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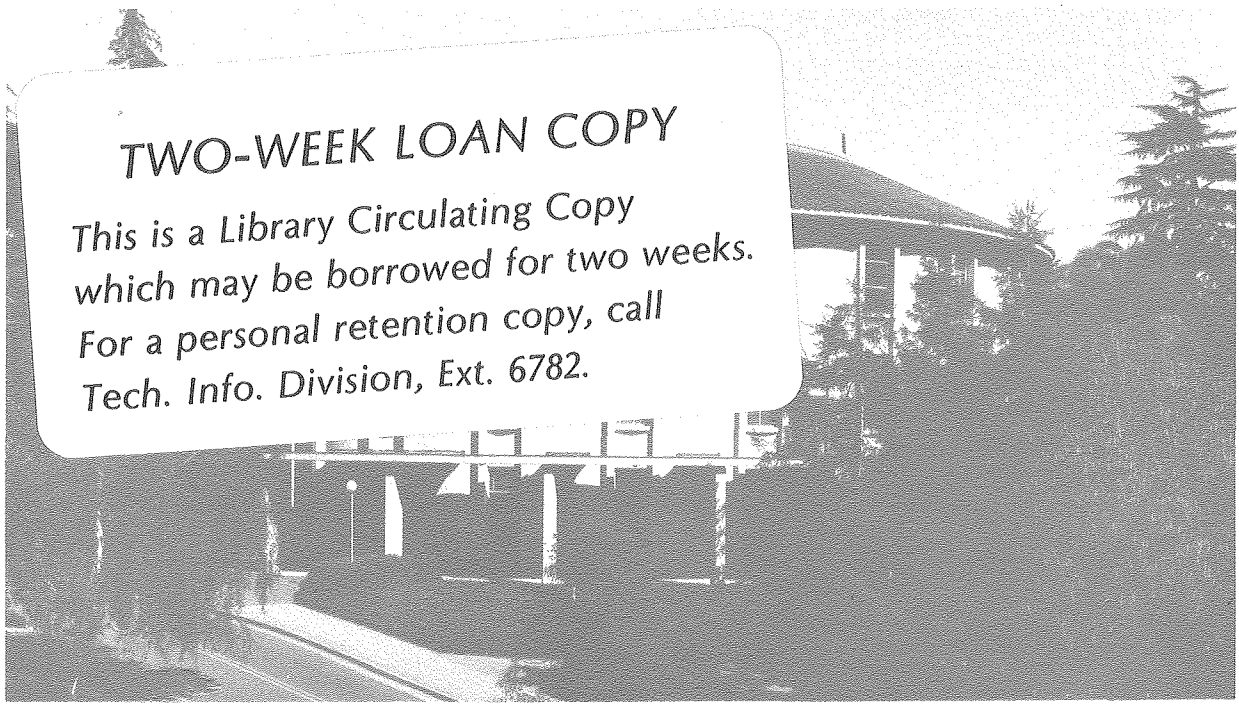

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A Comparative Study of Ribo-, Deoxyribo-, and Hybrid Oligonucleotide Helices by Nuclear Magnetic Resonance†

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Running title: NMR of Oligonucleotide Helices

[1]Abbreviations used: NMR, nuclear magnetic resonance; EDTA, ethylenediaminetertra-acetate; oligo A, oligoriboadenylic acid; oligo U, oligoribouridylic acid; TSP, sodium 3 trimethylsilylpropionate-2,2,3,3-d_4.

ABSTRACT

The nonexchangeable base protons and the hydrogen bonding N-H-N imino protons were used to study the conformations and the helix-coil transitions in the following oligonucleotides: (I) $dCT_5G + dCA_5G$, (II) $rCU_5G + rCA_5G$, (III) $dCT_5G + rCA_5G$, (IV) $rCU_5G + dCA_5G$. The first three mixtures all form stable double helical structures at 5°C, whereas IV forms a triple strand with a ratio of 2:1 rCU_5G:dCA_5G. The chemical shifts of the imino protons in the double strands indicate that I, II, and III have different conformations in solution. For example, the hydrogen bonded proton of one of the C•G base pairs is more shielded (a 0.4 ppm upfield shift) in helix I than in helix II or III. This implies a signficant change in helical parameters, such as the winding angle, the distance between base pairs, or overlap of the bases. The coupling constants of the H1' sugar protons show that helix I has 90% 2' endo sugar conformation, whereas helix III has greater than 85% 3' endo conformation for the observed sugar rings. The chemical shift and sugar pucker data are consistent with helix I having B form geometry, whereas II and III have A (or A') geometry. The chemical shifts of the base protons in system I were followed with increasing temperature. The midpoints for the transitions, T_m's, for all the base protons were 28-30°C; this indicates an all-or-none transition.

INTRODUCTION

Proton nuclear magnetic resonance[1] studies of oligonucleotides have greatly increased our understanding of the conformations and physical properties of nucleic acids in solution. The chemical shifts of the NH-N imino resonances, base proton resonances, and the H1' ribose or deoxyribose resonances have been used separately or in combination, to deduce RNA A or A' (Arnott et al., 1975) type conformations for double helical ribo-oligonucleotides (Arter et al., 1974; Heller et al., 1974; Borer et al., 1975; Hughes et al., 1978). Double stranded deoxyribo-oligonucleotides have shown spectra consistent with a B form geometry in solution (Cross & Crothers, 1971; Patel, 1974; Kallenbach et al., 1976; Early et al., 1977). These conclusions are based mainly upon comparison of the experimental chemical shifts with those computed from ring current effects for an assumed geometry, as well as the sugar pucker deduced from the H1' proton coupling constants.

X-ray studies of RNA-DNA hybrid duplexes have shown them to adopt A type geometries (Milman et al., 1967; Arnott et al., 1975). The only ^{1}H NMR study of an RNA-DNA duplex shows the structure of the hybrid to be different than the DNA-DNA duplex of the same sequence, and consistent with an A form in solution (Selsing et al., 1978).

Triple stranded structures are not uncommon in polynucleotide or oligonucleotide solutions (Bloomfield et al., 1974). Under conditions where triplexes were formed, Geerdes & Hilbers (1977)

observed the low field imino proton region of [oligo A-(oligo U)$_2$]. They saw separate resonances for the Watson-Crick and reverse Hoogsteen base pairs. Kallenbach et al. (1976) observed similar results for other triplexes including [AMP-(oligo U$_{15}$)$_2$].

In this work we report the ^{1}H NMR of oligonucleotides in a DNA duplex, an RNA duplex, a DNA-RNA hybrid duplex, and a DNA-RNA hybrid triplex, all of the same sequence. The molecules are (I) dCT$_5$G + dCA$_5$G, (II) rCU$_5$G + rCA$_5$G, (III) dCT$_5$G + rCA$_5$G, and (IV) rCU$_5$G + dCA$_5$G. The first three molecules form duplexes which all have different conformations in solution; IV forms a triple strand which is much less stable than the other structures. These results are consistent with optical studies previously reported on these molecules, which pointed out the importance of the instability of the DNA-RNA hybrid present at termination of transcription (Martin & Tinoco, 1980).

The thermodynamics of oligonucleotides have been studied by optical methods (Martin et al., 1971) as well as by proton NMR. The T_m's of the helix-to-coil transition from both methods have agreed fairly well (Borer et al., 1975; Kallenbach et al., 1976; Patel, 1979). Melting of the end base pairs before the rest of the helix has been seen in several of the systems with A·U or A·T base pairs on the ends of the helix (Borer et al., 1975; Patel, 1975; Kallenbach et al., 1976). In helices with C·G base pairs on the ends there seems to be no (Hughes et al., 1978) or little (Patel, 1979) differential melting of the ends of the helix before the

rest of the oligonucleotide. However, most of the systems studied thus far have been self-complementary oligonucleotides (except Hughes et al., 1978); this precludes measurement of the temperature dependence of the single strands. Ignorance of the temperature dependence of the single strand throughout the transition can lead to errors in the apparent T_m's; these errors will usually be larger for the interior base pairs than for the terminal ones. The oligonucleotides studied here are not self-complementary, so the single strand dependences have been observed. The measured T_m for each base proton is thus a more accurate representation of the melting of that part of the helix.

MATERIALS AND METHODS

The deoxyribo-oligonucleotides were synthesized by the diester method of Khorana (Khorana, 1968). The ribo-oligonucleotides were enzymatically prepared with polynucleotide phosphorylase (Martin et al., 1971; Uhlenbeck et al., 1971). Separation and purification of the oligomers were performed by RPC-5 column chromatography. Desalting of the samples was performed on Biogel P-2 columns (Bio-Rad). All samples were run in 8.0 mM Na_2HPO_4, 20 mM NaH_2PO_4, 0.18 M NaCl, 0.1 mM Na_2EDTA, pH = 7.0, unless otherwise noted. Concentrations of the oligonucleotides were calculated from the absorbance of the solutions at 260 nm. The extinction coefficients were calculated from extinction coefficients of dinculeoside monophosphates and mononucleotides with the assumption of only nearest neighbor interaction (Warshaw, 1965; Warshaw & Tinoco, 1966; Cantor & Warshaw, 1970). The values obtained in this way are 79×10^3, 58×10^3,

79 x 10^3, and 66 x 10^3 for dCA_5G, dCT_5G, rCA_5G, and rCU_5G, respectively, at 25°C.

