DNA OLIGOMERS

AND THEIR

INTERACTION WITH CISPLATIN

To Bob and Marg

and

dedicated to the precious gift of life

SHORT SYNTHETIC DNA OLIGOMERS AND THEIR INTERACTION WITH CISPLATIN

by

BRIAN DENNIS ALLORE, M.Sc.

A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

November, 1988

Doctor of Philosophy (1988) McMaster University
Department of Chemistry Hamilton, Ontario

Title: Short Synthetic DNA Oligomers and their Interaction with Cisplatin

Author: Brian Dennis Allore, B.Sc. (Laurentian University)
M.Sc. (University of Manitoba)

Supervisor: Professors C.J.L. Lock and T. Neilson

Number of Pages: xxii, 284

Abstract

The interaction between short oligodeoxynucleotides of specific sequence with cisplatin, cis-$Pt(NH_3)_2Cl_2$, has been examined under single stranded and duplexing conditions by ^1H nuclear magnetic resonance (NMR) spectroscopy. The oligonucleotides were synthesized by a modified phosphotriester technique adapting procedures initially used in oligoribonucleotide synthesis. The oligo sequences were designed to favour the intrastrand cis-$Pt(NH_3)_2$[d(-GpX_npG-)-N7(1),N7(n+1)] crosslink, where X was up to three thymine units. Variable pH NMR was used to determine the site of platination on the oligomer. Variable temperature NMR was used to determine changes in base stacking and duplex formation resulting from oligomer platination.

In the short oligomers, the intrastrand cisplatin crosslink formed between terminal guanines resulted in the internal section of the sequence being bulged out of a normal stacking orientation. The chemical shifts of the guanine H-8 resonances indicated that platination caused one of the guanine bases to flip from the normal anti geometry into a more syn-like orientation. This was observed for the three oligomers examined (X=thymidine, n=1,2,3). Variable temperature NMR of exchangeable imino protons in the oligomers with larger loops (n=2,3), indicated that the loop region of the complex was a random coil and that water access to the loop was restricted.

Longer sequences were examined which contained the cis-$Pt(NH_3)_2$[-d(GpX_npG)-] complex as well as external nucleotide units, 3′ and 5′ to the platinated region. Platination disrupted intrastrand base stacking of nucleotides externally adjacent to the platinum complex.

Complementary and partially complementary sequences were examined under duplexing conditions. Stabilities, reflected in the T_m of the duplex, of fully complementary hexamer, heptamer and octamer oligomer mixtures were obtained by variable temperature NMR. Changes in duplex stability resulting from oligomer sequence and length along with the nature of the duplex to coil transition were discussed.

Imperfect duplexes, designed to mimic the loop structures formed in platinated oligomers, indicated that the duplex containing one extra core thymine formed a helix resembling the fully complementary hexamer duplex with the extra base stacked out of the helix. With the core of the duplex containing two thymine units and one opposing complementary adenine, both thymines stacked into the helix with the adenine oscillating between the two thymines. When the oligomer mixture contained two extra thymine units with no opposing complements, duplex formation was not observed. A comparison between the duplex to coil transition in normal and imperfect duplexes was discussed.

Similar duplex experiments were carried out using the platinated oligomer sequences. In each case examined, aggregation of the platinated strand, coupled with the oligomer sequences employed contributed to the failure of duplex formation.

ACKNOWLEDGEMENTS

I would like to thank Dr. Colin Lock for his interest in this work and the free hand given to me during my research. I also thank Dr. Tom Neilson for his guidance in the nucleic acid studies, sadly missed but always remembered. A general, all around thanks to the Lock-Neilson-Bell conglomerate (families included), no finer a gathering of people to be found. And a thank you to Dr. Russell Bell for his guidance and encouragement in all my studies. Thanks to Dr. Brian McCarry for stepping onto my committee after the passing of Tom.

The contributions to my development and understanding of my research made by my many co-workers was greatly appreciated (or was it I'd talk to just about anybody about the stuff). Special thanks to Don Hughes, Dick Alkema, Alison Sinclair, Dan Visentin, Gary Buchko, and John Orban (and company) in the nucleic acids/NMR group. To the unforgettable inorganic section, Pierre Pilon (doot doot), Jim Britten and Deb Harvey, Mary Turner, Brenda Brown, and Mary Duarte, a special thanks.

Thanks to Steve Zweep for the studying time, for the physicists, Bruce, Karen and Roger for just liking electrons (why bother with the atoms), Sonia and Claude, and the baseball team. Thanks to HSC Research Development Corporation for their patience.

