockels cell alignment, indicating that the polarization was relatively intact. The alignment of the beam expander was the most difficult, since small angle deviations from normal incidence can be compensated for by wiggling the expander, producing a poorly polarized beam. Great patience is required for good alignment.

B. The Liquid Crystals

Cholesteric liquid crystals are helical arrays of molecules with both an intrinsic handedness and pitch. One model of the array is shown in Figure 3. This is the pile-of-plate-polarizers model, and is useful as a means of visualizing the order. (It is not useful in thinking about scattering, since a perfect pile of polarizers does not scatter.)

The intrinsic pitch of a cholesteric liquid crystal can be changed by adding a
Figure 3. The pile-of-plate polarizer model for liquid crystals. While it depicts the helical order of the cholesteric liquid crystal, it is misleading to use for scattering models, since such a pile of polarizers does not scatter light. The two orientations of the cholesteric helix axis with respect to the incident light are referred to as focal conic (top) and planar (bottom).
Cholesteric Liquid Crystals

Planar Orientation

Focal Conic Orientation

Incident Light

Scattered Light
nematic liquid crystal. (Nematic liquid crystals are not helical, but have linear order.) When the chemical structure of the nematic matches that of the cholesteric, the helical order of the cholesteric is nearly unperturbed, allowing the mixture to orient uniformly, and allowing the use of an empirical relation to relate the concentration of nematic and the pitch of the helix.

Some cholesteric liquid crystals are cholesteryl derivatives, and for these, the derivatives shown in Figure 4 have the indicated handedness. Binary cholesteric mixtures, such as cholesteryl chloride (CCI) plus cholesteryl nonanoate, are well studied, with phase diagrams of mole % CCl vs temperature showing right- and left-handed regions. These mixtures have several properties, however, that make them unsuitable for CIDS use. First, as oriented mixtures, they tend to develop local regions of solid crystals, where crystallization has occurred around an impurity in the mixture. The oriented mixtures are also optically inhomogeneous. Finally, these mixtures are very thermotropic, with temperature dependences on the order of sometimes tenths of degrees.

Some trinary cholesteric mixtures can form glasses, that appear physically like thin colored sheets of mica. They are formed by heating the mixture when it is sandwiched between two heavy heat-conducting weights (such as two heating plates) then allowing it to cool, continuing the application of pressure. Once formed, the glasses are very stable, easy to handle, and exhibit no thermotropy. However, forming glasses with a uniform pitch and good orientation is an art, and we never succeeded in producing a good sample.

For the CIDS experiments, we tried the binary mixture described above, as well as a mixture using cholesteryl oleyl carbonate. The sensitivity to temperature changes, whether from changes in room temperature or from local heating from the laser beam, resulted in wildly fluctuating CIDS signals. We tried to control the temperature using a flow of \( \text{N}_2 \) over the sample, but the flow was alter-
Figure 4. The chemical structures of the cholesteric liquid crystals used in the CIDS experiments.
Cholesteryl Derivatives

\begin{align*}
\text{CB15} & \quad \text{right-handed} \\
\text{Cl5} & \quad \text{left-handed} \\
\text{E7} & \quad \text{(eutectic mix; Nematic)}
\end{align*}
nately too weak to maintain a constant temperature, or so strong as to blow the liquid crystal out of its mount. These mixtures were abandoned.

The cholesteric liquid crystal that gave the best results, with no measurable thermotropy until the pitch was about 500 nm, is not a cholesteryl derivative. Professor Bruno Samori, University of Bologna, chose it, making an educated guess that it would work. The right-handed cholesteric liquid crystal CB15 is an optically active cyano-2-Me-butyl-biphenyl; the left-handed cholesteric C15, an optically active cyanoalkoxybiphenyl; and the nematic eutectic mixture E7, a cyanoalkylbiphenyl. (See Figure 4.) All are manufactured by BDH Chemicals and were purchased from E. Merck. The smectic mixture ZLI1612 was provided by B. Samori, purchased from E. Merck.