Experimental NMR

NMR spectra were taken on the HXS-360 MHz instrument at Stanford Magnetic Resonance Laboratory. Temperature was controled to ±1°C by a B-ST 100/700 Bruker temperature controller. Spectra of the non-exchangeable protons were measured in D_2O in the normal Fourier transform mode using 5 mm NMR tubes (Wilmad). Spectra of the exchangeable protons were measured in H_2O using the Redfield 214 pulse sequence (Redfield et al., 1975) to minimize the water signal. For these samples 5mm microtubes which hold 160 µl of solution (508 CP Wilmad) were used. All solutions were measured at concentrations of either 1.0 mM or 0.5 mM per strand. Spectra measured in D_2O were all referenced to the internal standard TSP, while the spectra in H_2O were referenced to the H_2O peak. The temperature dependence of the chemical shift of H_2O relative to TSP was calibrated for our buffer. The chemical shifts obtained in this way are accurate to ±0.005 ppm for the D_2O and ±0.05 ppm for the H_2O studies. All data were collected with a Nicolet 1180 computer with 16K data points obtained for the D_2O work and 8K data points for the H_2O. Spectra were taken every 10° from 35 to 65°C and every 5° from 5 to 35°C, for the spectra in D_2O. In H_2O measurements were made every 5° from 5 to 25°C or until peaks were no longer observed.

D_2O samples were prepared by three lyophilizations against 99.8% D_2O (Bio-Rad) and then dissolved in 100% D_2O (Bio-Rad). The internal reference TSP was added to the sample after the addition of the 100%

D_2O, since it had a tendency to remain insoluble after lyophilization with the oligonucleotides. Samples of the component oligomers titrated with Mn^{2+} were measured in D_2O with no buffer added at pD = 7.0. The pD was calculated by adding 0.4 to the measured pH, with the pD adjusted by the addition of aqueous NaOD or DCl.

RESULTS

Assignments of Nonexchangeable Protons

The assignments of the chemical shifts of the base protons were accomplished on the single strands. The method of incremental assignment was used at 65°C where there is less stacking of the bases in the single strands (Borer et al., 1975). The chemical shifts at other temperatures were obtained from the change in peak position with temperature. Chemical shifts of the single strand oligomers at 65°C are given in Table I.

The aromatic region (6.6-8.5 ppm) of rCU_5G was poorly resolved due to the H6 doublets on the uracil residues, so no assignments were made in solutions which contained this oligomer. In the other solutions, the cytosine resonances were the only doublets in the aromatic region and easily identified. The adenine H8 protons were differentiated from the adenine H2 protons because of the longer T_1 of the H2 protons (T'so et al., 1973). The H8 on guanine was distinguished from H8 of adenine by the fact that it exchanges much faster upon heating in D_2O at 80°C. (In one hour at 80°C the guanine

H8 intensity was decreased over 50% while the adenine H2 was decreased by about 10%.)

In the oligonucleotide dCA_5G, we unambiguously assigned all the base protons on the cytosine, the guanine, and the #2 adenine. Figure 1 shows the base proton spectrum of dCA_5G + dCT_5G in the single strands at 65°C, and also defines the numbering scheme. For the other penultimate adenine (#6), we were able to specifically assign the H2, but not the H8 proton. The four H8 adenine resonances which belong to the three internal adenines (#3-5) and the #6 adenine were identified, but not assigned to specific bases. The H2 protons on the three internal adenines all have very similar chemical shifts and we were unable to assign resonances to particular adenines in the sequence.

In order to make assignment of the penultimate adenine resonances (#2 and #6) it was necessary to study the smaller components of dCA_5G. The following compounds at 65°C were used for comparison with dCA_5G (dCpApApApApG): dCpA, dCpApA, $dC(pA)_6$, dpApA, $d(pA)_4$, dpApG, and dpApApG. The assignments on the dCpA and dpApG were made by inspection. The assignment of the base protons in dpApA required studying their linewidths when titrated with Mn^{2+}, which preferentially binds to the terminal phosphate at pD = 7 and thus broadens the 5' adenine protons relative to those of the 3' adenine (Chiao & Krugh, 1977). This study enabled the assignments of the dpApA to be made, with both H8 protons unambiguously assigned and the two H2 protons only tentatively assigned; tentative assignment

of the base protons on dApA has previously been made by Chang & Sarma, 1977. These assignments helped in the assignments of the dCpApA and dpApApG. Only partial assignments of the oligomers $dC(pA)_6$ and $d(pA)_4$ were possible. The chemical shifts of these component oligomers at 65°C are published elsewhere (Pardi, 1980).

The assignments of the base protons in dCT_5G were made in a manner similar to that for dCA_5G. The components used were dCpT, dCpTpT, $dC(pT)_4$, and dpTpTpG. Unambiguous assingments of the cytosine and guanine base protons as well as the H6 protons on the penultimate thymines were made. The three internal thymine H6 protons all had the same chemical shifts at 65°C. The assignments of the thymine methyl peaks to specific bases in the sequence were not made at any temperature.

Assignments of rCA_5G were made by comparison with the series $r(Ap)_nApG$ assigned by Shum (1977), as well as our analysis of the oligomers rCpA, rCpApA, and rCpApApApA. From this work we were able to assign the cytosine and guanine base protons as well as the H8 and the H2 on the penultimate adenines. Again, the assignments of the three internal H8 protons or the three internal H2 protons to specific bases were not attempted.

The assignment of the base protons at other temperatures was made by following each peak shift with temperature; this is illustrated in Figures 2 and 3. Spectra were taken every ten degrees at higher temperatures where there are small changes in chemical shifts

with temperature, and every 5°C in the range from 5 to 35°, where the peaks shift dramatically with temperature and exchange broadening is evident. The assignment of the chemical shifts at 5°C for the double strand dCA_5G + dCT_5G was aided by comparison of spectra taken before and after the guanine H8 protons were exchanged in D_2O by heating at 75°C for one hour. Chemical shifts of this double strand are given in Table II. A small four bond coupling of the thymine methyl protons with the H6 protons helps in the identification of the H6 resonances. Assignment of the adenine H2 protons was aided by the fact that these peaks were sharper than the rest of the resonances in the double strand, presumably because of their longer T_2 compared to the T_2 of the other protons. The base proton spectrum of dCT_5G + dCA_5G in the double strand at 5°C is shown in Figure 4.

The chemical shifts for the protons in the double strand hybrid helix (dCT_5G + rCA_5G) which could be unambiguously followed are given in Table II. In the complexes rCA_5G + rCU_5G and rCU_5G + dCA_5G the spectra were so poorly resolved because of the uracil doublets that we were unable to see individual peaks in the aromatic region at temperatures lower than 35°C.

The H1' protons in deoxyribose and ribose sugars resonate at 5.5-6.5 ppm. The H1' proton in deoxyribose sugars is coupled to the H2' and H2" protons while the ribose H1' is only coupled to the H2' proton. Thus the H1' protons on the two different rings are easily differentiated. We were not able to assign the H1' protons to particular sugars in the sequence, but only to either deoxyribose or ribose sugars.

Assignment of the Base Paired Imino Protons

The base paired imino protons for the duplex $dCT_5G + dCA_5G$ are shown in Figure 5a. Partial assignment of these resonances was made by the temperature dependences of the protons, as well as comparison with calculations of chemical shifts for the double strand. The guanine H1 proton involved in a hypothetical isolated base pair has been found to resonate 1 ppm upfield from the uracil or thymine H3 in the isolated base pair (Kearns & Shulman, 1974; Robillard & Reid, 1979). The numbers for these isolated base pairs derived from tRNA data are 14.4 ppm for the A·U base pair and 13.6 ppm for the C·G base pair (Robillard & Reid, 1979). Since there is only a limited amount of work available on the imino protons in deoxyribo-oligonucleotides of known sequence (see Sarma, 1979, Chapters 5 & 6), the numbers for isolated base pairs derived from the tRNA work have been applied to the DNA helices.

For the $dCA_5G + dCT_5G$ helix the two resonances found at 12.93 and 13.55 ppm broaden before the other peaks. Figure 6 shows that at 20°C these two peaks are extremely broad relative to the rest of the molecule, and by 25°C they have disappeared. The differential broadening observed is due to the faster exchange of the terminal base pair protons compared to those in the interior of the helix (Kan et al., 1975; Patel, 1975). Calculated chemical shifts of the two C·G resonances allow assignment of the resonance at 12.9 ppm to the C·G in the one position and the 13.55 ppm peak to the C·G in the seven position (Arter & Schmidt, 1976) (see Table III). The assignments of the A·T base pairs were made by comparison with

the calculated shifts for DNA B geometry. This enabled us to assign the A·T resonances at the 6 position to 14.56 ppm with the rest of the A·T base pairs assigned to region 13.9-14.4 ppm.

In the rCU_5G + rCA_5G system we were aided in the assignments by comparison with the system rCA_6 + rCU_5G. (Data not shown but see Figure 5b for the rCA_5G + rCU_5G spectrum.) This gives unambiguous assignment of the C·G(1) base pair at 12.6 ppm, A·U(6) at 14.6 ppm, and C·G(7) at 13.6 ppm. The other resonances in the rCU_5G + rCA_5G were assigned to the region 13.6-13.9 ppm. All chemical shifts at 5°C are given in Table III.