A special thanks to my parents and my brothers and sisters for their strength, faith and love throughout all my life, I'd be lost without them. And to Lynn, my wife, for her love and support, and for being there through it all, a special thank you.

TABLE OF CONTENTS

List of Figures

LIST OF TABLES

Abbreviations

A, dA	adenosine, 2'-deoxyadenosine
Å	angstrom (10^{-8}cm)
C, dC	cytidine, 2'-deoxycytidine
CD	circular dichroism
ce	cyanoethyl
cis-DDP	cisplatin, cis-dichlorodiamminoplatinum (II)
D_2O	deuterium oxide
DMF	N,N-dimethylformamide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
G, dG	guanosine, 2'-deoxyguanosine
GMP	guanosine monophosphate
gua	guanine
Hz	hertz (cycles/second)
HPLC	high pressure liquid chromatography
ICP	ion charged plasma
IR	infra-red
Ib	isobutyryl
MHz	megahertz
mmTr	monomethoxytrityl, p-anisyldiphenylmethyl
MSNT	1-mesitylenesulphonyl-3-nitro-1,2,4-triazole

MST	1-mesitylenesulphonyl-1,2,4-triazole
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
ORD	optical rotary dispersion
ppm	parts per million
R_f	ratio of distance travelled by solute to that of solvent
T	thymidine
TEA	triethylamine
tlc	thin layer chromatography
T_m	melting temperature of a duplex, point at which strands are 50% duplexed, 50% single stranded
UV	ultraviolet
VT	variable temperature

Abbreviations used in synthesis diagrams:

*	represents protected amino group
p	represents phosphate triester protected by 2,2,2-trichloroethyl group

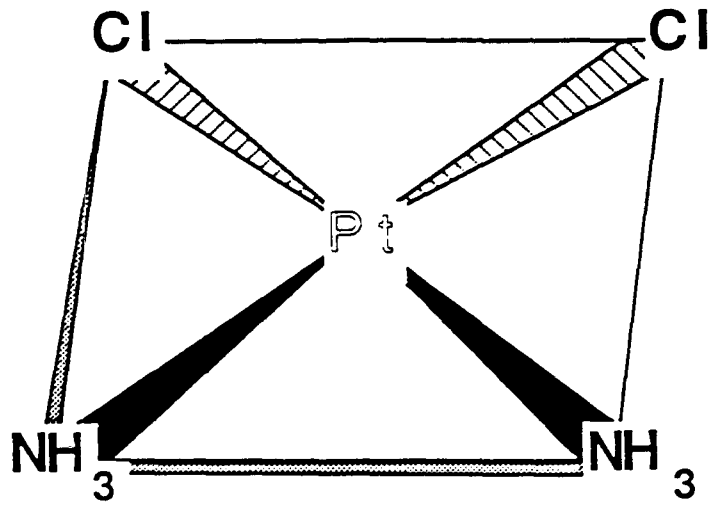
Chapter One

Introduction

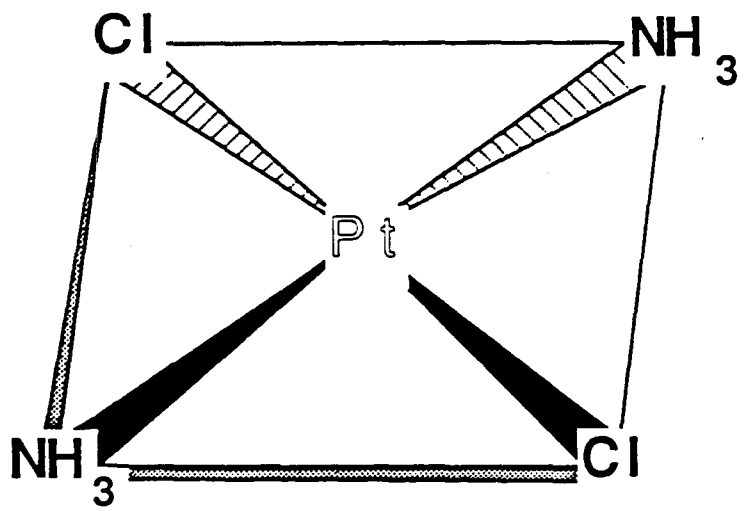
The use of *in vitro* modelling systems to examine and elucidate the mechanisms of biologically significant pathways has been a common course for much chemical and biochemical research. Physical studies on the molecular interaction between a drug and its biological target are useful not only for understanding drug action but results also aid in the improvement of existing treatments as well as the design of new drugs.