Orientation

The cholesteric liquid crystals were oriented with their helix axis either parallel (planar) or perpendicular (focal conic) to the incident beam. The orientation was determined by the coating applied to the plates: polyvinyl alcohol (PVA) for planar and RBS 25 for focal conic. Two sets of plates were used, one for each coating, as the coatings were difficult to remove entirely and using the same plates for both resulted in poor orientation of the liquid crystals. Polyvinyl alcohol was purchased from Kodak. RBS 25, \((\text{Na})_p\)-stearyl-benzenesulfonate, was provided by B. Samori. The quartz plates used as a cell for the liquid crystals had a scinted glass spacer fused to one of the plates, creating a pathlength of either 30 microns or 10 microns, when the two plates were pressed together. The plates were purchased from Hellma, Inc.

The plates were coated with PVA by placing them in a saturated filtered solution of PVA, and very slowly and steadily pulling them out, using a variable speed stepping motor. This dipping was done 5-6 times for each plate. The two plates
were then placed flat on a clean Kimwipe to dry, with the side that was to contain
the liquid crystal exposed to air. After drying, they were stroked in one direction
with a piece of styrofoam to orient the PVA. The liquid crystal was placed on one
plate and the second plate placed on top, being careful to avoid trapping air bub­
bles. To avoid linear birefringence, the second plate was oriented so that the
stroked PVA was at right angles to the stroked PVA of the first plate. The plates
were placed in the cell holder and the preparation allowed to stand for 12 to 24
hours before measurement was made, since the orientation of the helices was
slow.

The other set of plates was coated with RBS 25 by placing them in a 10% solu­
tion at 65°C for about 3 minutes. The plates were then washed with distilled
water and dried with N₂. The liquid crystal was placed between them and the cell
was mounted in the cell holder. The mounted liquid crystal was then melted with
a heat gun until it became isotropic, and allowed to cool. This was repeated two
or three times, until the texture was uniform. A well-oriented focal conic align­
ment displayed a texture like frosted glass.

The cell holder was designed to enclose the edges of the two plates, leaving a
slit in the middle approximately one cm high by 3 cm long. This puts a uniform
pressure on the liquid crystal as it is pressed between the two plates, to ensure
that its orientation was homogeneous. The quartz plates were mounted in the
holder in such a way that no translation or rotation was possible, but that the
only pressure was exerted normal to the plate surface by the washers of the
holder. The holder itself was of two pieces of anodized aluminum, held together
by four screws. It should be pointed out that this sample holder restricts the
angles of measurement to those less than about 60° and those greater than about
110°. This can be seen by observing the placement of the cell holder as shown in
Figure 2. The measured scattering angle does not correspond to the true
scattering angle, due to refraction at the quartz-air interface. The angle was corrected using Snell's law with a refractive index of \( n = 1.5 \) for quartz and \( n = 1 \) for air.

The CB15, E7, and ZLI1612 had a specific gravity of 1 gm/cm\(^3\), so they could be mixed by volume to give volume or weight percentage. After addition of the nematic or smectic to the cholesteric, the mixture was heated to its clearing point and allowed to cool to ensure its homogeneity. C15, the left-handed cholesteric, is a solid at room temperature. The amount of added nematic that was necessary to keep it fluid gave a pitch of about 4 microns. Any greater C15 concentration caused the mixture to crystallize. This difficulty made it impossible to use C15 in the planar orientation, since a 4 micron pitch is too long for stable orientation in a 10 micron pathlength cell. Attempts to use a 30 micron pathlength cell gave no reproducible results, presumably because 30 microns is too great a distance to maintain a helical path that is perpendicular to the plates. The C15 was used in the focal conic orientation, where its CIDS pattern could be compared to that of CB15 with a pitch of 4 microns. These two compounds are nearly identical, so a comparison of the two CIDS patterns should not be confused by signals that might arise from chemically different compositions. The nematic mixture E7 is chemically similar to the cholesteric liquid crystals, which is a critical factor in obtaining a well-oriented homogeneous sample. When the chiral component is very different from the nematic, the two compounds will not form smooth layers, but will have discontinuities around the different shapes. This was an especially important consideration for scattering experiments, since these potential discontinuities could cause diffraction of the light.

The magnitude of the CIDS signal can be influenced by a number of factors, and it is not obvious which factor(s) have the greatest contributions. It is clear that the degree of orientation is critical to obtaining large signals. Figure 5 illus-
trates the changes in the scattering pattern for the same sample immediately after preparation and after 24 hours. The main features of the pattern (the sign of the lobes and the position of the zeros) are constant, but after allowing the sample time to orient, the magnitude of the signals increased. On the basis of this observation, samples were measured 24 hours after preparation.