In the duplex rCA_5G + dCT_5G, assignments were made in an analogous manner to the procedure used for the dCA_5G + dCT_5G helix. The C·G resonances again show very different temperature dependences than the A·T protons. The other peak assignments were then made using an RNA A geometry to calculate ring current shifts for each resonance. For this hybrid the calculated ring current shifts are shown for RNA A and A' as well as DNA B form geometry in Table III.

Temperature Dependence of the Nonexchangeable Protons in the Single Strands

The temperature dependence of the base protons of the single strands dCA_5G, dCT_5G, and rCA_5G has been studied. The cytosine H6 and H5, the adenine H2 and H8, the guanine H8, and the thymine H6 and methyl proton resonances were observed. The chemical shifts for all the base protons on dCT_5G show little change with temperature (<0.1 ppm from 5-65°C). The largest changes are on the adenine H2 protons in dCA_5G and the adenine H8 protons on rCA_5G as shown in Figure 7. Most of the proton chemical shift changes observed are

not linear with temperature; this is important to note when analyzing melting curves, as will be discussed in a later section. There are large differences in chemical shift changes with temperature between dCA_5G and rCA_5G. The temperature dependences of the chemical shift for a particular proton are sometimes in opposite directions for the two strands. For example, compare the H6 of C(#1) and the H8 of A (#3, 4, 5 or 6) on the two strands in Figure 7. These observations indicate different conformations for the deoxyribo- and ribo- strands of CA_5G.

The best measures of base-base stacking from chemical shifts are obtained from the H2 of adenine and the H5 of the pyrimidines (Lee & Tinoco, 1980). The H8 of adenines and the H6 of pyrimidines have significant factors besides ring currents, which affect their chemical shifts (Ts'o et al., 1969; Lee & Tinoco, 1980), such as the glycosidic torsion angle, the proximity of the sugar ring oxygens, or phosphate groups. Therefore, in order to get an idea of the relative base-base stacking in the dCA_5G and rCA_5G strands, we compared the chemical shifts of the adenine H2 protons. The adenine H2 protons on the ribose strand are always downfield (less shielded) from the same protons on the deoxyribose strand; this is indicative of more base-base stacking in dCA_5G than in rCA_5G.

The Helix to Coil Transition of $dCT_5G + dCA_5G$ and $dCT_5G + rCA_5G$ as Followed by the Chemical Shift of the Base Protons

The temperature dependence of the chemical shifts of the base protons were studied in the oligonucleotides, $dCT_5C + dCA_5G$, and

dCT_5G + rCA_5G. In the helix to coil transition of the dCA_5G + dCT_5G, all the base proton chemical shifts were followed from 5-65°C. In the intermediate states from 20-35°C, it is difficult to obtain exact chemical shifts of some of the protons due to extensive broadening of the resonances undergoing chemical exchange. In the other helix, dCT_5C + rCA_5G, the chemical exchange broadening over the 20-30°C temperature range prevented the assignment of many of the resonances at lower temperatures. The chemical shift changes with temperature for the deoxyribose helix is shown in Figure 3.

In order to monitor the helix to coil transitions in these oligomers, base protons were chosen which show large changes upon formation of the helical state (>0.1 ppm). The curves for many of these protons have sigmoidal shapes and characteristics which are very similar to absorbance versus temperature curves for these oligomers. However, the NMR data allow one to follow the properties of individual bases throughout the double to single strand transition. Thus NMR is more useful than absorbance studies for finding properties such as differential melting of the helix (Borer et al., 1975; Kan et al., 1975; Patel, 1975).

The melting temperatures, T_m's, of different bases were compared to learn if there is any melting on the ends of the dCT_5G + dCA_5G helix. The analysis was the same as that used in optical studies (Martin et al., 1971) which requires the melting behavior of the single strands. The single strand molecule gives the upper baseline in Figure 3 and the lower baseline is the chemical shift measured at 5°C. For most of the protons there are very small

chemical shift changes between 5 and 15°C so that the chemical shift of the double strand was taken to be temperature independent. The T_m values are all very similar for protons in different parts of the helix, with an average value of 29±2°C at a concentration of 1.0 mM per strand. Since all the base protons melt at approximately the same temperature, the concentration of partially formed helices is small.

Optical melting under identical conditions gives a T_m of 34±2°C. The difference is probably due to assumptions about the temperature dependence of the properties of the double strand and also errors involved in assuming fast exchange, on the NMR time scale, throughout the single to double strand transition. These effects will be discussed in a later section.

For the helix $dCT_5G + rCA_5G$ the temperature dependence of only some of the base protons were followed, since exchange broadening and overlap of too many protons hindered the identification of many of the protons in the double strand. In this helix we were unable to follow any of the guanine or cytosine protons throughout the whole melting transition, so that it was not possible to tell if there was significant melting of the ends of the helix. The average T_m for all the base pairs was 23.5°C at 0.5 mM per strand. The T_m obtained under similar conditions from optical data extrapolated to the same concentration was 25.7°C (Martin, unpublished).

The validity of T_m or enthalpy values extracted from melting curves is often dependent upon the method of analysis. In order

to get useful results from a melting curve, one must estimate the temperature dependence of the measured property of the double and single stranded molecules (Martin et al., 1971). One major advantage in using non-selfcomplementary oligonucleotides is that the properties of the pure single strand can be studied separately from the double to single strand transition. The temperature dependence of the chemical shifts in the single strands often show large deviations from linearity (see Figure 7 and Shum, 1977). In self-complementary molecules one is forced to assume linearity which can lead to large errors in the T_m. For example, in the system $dCA_5G + dCT_5G$ the analysis of the melting curves with and without the experimental temperature dependence of the single strand gives T_m values which differ by up to 5°C for some of the protons. This problem seems to be much more important in NMR than in optical studies, and should be taken into account when interpreting thermodynamic parameters derived from NMR melting curves.

Difficulties involved in obtaining equilibrium constants from chemical shift data have recently been discussed (Feeney et al., 1978). One usually assumes that the system is always in fast exchange, on the NMR time scale, and that the measured chemical shift is a weight average of all the states. The validity of this approach breaks down when the system is not in the fast exchange domain, which happens during melting of most oligonucleotides. We have made calculations of the lineshapes of the resonances for any exchange rates between two states, in order to estimate the errors made in extracting thermo-

dynamic data with the assumption of fast exchange (Pardi, 1980). For parameters which reasonably mimic the behavior of the dCA_5G + dCT_5G system, we found that the system clearly is not in fast exchange throughout the helix-to-coil transition for many of the base protons. The effect of assuming fast exchange in our model system was to change the shape of the melting curve, especially below the T_m. The model calculations show that the assumption of fast exchange in analyzing experimental melting curves will lead to errors in drawing of the lower baseline, and can give T_m values which are in error by up to 4-6°C.

In the system dCA_5G + dCT_5G, the T_m found optically had a value ~5°C higher than the T_m obtained by NMR, which showed all the resonances melting at 28-30°C. We think the reason for this difference is the invalid assumption of fast exchange throughout the transition, which causes an incorrect estimation of the lower baseline of the NMR melting curve. In these molecules, we took all the lower baselines to be flat, so that the error is probably systematic and will be similar for all the protons. Modeling this system gives the same conclusion, that the errors due to assuming fast exchange are approximately the same for all the protons. Therefore, we think that the terminal and interior base pairs melt at the same temperature, within 2-3°C, and that an all-or-none transition is a good approximation for this duplex.

Temperature Dependence of the Exchangeable Imino Protons

The temperature dependence of the imino base protons in dCT_5G + dCA_5G are shown in Figure 6. As already discussed in the section

on the assignments of the imino protons, it is found that the base pairs on the ends broaden and disappear before the rest of the base pairs in the helix. Similar broadening is observed for the other helices $dCT_5G + rCA_5G$, and $rCU_5G + rCA_5G$ (Pardi, 1980). The broadening phenomenon observed is due to chemical exchange (Gutowsky & Holm, 1956; Kaplan & Fraenkel, 1980), as will be discussed in a later section. The C·G imino protons for the three systems broaden and disappear in the range from 17-23°C. There is little chemical shift change with temperature for any of these resonances. Differences in the extent of broadening for a particular proton at a given temperature are seen in the three systems; this is due to different lifetimes for exchange of the imino protons with H_2O. We are investigating the lifetimes of the imino protons in these systems by the saturation recovery method used by Redfield (Johnston & Redfield, 1978). Preliminary results on the $dCA_5G + dCT_5G$ helix show that the lifetimes of the terminal C·G base paired protons are between 15-50 msec and that the lifetimes of the internal protons range from 150-250 msec at 5°C (Pardi, 1980).

Sugar Pucker of the Furanose Ring in the Double Strand

In two of the oligomer systems, the sugar pucker for some of the ribose or deoxyribose rings were calculated. The conformation of the ribose or deoxyribose ring has been described by Altona & Sundaralingam (1973) as a two-state equilibrium between type N[C2' exo, C3' endo] and type S[C2' endo, C3' exo]. The ribose ring conformation can then be calculated from the fact that in N type conformation $J_{1'2'} = \sim 0$ Hz and the S type has $J_{1'2'} = \sim 10$ Hz. For the deoxyribose ring the N

type conformation has the property of $J_{1'2'} + J_{1'2''} = \sim 7$ Hz while the S type has a value of ~16 Hz for this sum (Altona & Sundaralingam, 1973).