1.1 Cisplatin: Discovery and Physiological Application

The serendipitous discovery of the biological activity of *cis*-$Pt(NH_3)_2Cl_2$, (*cis*-DDP, cisplatin), Figure 1, by Rosenberg and co-workers in 1965 (Rosenberg *et al.* 1965), and its subsequent importance in cancer therapy (Rosenberg *et al.* 1969) has led to hundreds of publications involving the platinum complex. The beginning for the platinum compound was the study by Rosenberg and co-workers on the effect of electric current on cell growth. The passage of a low alternating current through cell growth medium via platinum electrodes was shown to inhibit cell division while cell growth was unaffected. Rosenberg found that it was not the direct effect of the electronic current which altered the normal cell growth behaviour but rather a product of platinum metal leaching from the electrodes into the culture media. Since the identification of *cis*-DDP as the actual platinum compound which possessed



CIS



TRANS

Figure 1

Structures of the square planar, cis- and trans-dichlorodiamminoplatinum (II).

the biological activity, it has been shown to have activity against a wide range of mammalian tumors, the most sensitive being testicular, head and neck, and ovarian carcinomas (Wallace et al. 1974; Krakoff et al. 1974; Wiltshaw et al. 1974). Clinically, cis-DDP therapy may involve the administration of the platinum drug alone or in combination with other drugs such as adriamycin, vinblastine, or bleomycin (Bruckner et al. 1977; Einhorn et al. 1976). Bacterial studies involving cis-DDP have demonstrated that the platinum compound is a radiation sensitizer and some work has been carried out on mammalian tumors where the drug is combined with radiation therapy (Richmond & Powers, 1976).

1.2 In vivo Chemistry

Administration of cis-DDP is done in a physiological saline solution which has a chloride ion concentration of approximately 150 mM. Under these solution conditions, the platinum-containing molecule retains its neutral charge and there is no loss of either of the two chloride ligands (Greene et al. 1979; Rosenburg, 1978; Cleare, 1977), Figure 2. Once injected into the body, while the drug remains in the blood or extracellular fluid, where the chloride ion concentration remains at approximately 110 mM, there is no hydrolysis or ligand exchange with cis-DDP. The result of this is that the drug maintains its neutral charge, allowing for easier passage across cell walls. Once inside the cell the fluid environment changes, with the chloride ion concentration dropping to approximately 4 mM, and as a result hydrolysis of the platinum compound occurs (Rosenberg, 1977; Lim & Martin, 1976). The chloride ligands on the platinum are replaced by water molecules which in