The pitch of the CB15 was controlled by adding varying amounts of E7 or ZLI1612. Neat CB15 has a pitch of 150 nm (from the E. Merck data sheets) and using the two mixtures, the pitch was varied from 4 microns to 468 nm. The smaller pitches were observed only with the ZLI1612, since the CB15/E7 melted at room temperature at pitches less than about 500 nm. The pitch was measured for the planar orientation using the reflection spectrum, or pitch band. Since cholesteric liquid crystals reflect light of the same handedness when the pitch matches the wavelength (7-10), it was possible, using either visible or infrared spectrophotometers, to measure the pitch of the planar samples. The sample in the cell holder was placed in the spectrophotometer with the face of the quartz plate perpendicular to the incident beam. By scanning over wavelength, the reflection spectrum of the sample was measured. The amount of reflection (or apparent absorption) and the width of the band correspond to the degree of orientation of the sample and to the distribution of pitches present. Conversely, the absence of a pitch band for the focal conic orientation provided evidence that there was no planar component present. For those samples whose pitches were not measurable (due to the IR absorbance of the quartz plates) the pitch was calculated using the empirical relation that 5% w/w CB15 in E7 gave a pitch of 3 microns, and 20% CB15 in E7 gave a pitch of 750 nm. This inverse relation was found to work quite well. The caveat is that it applies only to liquid crystals of similar chemical composition and so presumably of similar hydrodynamic structure. Using ZLI1612, the relation was approximate.
Figure 5. The CIDS patterns of a sample in the planar orientation, as a function of time. While the general shape of the pattern does not change with time, the magnitude of the CIDS signals does change. Because the magnitude of the signal is related to the degree of orientation of the sample, all preparations, both focal conic and planar, were allowed to orient in the sample holder for 24 hours before measurement.
The pitch of the focal conic samples can in theory be measured by using the laser diffraction patterns. The pitch is calculated using the formula 
\[ P(\sin \theta_m)/2 = (m\lambda)/n, \]
where \( n \) is the refractive index, \( \lambda \) is the incident wavelength, \( P \) is the pitch, \( \theta_m \) is the angle between the normal and the position of the diffraction ring, and \( m \) is the order parameter. The difficulty is in the determination of the correct value of \( m \). The diffraction pattern consists of a series of concentric (and frequently eccentric) rings, each corresponding to a different value of \( m \). As the pitch increases, the rings move in toward the center. They were visualized by placing a projector screen about 3-4 feet beyond the cell holder. The blue diffracted light created blue rings on the screen, but where the laser hit the screen directly downbeam from its point of origin, the screen would develop a hole, so that point was covered by a piece of black tape. The patterns were sometimes easier to see after heating the sample to its isotropic point and watching the pattern change as the liquid crystal cooled and oriented. We were never able to consistently identify the order of the rings, so the diffraction method was not used for quantitation.

It should be noted that obtaining a uniformly ordered sample is an art. The best (and perhaps only) sure way of learning to handle these systems is to work with someone who knows what to look for. Bruno Samori provided the instruction necessary for me to do the sample preparation. At his suggestion, the texture of the oriented samples was checked with a microscope to look for regions of disorder or dust particles. If the offending regions were very small, then that region was covered on both sides with a piece of black tape, and the CIDS measured. Sometimes, with time, those areas would disappear. Because of the sensitivity to long range ordering effects, though, those samples that had imperfections usually were not used. Also, the preparations were checked for regions of local linear birefringence (disorder) in the microscope by placing the preparation in the cell mount on a polarizer, then rotating a second polarizer parallel to
the first in the plane between the objective and the sample. This way of visualizing discontinuities was very sensitive to orientation defects and to dust. Only after passing the microscopic examination (12-24 hours after preparation) was the sample used for CIDS measurement. With practice, the rejection percentage decreased to only about 20%. The most difficult task was keeping out dust, since dust particles destroyed orientation.