The assignment of the H1' protons to particular sugars in the oligomers was not possible, and coupling constants were obtained only for those resonances which were well separated from the main group of H1' protons. For the $dCA_5G + dCT_5G$ helix at 5°C, only two H1' deoxyribose protons at 5.64 and 5.48 ppm were separated from the rest. Both protons had coupling constants of $J_{1'2'} + J_{1'2''} = 15 \pm 1$ Hz, which gives 90% S type (2' endo) conformation for these sugars. The temperature dependence of these peaks, as well as comparison with the single strands at 65°C, shows that both protons are from the dCA_5G strand. The chemical shifts of the H1' protons in the components of dCA_5G studied indicate that these two protons are due to deoxyadenosine resonances. The fact that these deoxyribose sugars are in 90% S type (2' endo) conformation is consistent with a B form type geometry for this helix.

In the hybrid oligonucleotides $rCA_5G + dCT_5G$, all seven ribose H1' protons were resolved, and had coupling constants $J_{1'2'} \leq 1.5$ Hz. The ribose ring sugar pucker is then calculated to be $\geq$85% N type (3' endo) conformation. This 3' endo sugar pucker in the ribose strand of the double helix is indicative of an A type geometry for the helix.

Linewidths of the Base Protons and Chemical Exchange

For the $dCA_5G + dCT_5G$ system we see extensive broadening of some of the base protons during the melting transition. The fact that some

of the resonances broaden while others stay very sharp is clearly seen in Figure 2. The reason for this broadening is the fact that the system is undergoing chemical exchange between the double stranded helix (helix) and the single strands (coil). The rate of exchange, the population difference, as well as the difference in chemical shift between the two states are the factors which lead to broadening of the resonances (Gutowsky & Holm, 1956). Resonances such as the guanine (#1) H8 at 7.95 ppm, the thymines (#3-5) H6 at ~7.6 ppm, all shift little, and therefore are sharp throughout the transtion. The three interior adenine H2 protons (at 7.09, 7.08, and 6.98 ppm at 5°C) have such large chemical shift differences for the two states that their resonances are almost broadened into the baseline at 25°C.

The (#2) adenine H8 proton (8.32 ppm at 5°C) is well separated from its neighbors and broadens to a moderate extent, which makes it possible to extract rate constants for the helix-to-coil transition (Patel, 1979; Patel, 1975). Equation (1) applies when the system is close to the fast exchange limit (Kaplan & Fraenkel, 1980), and has been used to calculate rate constants for the helix-to-coil transitions in other oligonucleotides (Patel, 1979; Patel, 1975).

$$(1) \qquad \frac{1}{\pi T_2(\text{obs})} = \frac{P_C}{\pi T_2(C)} + \frac{P_H}{\pi T_2(H)} + 4\pi P_C^2 P_H^2 (\nu_H - \nu_C)^2 (\tau_H + \tau_C)$$

$1/\pi T_2(H)$ and $1/\pi T_2(C)$ are the measured linewidth of the helix and coil states, respectively, while $1/\pi T_2(\text{obs})$ is the observed linewidth in the broadened spectrum. The linewidth of the helix state for a particular proton is assumed to be independent of temperature

and is thus the measured linewidth of the helix at 5°C. The linewidth of the coil was taken from the measured linewidth during the melting of the single strand. ν_H and ν_C are chemical shifts of the protons in the helix and coil given in Hz, again ν_H is assumed to be the chemical shift of the helix at 5°C, while ν_C is measured from the melting of the single strand. P_H and P_C are the populations at a given temperature for the double and single strands, respectively. The populations were obtained from analysis of the absorbance versus temperature melting curve. τ_H and τ_C are the lifetimes of the helix and coil states; by the definitions, $P_H\tau_C = P_C\tau_H$. At 25°C for the $dCA_5G + dCT_5G$ helix and the #2 adenine H8 proton, the following parameters were obtained: $1/\pi T_2(H) = 6$ Hz, $1/\pi T_2(C) = 2$ Hz, $P_H = 0.88$, $P_C = 0.12$, $1/\pi T_2(obs) = 15$ Hz, and $(\nu_H - \nu_C) = 72$ Hz. Using Equation (1) we calculate the lifetimes to be $\tau_H = 14$ msec and $\tau_C = 2$ msec. Since the measurements of these lifetimes are fairly indirect, the errors in the numbers are of the order of 30-40%. These lifetimes and their rate constants are consistent with kinetic results obtained on other oligonucleotides by temperature jump methods (Porschke et al., 1971; Ravetch et al., 1973).

DISCUSSION

Comparison of the Structures of Oligonucleotide Complexes in Solution

The Double Stranded Structures. Figures 5a, 5b and 5c show low field spectra of the three double stranded helices at 5°C. There are seven imino protons in each spectrum, one for each base pair in the

double helix. From examining the chemical shifts of the imino protons in the three double strands, one sees distinct differences in the peak positions of many protons. This is indicative of different conformations for each system. The best fits for calculated and experimental results are for the $dCT_5G + dCA_5G$ in a DNA B type geometry, while the $dCT_5G + rCA_5G$ and $rCU_5G + rCA_5G$ are in an RNA A or A' type geometry.

The fits of the DNA B form to the deoxyribose double helix is excellent in the relative positions of the imino protons, but are not as good in their predictions of the exact positions. The predicted resonances are all about 0.3 ppm higher field than the experimental results. Since the conformations of RNA and DNA helices are different, it is likely that the intrinsic positions of these isolated base pairs are also different. As more proton NMR work is done on deoxyribo-oligonucleotides, it may be possible to obtain empirical results for the chemical shifts of the isolated A·T and C·G imino protons in deoxyribose systems.

RNA structures are found to be more rigid and less susceptible to conformational changes than their DNA counterparts (Arnott et al., 1975). We would then expect that the RNA helix in our study would be close to an RNA A form geometry. As seen in Table III, there are large differences between the experimental results and the calculations of the chemical shifts for an RNA A or A' geometry. These differences can be due to several factors, such as inaccurate values for the ring currents used in the calculations, incorrect

geometries assumed in the calculations, sequence dependent geometries, or other parameters besides ring currents having significant effects on the chemical shifts of the imino protons (see Borer et al., 1975, for discussion of these effects). Aggregation will also directly effect the chemical shifts of the terminal base pair protons. Optical studies on these oligonucleotides have shown that there is significant aggregation at these concentrations, so the chemical shifts from the terminal base pairs are more difficult to interpret.

The chemical shifts of the nonexchangeable base protons are compared with the calculated results for several of the oligonucleotide double helices in Table II. One sees large discrepancies between the experimental and calculated chemical shifts for the dCA_5G + dCT_5G system. We are investigating the discrepancies to see if the differences are due to the oligonucleotide being in a slightly different geometry than the classical DNA B form. Calculations of the chemical shifts for other energy minimized geometries are presently being performed to see how slight changes in helical parameters, such as winding angle, twist, and tilt, effect the chemical shifts of the base protons.

The coupling constants and chemical shifts of the ribose and deoxyribose protons have been extensively used in the past to obtain conformational information on oligonucleotides in solution (Lee et al., 1976; Ezra et al., 1977). One of the most meaningful parameters in the geometry of the nucleic acid is the conformation of the sugar ring (Arnott & Hukins, 1973). In the dCT_5G + dCA_5G double helix, two of the deoxyribose sugar rings in the dCA_5G strand were found

to be 90% in a 2' endo conformation. The 2' endo sugar puckers are found in B form geometries which is consistent with this helix being in a B type conformation.

The hybrid dCT_5G + dCA_5G was also well enough resolved in the H1' proton region to obtain coupling constant data. The evidence of a 3' endo conformation of the ribose strand is indicative of an A type geometry. Knowing the sugar pucker of the helix greatly restricts the range of conformations for the sugar phosphate backbone, thereby ruling out many possible geometries for the helix. This fact is especially useful in conjunction with the ring current calculations, and may make it possible to define conformations more exactly than the qualitative "A" type or "B" type geometries.

Triple Strand. The system rCU_5G + dCA_5G forms a triple strand under our conditions. The low field imino proton spectrum of this mixture is quite different than the other spectra shown in Figure 5. For example, there is a broad resonance at very low field around 15.0 ppm in Figure 5d, and the normal Watson-Crick imino protons are not found to resonate higher than ~14.6 ppm. This resonance as well as those centered at 14.6 ppm are most likely due to reverse Hoogsteen base pairs involved in the triple strand.

Geerdes & Hilbers (1977) have studied [oligo A -(oligo $U)_2$] triplexes in solution by ^1H NMR. From the chemical shifts of the imino protons they proposed ~14.3 ppm for the intrinsic position of the Watson-Crick A·U pair in the triple strand and 14.8 ppm for the reverse Hoogsteen pair. Robillard & Reid (1979) have recently attempted calculations which empirically optimize the magnitudes of the chemical shifts of the isolated base pairs by a method which

assumes a strong similarity between the crystal and solution structure of tRNA. They found the optimized intrinsic position of the reversed Hoogsteen A·U to be at 14.9 ppm. Kallenbach et al.(1976) have studied triple stranded structures in which oligo U_{15} and rAMP showed two sets of imino resonances, presumably one for the Watson-Crick and one for the reverse Hoogsteen base pairs. They concluded that the most probable intrinsic chemical shift was 14.1 for the reverse Hoogsteen base-pair, but were unable to rule out an alternate assignment in which the intrinsic chemical shift was ~14.8 ppm.