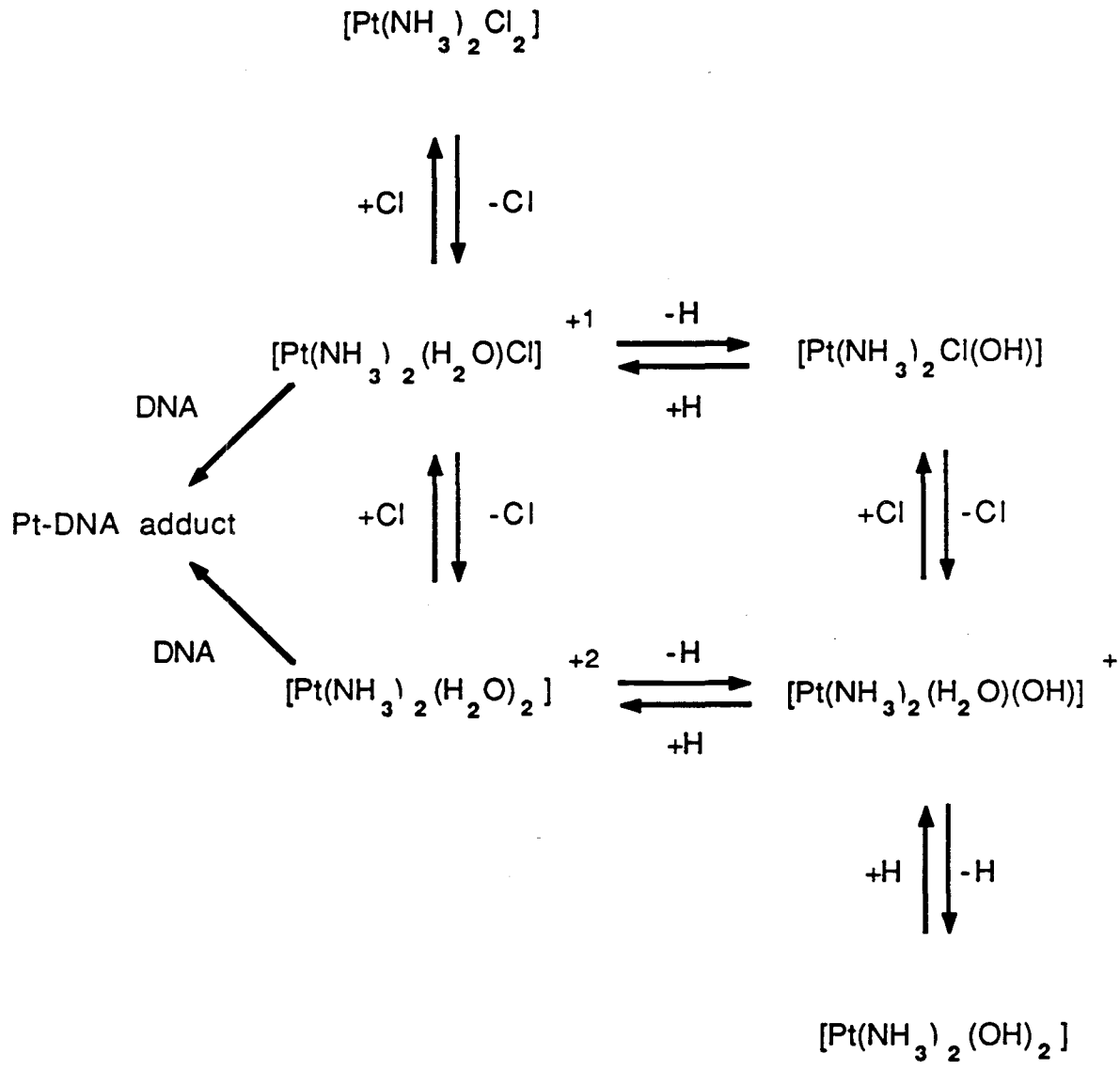


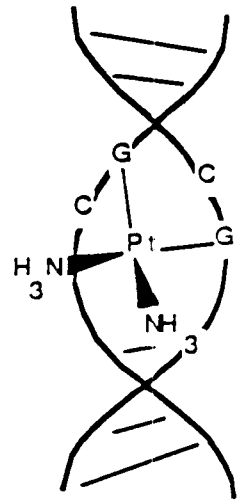
Figure 2 (a)

Hydrolysis of $[Pt(NH_3)_2Cl_2]$, formation of the primary intermediates involved in DNA reaction.

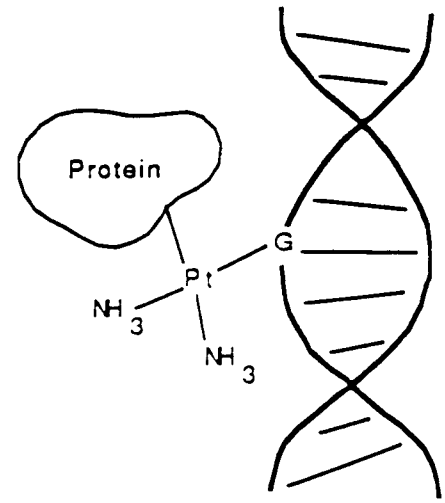
(a) adapted from Johnson et al. (1980).

subsequent reactions may loose protons to form mono- or di-hydroxy compounds. A distribution of species in solution involving the unhydrolysed parent compound, the partially hydrolysed adduct and the fully hydrolysed species will exist, depending on the pH, and the chloride ion concentration of the fluid, (Aggarwal et al. 1980; Macquet et al. 1983). It is mono- and di-aquo substituted compounds which are the biologically active species, Figure 2 (Aggarwal, 1979; Johnson et al. 1980).