C. CIDS Measurements

Focal Conic Orientation

The CIDS patterns for the focal conic orientation of left-handed C15 and right-handed CB15 are shown in Figure 6. These mixtures have similar pitches (approximately 4 microns) calculated using the inverse relation of pitch to cholesteric concentration. The pathlength of the sample cell here is 30 microns. The lobes and zeros of the patterns are at nearly the same angles, but the sign of the lobes is opposite. Scattering intensities are nearly identical for the two samples, providing some indication that the relative degree of orientation is the same for both preparations.

CIDS is predicted to be sensitive to the pitch of the helix. To measure the scattering of the focal conic orientation as a function of pitch, only the mixture of CB15 with E7 was used, since C15 was a solid at small pitches. Figure 7 shows the result of varying the pitch from 4 microns to 670 nm. The zeros of the scattering pattern in the forward direction move toward 0° as the pitch is increased. It appears that the forward lobes also move as the pitch increases, but it is not clear if they are moving forward or backward. If there were more data between P/λ=4.5 and P/λ=1.5, it might be possible to determine the direction of movement. As a guess, they appear to be moving backward, so that at an intermediate value of P/λ, the pattern would have two zeros, one near 5° and the
Figure 6. The helical handedness determines the sign of the lobes of the scattering pattern. For two focal conic preparations with equal pitch but opposite handedness, the sign of the CIDS pattern reverses.
Focal Conic

(helix axis perpendicular to incident light)

pitch = \(4 \mu\)

Right-handed

Left-handed

Changing the helix handedness reverses the sign of the CIDS.
Figure 7. The CIDS patterns change as a function of pitch for the focal conic orientation. The zeros in the forward direction move toward 0° as the pitch is increased. The magnitude of the signals decreases as the pitch approaches the wavelength of the incident light.
other near 25°, with three lobes in the order positive-negative-positive.

For the focal conic texture, the magnitude of the CIDS signal decreases as the pitch approaches the wavelength of light. From theory, large CIDS signals are expected when the distance between repeating scattering elements in a chiral arrangement matches the wavelength of light. So in one sense, these results are paradoxical. If the theory were better developed to describe liquid crystals, or if the present theory had been applied to the focal conic orientation, then perhaps these results could be used to say something about either the nature of the scattering unit in the liquid crystal, or the contribution of relative orientations to the scattering pattern as a function of pitch. As things stand, the results remain anomalous.

**Planar Orientation**

It was important to show that the sign of the CIDS changed as a function of handedness independent of the orientation of the helix. Because the left-handed C15/E7 mixture could only be used at long pitches, and because the 30 micron cell was too long to allow good planar orientation, another left-handed cholesteric mixture was used. The mixture of cholesteryl-oleyl-carbonate (COC) and a nematic (1167, from B. Samori) was used to give a pitch of 1.85 microns. The CIDS pattern of the planar orientations of COC and CB15 are compared in Figure 8. Again, it is clear that the sign of the CIDS signal reverses when the handedness of the helix is changed.

For the planar orientation, the magnitude of the CIDS signal increases as the pitch approaches the incident wavelength. This is illustrated in Figures 9 and 10. This increase in magnitude is a consequence of the property of planar cholesteric liquid crystals to reflect light of the same handedness as the helix, when the pitch matches the wavelength. This correspondence is illustrated in Figure 11, where the CIDS at 0° and at 160° are plotted vs pitch/wavelength (P/λ).
Figure 8. The helical handedness again determines the sign of the CIDS signal. For two planar preparations with equal pitch, but opposite handedness, the sign of the CIDS is reversed.
Right-Handed Helix
\[ P \approx 2 \mu \]

Left-Handed Helix
\[ P \approx 1.8 \mu \]
Figure 9. The pattern of the CIDS changes as a function of pitch of the liquid crystal. The sign of the CIDS in the back direction is constant for a given handedness, independent of pitch and chemical composition of the liquid crystal. The magnitude of the CIDS signal increases as the pitch approaches the wavelength, and seems to be related to the reflection by planar liquid crystals of light when its wavelength matches the pitch.
Planar, Right-handed, Cholesteric liquid Crystal

\[
\begin{align*}
P/P/\lambda & \\
\bullet & 2\mu & 4.5 \\
\circ & 1\mu & 2.3 \\
\triangle & 0.67\mu & 1.5 \\
\triangle & 0.52\mu & 1.2 \\
\end{align*}
\]