In our system we are unable to make unambiguous assignments of the imino protons, but we think that the broad resonance at ~15.0 ppm is due to an A·U reverse Hoogsteen base pair. It seems to resonate at too low field to be from a normal Watson-Crick type A·U resonance, and the above studies on triplexes indicate that the reverse Hoogsteen base pair's intrinsic shift may be in the region of 15.0 ppm. This would indicate that the other resonances at 14.2-14.5 ppm would also be mostly from Hoogsteen base pairs. The resonances below 14.0 ppm are then due to Watson-Crick type base pairs. The intrinsic shift of an rU·dA·rU reverse Hoogsteen pair would then be $\geq$15.0 ppm

The fact that there are so many types of resonances in the low field region of the $dCA_5G + rCU_5G$ system, many more than the seven protons one sees in the double helical systems, indicates that the oligomers are fully or partially in the triple strand conformation.

Fraying Versus Melting of Oligonucleotides

It is important to understand the distinction between fraying of the ends and the differential melting of the ends of an oligonucleotide. Here we define the melting of a base pair in the oligomer with respect to the fraction of the base pair which is formed, or involved in hydrogen bonding. An 80% melted base pair would have an equilibrium concentration of oligonucleotides with 80% of that base pair broken and 20% of that base pair formed. Melting thus reflects an equilibrium effect and is dependent only upon the concentrations of the two states.

Fraying is defined as the rapid opening and closing of a base pair (Patel & Hilbers, 1975; Hilbers, 1979). It is thus a kinetic effect and the important parameters involved are the rate constants linking the open and closed states. Fraying can manifest itself in the H-bonding imino protons where the exchange rate of the proton is reflected by the linewidth of the resonance (see Crothers et al., 1974; Hilberts, 1979, for discussion of exchange of imino protons with H_2O). In our oligomer systems the C·G base-paired imino protons broaden and disappear before the interior base pairs, which is indicative of fraying of the ends of the helix. This does not mean the end base pairs are melted at the point where the resonance has disappeared. For example, in the helix $dCT_5G + dCA_5G$ at 25°C, the imino proton region is extremely broad, and by 30°C the resonances have disappeared into the baseline (see Figure 6). This is not representative of the equilibrium concentrations of these states, the oligomer is fraying at this point, but it is not melted. In fact, the chemical shift

versus temperature data of protons on all bases, and optical studies of this oligomer show that it is >50% in the double strand at 30°C, where all the imino protons have disappeared. The temperature dependence of the broadening of the imino protons is mainly a kinetic, not an equilibrium, effect. Thus an end base pair can be fraying, but not "melted" at the same time. In our system, although we see fraying of the ends of the helix, i.e., the end bases are opening and closing faster than the interior base pairs, the ends do not melt appreciably lower than the rest of the helix. It is worth noting that fraying and melting reflect not only different processes, but there may also be different states involved in the two processes. For example, in order for an imino proton to exchange with water, the base pair must open to some extent. This opened state most likely differs from the "melted" state in a molecule with differential melting on the ends of the helix. Care should be taken in distinguishing between fraying and melting, as well as the effects ascribed to them.

CONCLUSION

We have studied the following oligonucleotides separately, and in their complementary mixtures by proton NMR: dCA_5G, dCT_5G, rCA_5G, and rCU_5G. Results on the single strands show that the ribose and deoxyribose strands of CA_5G have different conformations in solution, and specifically that there is more base-base stacking in the deoxyribose strand.

Results on the mixtures indicate that rCU_5G + dCA_5G at least partially forms a triple strand with a 2:1 rCU_5G:dCA_5G helix, while rCU_5G + rCA_5G, rCA_5G + dCT_5G, and dCT_5G + dCA_5G form double helical structures. Comparison of the three double strands shows that they all have different structures in solution. The chemical shifts of the imino protons and the calculations of sugar ring pucker in these systems indicate that the deoxyribose helix (dCA_5G + dCT_5G) is in a B type geometry, whereas the ribose (rCA_5G + rCU_5G) and hybrid (rCA_5G + dCT_5G) helices are in an A or A' type geometry.

The melting of the helix dCA_5G + dCT_5G as followed by the chemical shift changes of the nonexchangeable base protons shows that base pairs on the ends of the helix melt at approximately the same temperature as the interior base pairs. The temperature dependence of the imino protons indicates there is fraying of the ends of the helix. Thus this helix exhibits fraying of the ends, but very low concentrations of partially formed helices at the same time. These results indicate that a two-state model is a good approximation of the helix-to-coil transition in this oligonucleotide, as often assumed in optical studies. Some of the biological implications due to the differences in stability of these oligonucleotides have been discussed elsewhere (Martin & Tinoco, 1980). The differing conformations for these DNA, RNA, and hybrid helices may also be important in enzymatic recognition of different types of helices.

Studies on the kinetics of these oligonucleotide helices, as well as the changes in the conformation and stability of double helical oligonucleotides when there is a mismatched base on one strand, or when frameshift mutagens are bound, are presently being pursued.

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Table I. Chemical Shifts of Single Strands at 65°C[a]

		#1C	#2A	#3A	#4A	#5A	#6A	#7G
	AH2	--	7.985	(7.752	7.725	7.696)	7.854	--
dCA_5G	CH6	7.489	--	--	--	--	--	--
	A or GH8	--	8.120	(8.081	8.052	8.016	8.012)	7.872
	AH2	--	8.091	(7.982	7.971	7.953)	8.034	--
rCA_5G	CH_6	7.632	--	--	--	--	--	--
	A or GH8	--	8.265	(8.175	8.165	8.152)	8.190	7.878

		#7C	#6T	#5T	#4T	#3T	#2T	#1G
	C or TH6	7.770	7.532	7.627	7.627	7.627	7.645	--
dCT_5G	TCH_3	--	1.878	1.878	1.878	1.878	1.878	--
	GH8	--	--	--	--	--	--	7.993

[a] Spectra were taken on samples containing 8.0 mM Na_2HPO_4, 20 mM NaH_2PO_4, 0.18 M NaCl, 0.1 mM Na_2 EDTA, pD = 7, at a concentration of 1 mM per strand. The values in parentheses indicated that we were unable to assign these peaks to particular bases in the sequence.

Table II. Experimental and Calculated Chemical Shifts of the Nonexchangeable Base Protons in the Double Helix[a]

		#1	#2	#3	#4	#5	#6	#7
		C	A	A	A	A	A	G
		G	T	T	T	T	T	C
dCA_5G + dCT_5G helix	A H2	--	7.337	(7.084	7.082	6.976)	7.590	--
	C or T H6	7.630	7.356	(7.640	7.617	7.590)	7.760	7.910
	G or A H8	7.964	8.324	(8.173	8.085	8.012	7.920)	7.870
rCA_5G + dCT_5G helix	A H2	--	7.766	(7.229	7.229	6.876)	7.317	--
	C or T H6	*b*	7.860	(7.676	7.621	7.574)	7.502	~8.0
	G or A H8	*b*	8.163	*b*	*b*	*b*	*b*	*b*
Calculation for DNA B form.	A H2	--	6.78	6.92	6.99	7.07	7.73	--
	C or T H_6	7.93	7.83	7.84	7.84	7.85	7.83	7.94
	G or A H8	8.14	8.40	8.32	8.29	8.30	8.30	7.96
Calculation for RNA A form.	A H2	--	6.89	7.12	7.15	7.17	7.61	--
	C or T H6	8.13	7.72	7.73	7.72	7.77	7.77	8.10
	G or A H8	8.05	8.46	7.89	7.83	7.83	7.80	7.98
Calculation for RNA A' form.	A H_2	--	6.80	7.06	6.82	6.89	7.57	--
	C or T H6	8.13	7.71	7.72	7.71	7.76	7.76	8.10
	G or A H8	8.04	8.42	7.79	7.72	7.72	7.69	7.45

Table II. (continued)

[a] Spectra were taken on samples containing 8.0 mM Na_2HPO_4, 20 mM NaH_2PO_4, 0.18 M NaCl, 0.1 mM Na_2EDTA, pD = 7.0, at a concentration of 1 mM per strand of dCT_5G + dCA_5G and 0.5 mM per strand for dCT_5G + rCA_5G. The calculations were done using numbers derived by Arter & Schmidt (1976), and we assumed thymine has the same ring current as uracil. The values in parentheses indicate that we were unable to assign these peaks to particular bases in the sequence (see text).

[b] The poor resolution of the spectrum made assignment of this proton impossible at this temperature.

Table III. Experimental and Calculated Chemical Shifts of the Imino Protons in the Double Helix [a]

	→ C	A	A	A	A	A	G
	G ←	T	T	T	T	T	C
dCT_5G + dCA_5G	12.94	(13.95	14.30	14.08	14.08)	14.56	13.51
dCT_5G + rCA_5G	12.38	13.40	(14.02	13.88	13.80)	14.53	13.52
rCU_5G + rCA_5G	12.52	(13.60	13.76	13.82	13.82)	14.60	13.60
DNA B Form Calculation	12.53	13.50	13.80	13.80	13.85	14.15	13.30
RNA A Form Calculation	12.80	13.46	13.70	13.70	13.70	14.10	13.50
RNA A' Form Calculation	12.72	13.27	13.58	13.58	13.6	14.11	13.47

[a] Spectra were taken on solutions containing 8.0 mM Na_2HPO_4, 20 mM NaH_2PO_4, 0.18 M NaCl, 0.1 mM EDTA, pH = 7.0, at a concentration of 1.0 mM per strand. The calculations were done using numbers derived by Arter & Schmidt (1978), and we assumed thymine has the same ring current as uracil. The values in parentheses indicate that we were unable to assign these peaks to particular bases in the sequence.