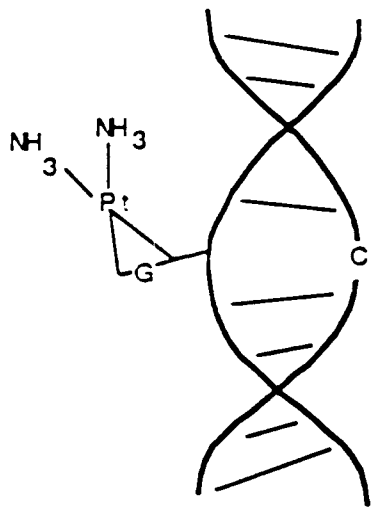
One of the first questions put forth by Rosenberg in his original 1965 Nature publication has not yet been completely answered in 1986: What is the mechanism by which the metal ions inhibit cell division in E. coli, while not interfering with cell growth? Examination of the interaction of cis-DDP with the DNA-containing bacteriophage T7 and with the RNA-containing bacteriophage R17 showed several sites of platinum reaction (Shooter et al. 1972). Minor reaction products included the inter-molecular cross-linking of complementary strands of nucleic acids as well as cross-linking of nucleic acid to protein, Figure 3. It is now generally agreed upon that the most important product from the in vivo platinum reaction is the intrastrand cross-linking of nucleic acids, in particular DNA. Studies on the cellular synthesis of RNA, DNA, and protein showed that cis-DDP treated cells exhibited a selective inhibition of DNA synthesis, while protein and RNA synthesis were not affected (Harder et al. 1976; Pascoe & Roberts, 1974a, 1974b; Howle & Gale, 1970). With these, and the results from many other biological studies, it was proposed that the primary, biologically important site of the platinum reaction was DNA (Roberts & Thompson, 1979; Rosenberg 1977).



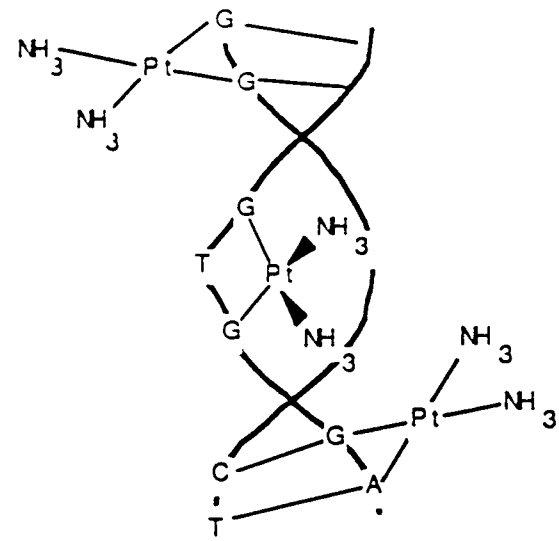
INTERSTRAND CROSSLINKS



DNA-PROTEIN CROSSLINKS



BIFUNCTIIONAL BINDING TO
A SINGLE GUANINE



INTRASTRAND CROSSLINKS

Figure 3

Examples of proposed cisplatin-DNA covalent interactions.

This conclusion is in line with the observed filamentous bacterial growth in Rosenberg's original work. Similar growth patterns have been observed with other agents, i.e. cytotoxic alkylating agents, and UV and X-ray irradiation, all of which react with and damage DNA.

1.3 DNA Structure and Dynamics

Prior to the examination of intermolecular interactions between a drug and its biomolecular target, the two systems must be studied individually to characterize their unperturbed, natural states. This is particularily true in cases involving large macromolecules such as nucleic acids.

The discovery of the double helix conformation of nucleic acids by Watson and Crick in 1953, (Watson and Crick, 1953), has been followed by a multitude of publications whose purpose has been to disentangle the finer structural details of this biochemically important macromolecule. Research concerning the relationship between base sequence, duplex stability and three dimensional structure of nucleic acids to biological activity is a continuing effort. Nucleic acids possess the ability to attain several different conformations, all of which may exhibit a dependence on the experimental conditions employed. Variations in the concentration of the nucleic acid, as well as changes in pH, ionic strength, and temperature are major contributors to the observed behaviour of the nucleic acid in both solution and the solid state (Saenger, 1984).

1.3.1 Basic DNA Structure

In single stranded nucleic acids the individual nucleotide moieties, which are the repeat units in the oligomer, are joined to one another through the 5′-hydroxyl group of one nucleotide to the 3′-hydroxyl group of the neighbouring unit via a phosphodiester linkage, Figure 4. By convention, the base sequence of the nucleic acid strand is written starting from the 5′ end of the strand in the direction of the 3′ terminus. There are two types of heterocyclic, aromatic bases present in nucleic acids, the purines, adenine and guanine, and the pyrimidines, thymine and cytosine. The nucleotide unit consists of a 2′-deoxyribose ring which contains, a phosphate group located at either the 3′ or 5′ hydroxyl function, and an aromatic heterocyclic base located at the anomeric C1′ carbon of the furanose ring. The plane of the aromatic heterobase is approximately perpendicular to the plane of the furanose ring.