Scattering Angle (degrees)
Figure 10. Planar preparations of equal pitch but different chemical composition have CIDS patterns that differ in magnitude but not in the position of lobes or zeros.
Planar

Mixture  Pitch  P/λ

○ CBI5 + E7  P = 670  1.5
● CBI5 + 1612  P = 680  1.5
▲ CBI5 + E7  P = 520  1.2
▲ CBI5 + 1612  P = 485  1.09
× CBI5 + 1612  P = 468  1.06

Scattering Angle (degrees)
Figure 11. A plot of the magnitude of the CIDS signal at 0° and at 160° as a function of the ratio of pitch to wavelength. The increase in magnitude as $P/\lambda - 1$ is a result of the total reflection of light of the same handedness as the helix when the wavelength matches the pitch.
As the pitch approaches the wavelength, CB15 will reflect right-circularly polarized light, and transmit the left-circular components. This is clearly what is observed here.

As \( P/\lambda \) approaches one, the CIDS for the planar orientation in the front direction is also predominantly \( I_R \), probably because most of \( I_L \) is being transmitted. The source of the forward scattering may be inhomogeneities in the orientation, or the scattering from helices whose pitch is not equal to the wavelength. Alternatively, because the first Born approximation does not hold for the CIDS of liquid crystals, the nonzero forward CIDS could arise from interactions due to induced electric fields described by the second Born effect. This in fact has been shown to be the case (11).

The sign of the back scattering is a constant for a given handedness in the planar orientation. Thus right-handed helices scatter right-circularly polarized light in the back direction, independent of \( P/\lambda \) and independent of chemical composition. Because CB15/E7 is isotropic at room temperature for pitches less than 500 nm, it was necessary to use CB15/ZLI1612. In Figure 10, the CIDS patterns are shown for the two mixtures of nearly identical pitch. The sign of the back scattering is the same, although the magnitudes vary. The general pattern of front scattering is the same, but the zero has shifted. From Figure 9, it appears that the zero of the front scattering is relatively independent of the pitch, until there begins to be preferential transmission of \( I_L \). The change in the angular position of the zero for the two mixtures may arise from the different optical properties, a slightly different packing, or more inhomogeneity in alignment.

The theory predicts that the planar orientation should be symmetric about the direction of propagation of the light. This was found to be true.
D. Conclusions

There are three main conclusions to be drawn from these results. First, that CIDS can indeed indicate the sense of a helix, i.e., right- or left-handed. The change in the sign of the CIDS pattern occurs for liquid crystals oriented with the helix parallel or perpendicular to the incident beam. The theory predicts this result, and the observation confirms the theory.

Second, the CIDS pattern is sensitive to changes in the pitch, although the character of the response is dependent on the alignment of the system. For the focal conic orientation, the front lobes appear to move backward while the back scattering changes sign as the pitch is decreased. For the planar orientation, the scattering dependence on pitch seems to be related to that peculiar property of cholesteric liquid crystals of reflecting light of the same handedness as the helix. As the pitch approaches the wavelength, for the CB15 system, the magnitude of the scattering of $I_R$ increases, as more $I_L$ is transmitted.

This last observation is the third main conclusion of the experiments: that under these experimental conditions, the scattering of the planar liquid crystal agrees with previous experiments which used dramatically different techniques.\(^{(7)}\) When the pitch exactly equals the wavelength, there is total reflection of the light of the same handedness as the helix. Here, for the right-handed CB15 system, as $P/\lambda$ approaches one, we see an enormous positive increase in the apparent CD (measured at $0^\circ$), indicating that $I_L$ is being transmitted. The negative back scattering ($I_R$) increases as $I_L$ is transmitted, indicating that predominantly right-handed light is reflected.

The contributions to the CIDS pattern from inhomogeneities in the sample arising from density fluctuations, imperfect alignment, or variation in pitch, is not clear. The main features of the patterns, particularly for the planar orientation, are empirically predictable, and the theory is able to predict the sign of the
back scattering. The details of the patterns, however, while constant for a preparation over time, are more problematic. The position of the lobes for the focal conic orientation is unpredictable. It may be that the zeros in the front direction for the planar orientation depend on the chemical composition of the mixture.
References


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