FIGURE LEGENDS

Figure 1. ^1H NMR (360 MHz) spectrum of the nonexchangeable base proton region of $dCA_5G + dCT_5G$ in the single strands at 65°C. The sample concentration was 1.0 mM per strand.

Figure 2. Temperature dependence of the nonexchangeable base protons in $dCA_5G + dCT_5G$. Sample concentration was 1.0 mM per strand.

Figure 3. Melting curves (chemical shift versus temperature) for the nonexchangeable base protons on $dCA_5G + dCT_5G$. The sample concentration was 1.0 mM per strand. The single strand melt (X——X) and the double strand melt (●——●) are shown.

Figure 4. Assignments of the nonexchangeable base protons of $dCA_5G + dCT_5G$ in the double helix at 5°C. The sample concentration was 1.0 mM per strand.

Figure 5. Comparison of the imino proton region of the four different helices: (a) $dCA_5G + dCT_5G$, (b) $rCA_5G + rCU_5G$, (c) $rCA_5G + dCT_5G$, and (d) $dCA_5G + rCU_5G$ at 5°C. Spectra (a), (b) and (c) were measured at concentrations of 1.0 mM per strand, whereas (d) was measured for a mixture of 1.0 mM dCA_5G plus 2.0 mM rCU_5G.

Figure 6. Temperature dependence of the imino protons in the dCA_5G + dCT_5G double helix. The sample concentration was 1.0 mM per strand.

Figure 7. Comparison of the temperature dependences of the nonexchangeable base protons for rCA_5G (X——X) and dCA_5G (●——●) in the single strands.

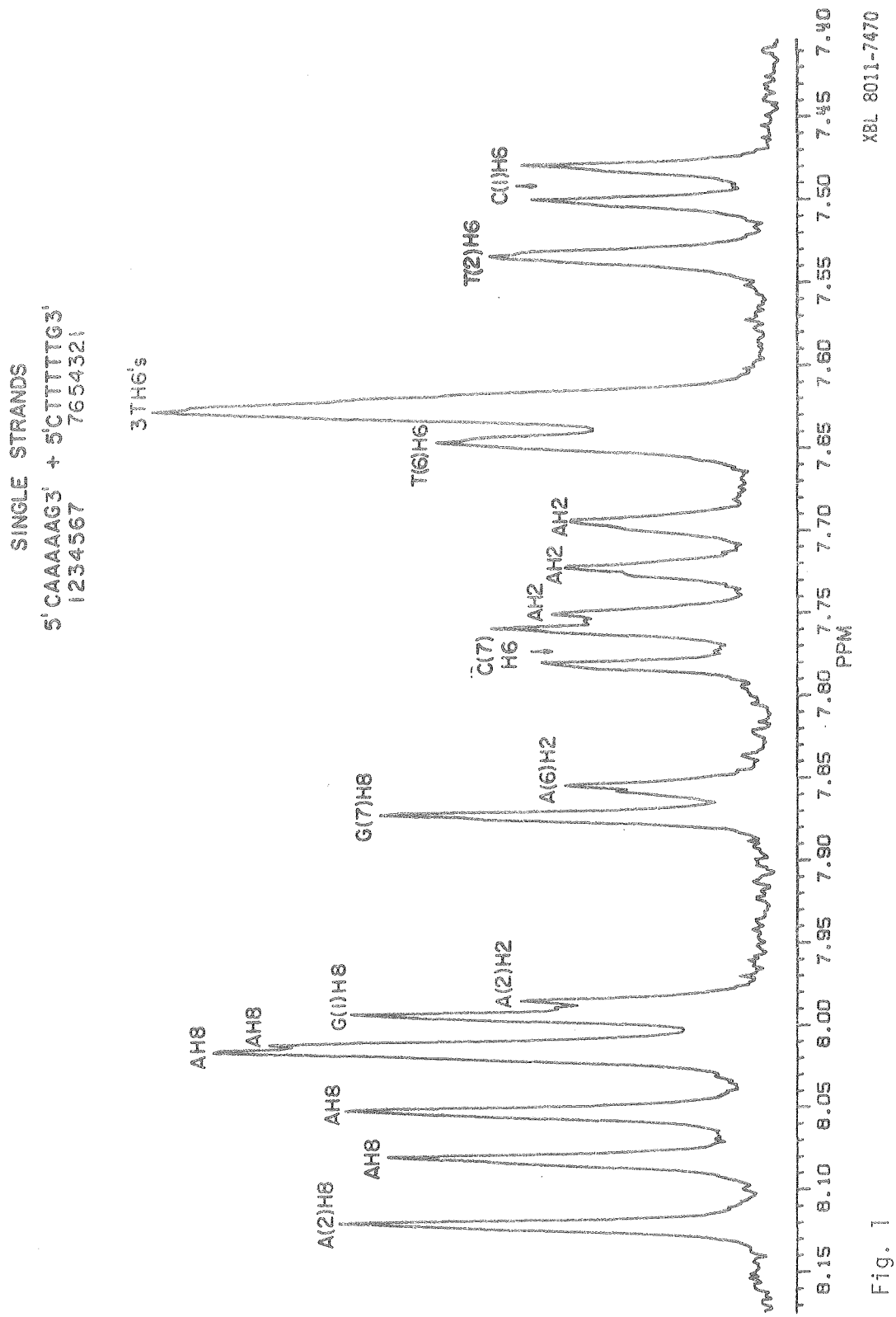

SINGLE STRANDS
5'CAAAAG3' + 5'CTTTTTG3'
1234567 7654321
3 T H6's
T(6)H6
T(2)H6
C(1)H6
C(7) H6
A H2
A H2
A H2
G(7)H8
A(6)H2
A(2)H2
G(1)H8
A H8
A H8
A H8
A H8
A(2)H8
8.15
8.10
8.05
8.00
7.95
7.90
7.85
7.80
7.75
7.70
7.65
7.60
7.55
7.50
7.45
7.40
PPM
XBL 8011-7470