1.3.2 Furanose Ring Conformation

The 2′-deoxyribose ring systems are never flat but exist in puckered envelope or twist forms, with the predominant conformations being either C2′-endo or C3′-endo, Figure 5, (Sundaralingam, 1973; Altona and Sundaralingam, 1972). The primary purpose of the pucker in the furanose ring is to minimize the non-bonding interactions between the different substituents on the ring. Solution studies of nucleic acids indicate a rapid equilibrium between the two conformations, with DNA showing a preference for the C2′-endo structure. In the C2′-endo form, the major furanose conformation in B-DNA, the 3′-hydroxyl group is in a pseudo-

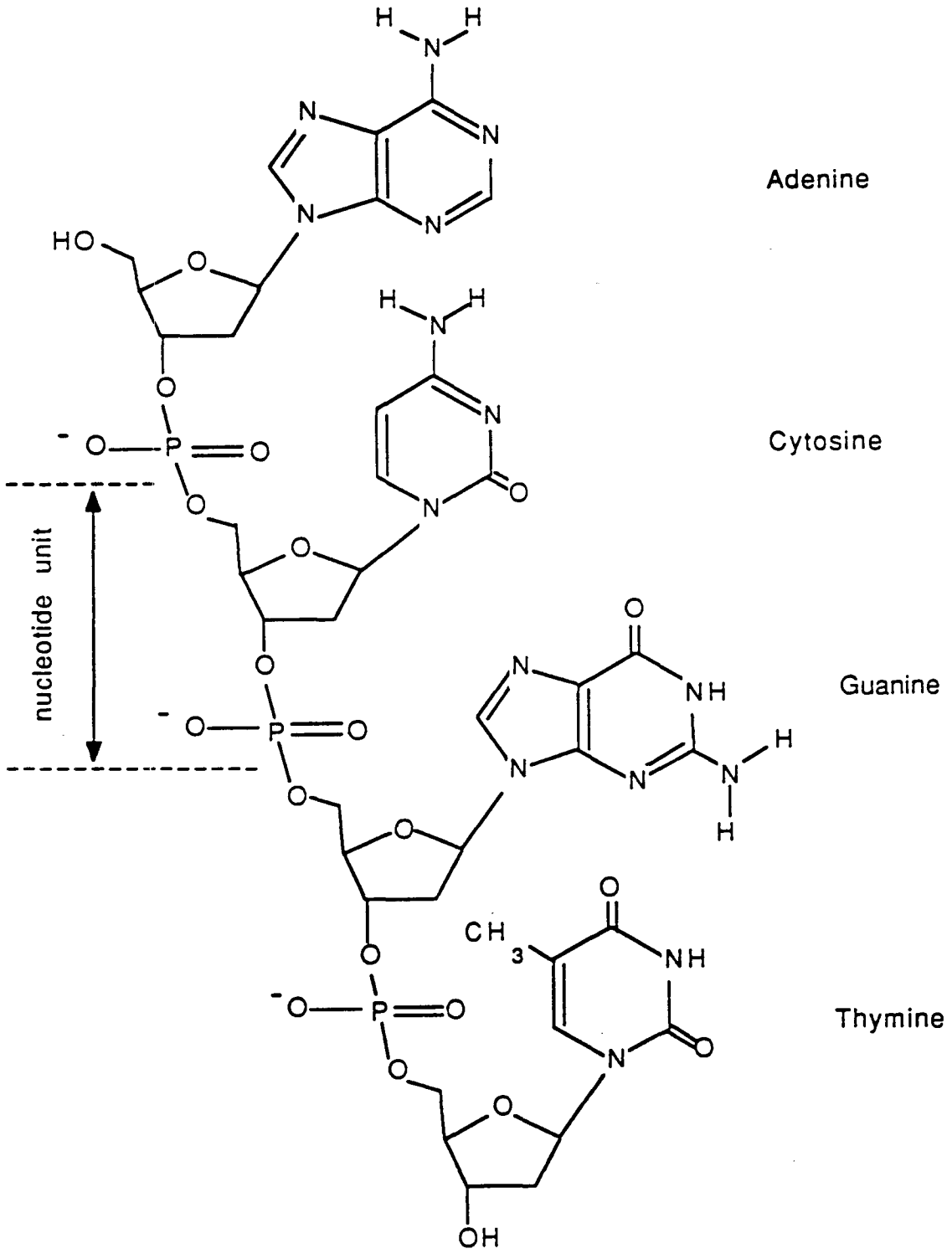


Figure 4

Chemical structure of a DNA tetramer of sequence

5' HO-A-p-C-p-G-p-T-OH 3'.

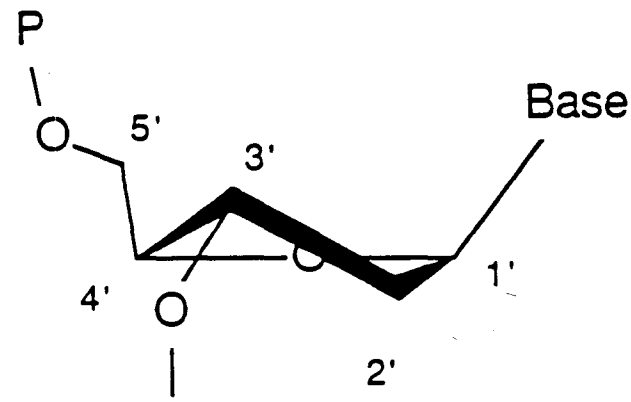
axial position which increases the distance between the 3′ and 5′ phosphodiester groups and stretches out the helix backbone.

1.3.3 Heterocyclic Base Conformation

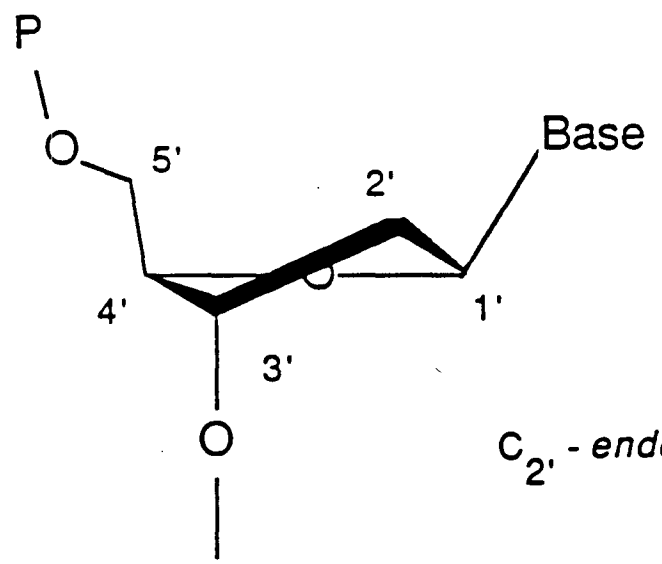
The heterocyclic base has some freedom to rotate about the glycosyl link which joins the furanose ring and the heterobase together. The position of the heterobase relative to the deoxyribose ring is, however, sterically hindered to rotation, restricting the populations of the syn and anti conformers, Figure 6, (Sarma, 1980; Sundaralingam, 1973). In the favoured, lower energy anti conformation, the bulky region of the heterobase is located out, away from the deoxyribose ring resulting in the least amount of intramolecular steric interaction. In the less favoured, higher energy syn conformation, the bulky region of the base sits over the furanose ring and, in situations where the syn conformation occurs the 2′-deoxyribose moiety maintains a C2′-endo configuration which decreases steric interactions. The functional groups present on the pyrimidine heterobases result in a greater hindrance to rotation than that found for the purines.

1.3.4 Watson-Crick Base Pairing and Duplex Structure

Watson-Crick base pairing occurs through hydrogen bonding interactions between specific purine-pyrimidine pairs, either guanine and cytosine, or adenine and thymine, Figure 7, (Donohue and Trueblood, 1960; Watson and Crick, 1953). Hydrogen bonding interactions between two complementary nucleic acid strands running in anti-parallel directions, as well as interstrand strand stacking between adjacent bases, and



$C_{3'}$ - *endo* (N)



$C_{2'}$ - *endo* (S)

Figure 5

Principle puckering conformations of the furanose ring in 2'-deoxy nucleic acids.