Fig. 1

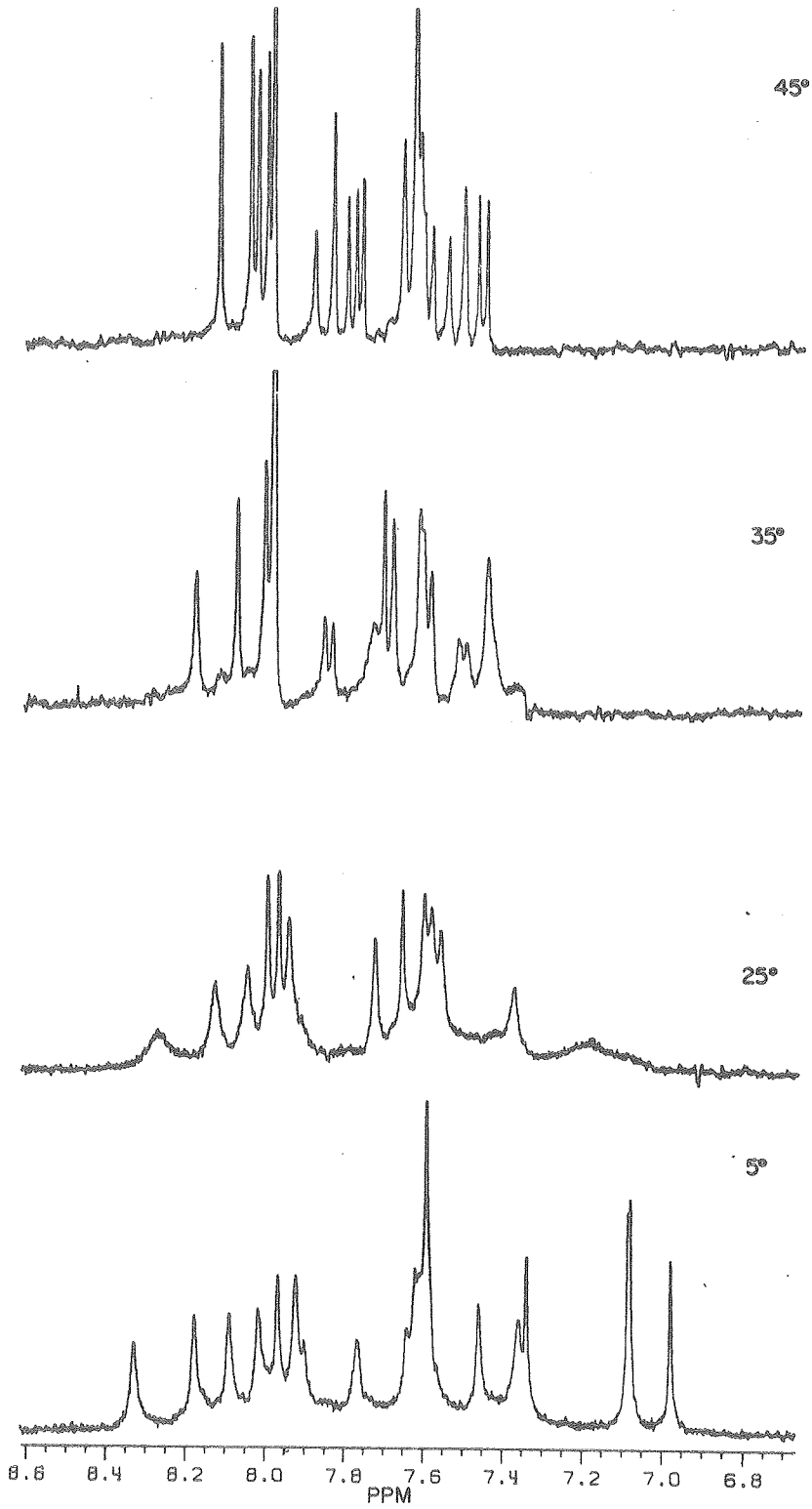


Fig. 2

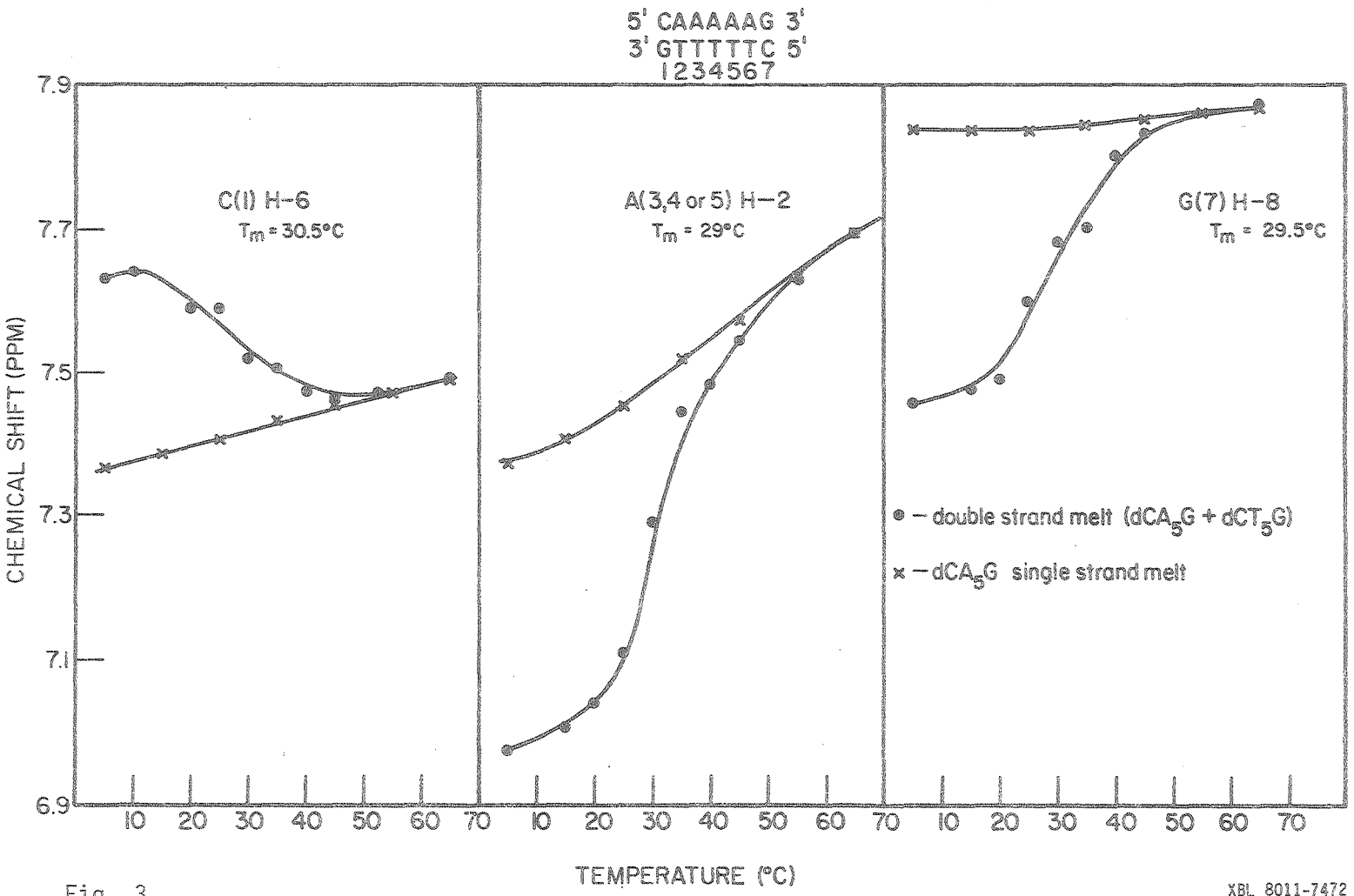
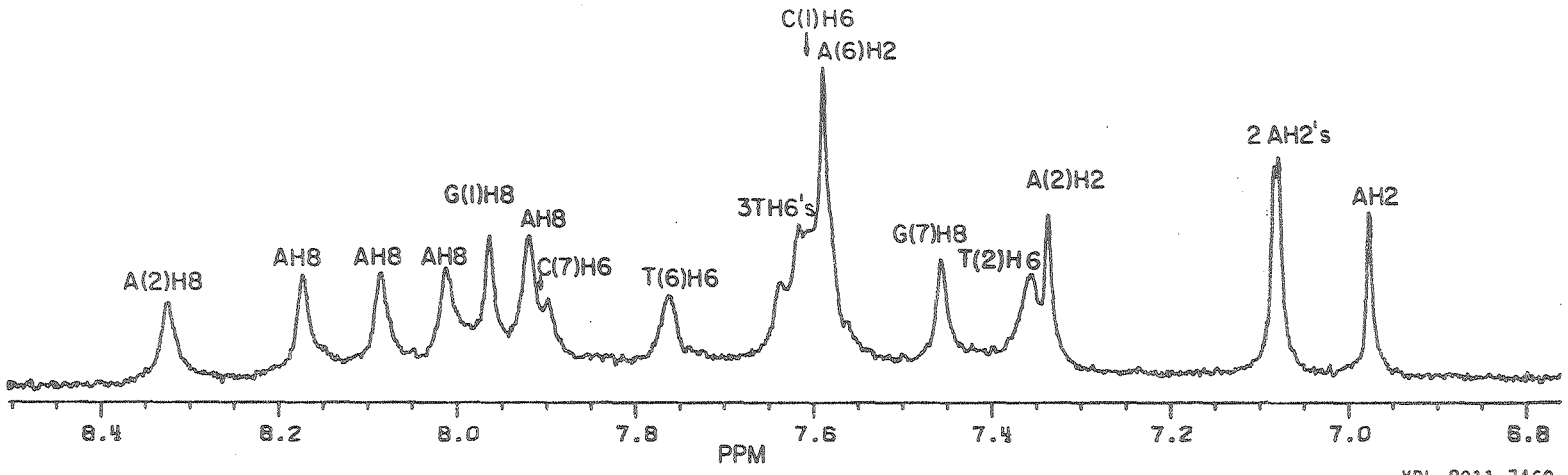


Fig. 3

XBL 8011-7472

DOUBLE STRAND

5'CAAAAAG3'
3'GTTTTTC5'
1234567



XBL 8011-7469

Fig. 4

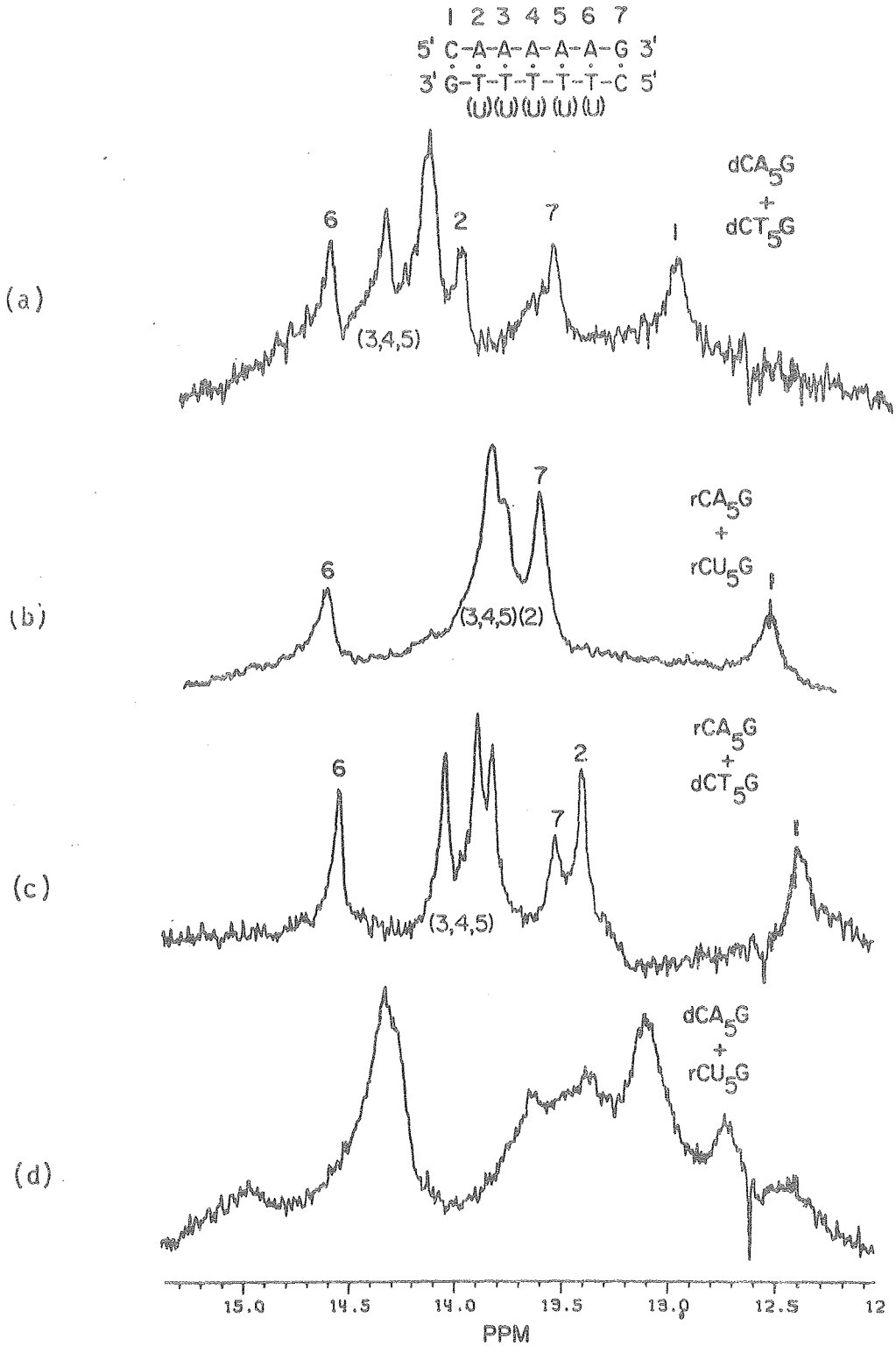


Fig. 5

XBL 7911-13518

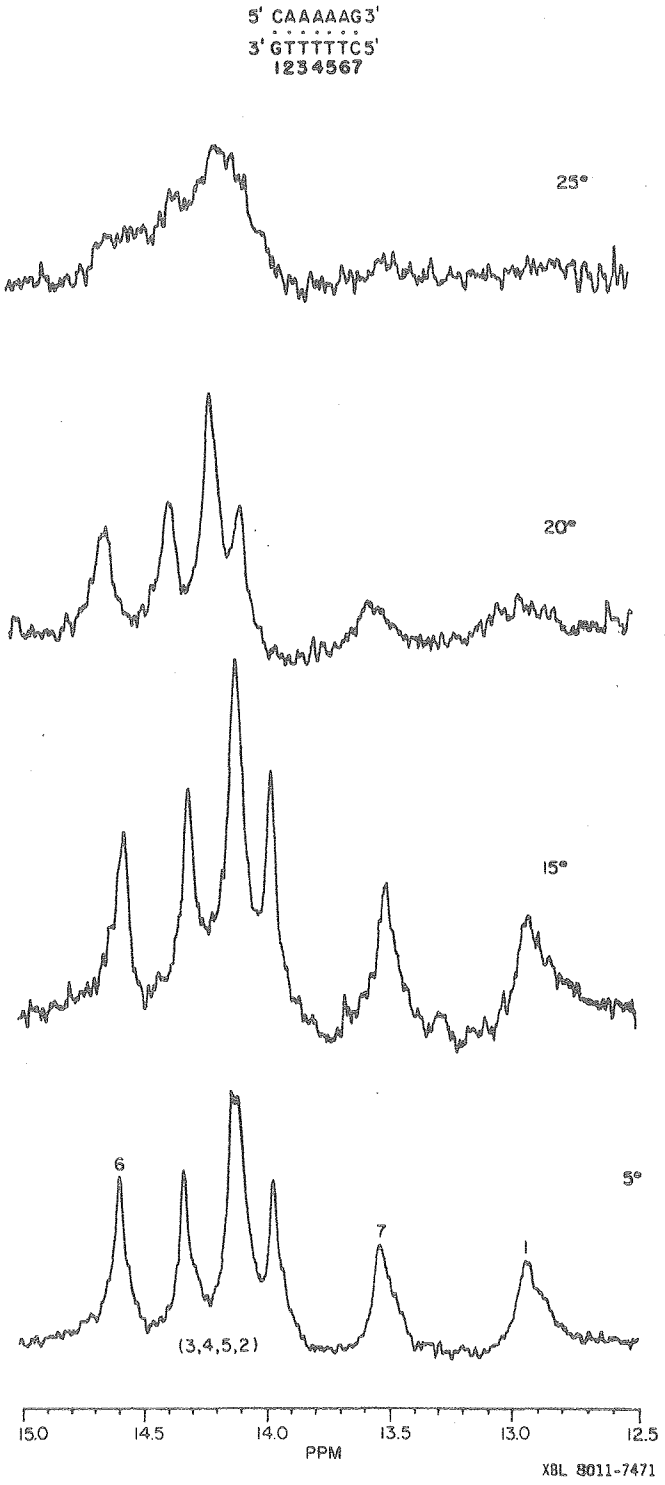

5' CAAAAAG3'
3' GTTTTTC5'
1234567
25°
20°
15°
5°
6
7
1
(3,4,5,2)
15.0
14.5
14.0
13.5
13.0
12.5
PPM
XBL 8011-7471

Fig. 6

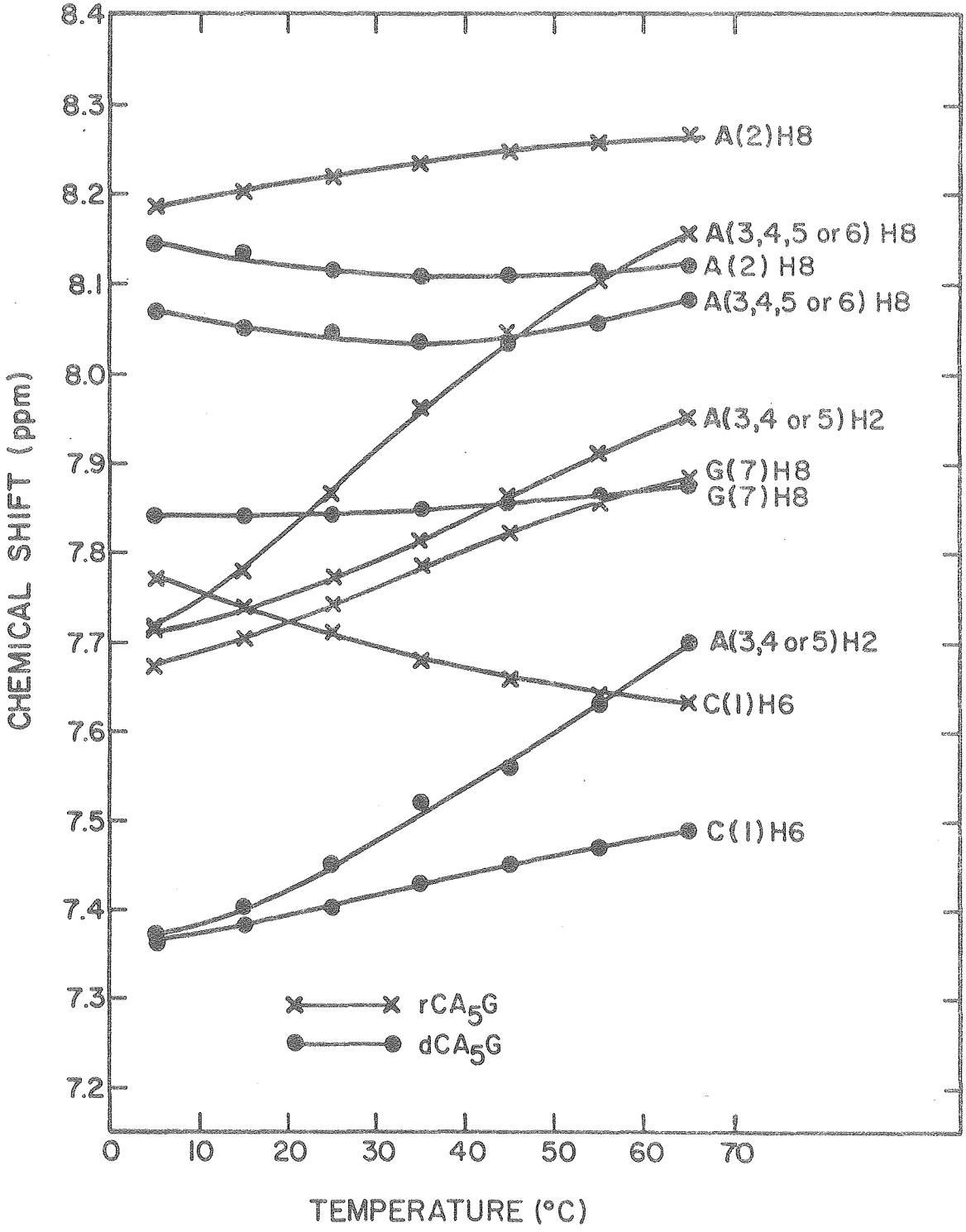


Fig. 7

XBL 8011-7467