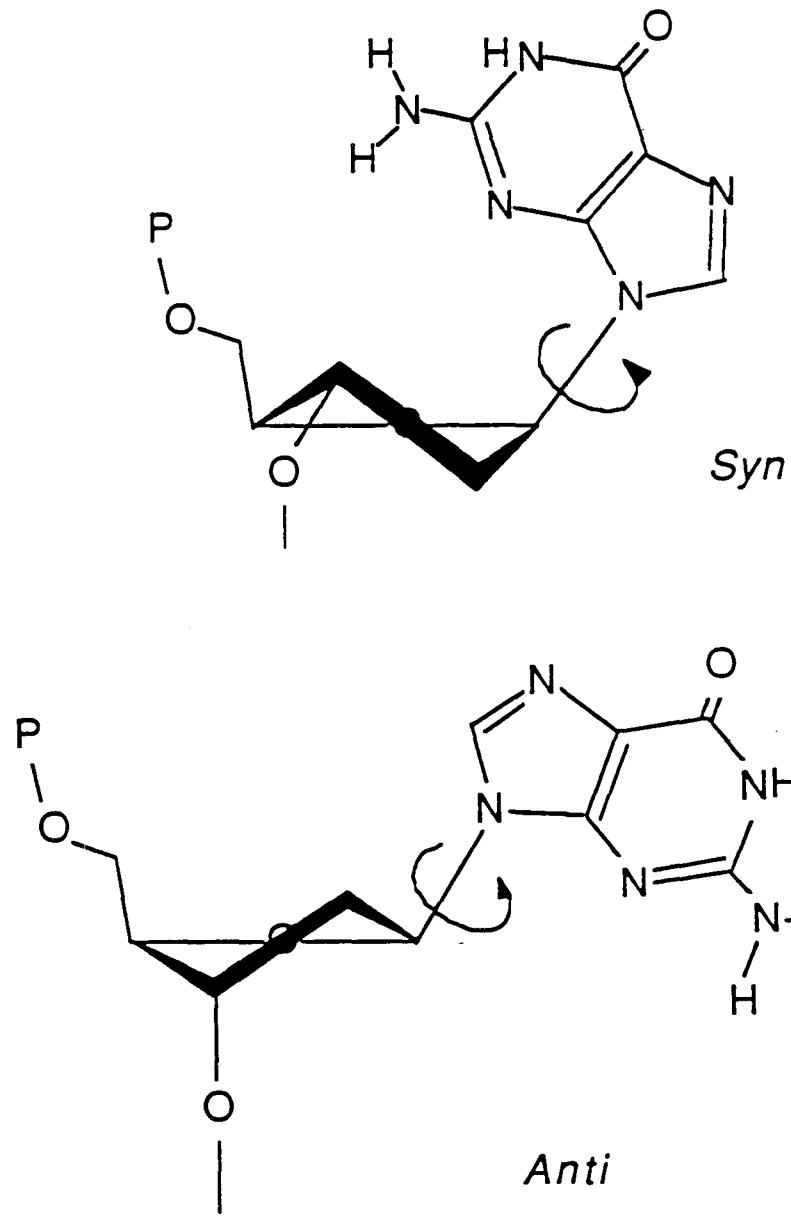


Figure 6

Two predominant base conformations relative to the furanose ring via rotation about the C1'-N glycosyl bond.

solvation effects all contribute to duplex formation. In general, the duplex contains a hydrophobic core region made up of the neutral aromatic bases and, a charged, hydrophylic sugar-phosphate backbone on the outside of the helix. DNA, unlike RNA, has the ability to attain several different helical types. In the right handed forms, DNA is divided into two families, A and B, (Saenger, 1984a). When DNA is in the B form, the helix axis runs up the center of the helix and through the middle of the base pairs. The core of the B-DNA duplex is filled with the hydrophobic bases and the sugar moities are in the C2'-endo conformation. In the A helix, the helix axis runs up the major groove of the duplex with the core of the structure being open and the deoxy sugars in the C3'-endo conformation. Although each component of the repeating nucleotide unit contributes to the three dimensional structure of the duplex, it is the sugar-phosphate backbone which affords the flexibility to the system. The difference between the two sugar puckers in the A and B-DNA forms results in the A structure having a shorter distance between the backbone phosphate groups, overall the A-helix being shorter and fatter than the B structure.

1.3.5 Helix to Coil Transitions

In the simplest model, the two complementary strands of nucleic acids exist in one of two states, the stacked double helix, or the unstacked, single stranded random coil (Cantor and Schimmel, 1980a; Kan *et al.* 1982, Patel *et al.* 1982a). The formation of a perfect duplex occurs via a cooperative interaction between the two complementary random coils. Initiation of duplex formation is dependent on the nucleation of

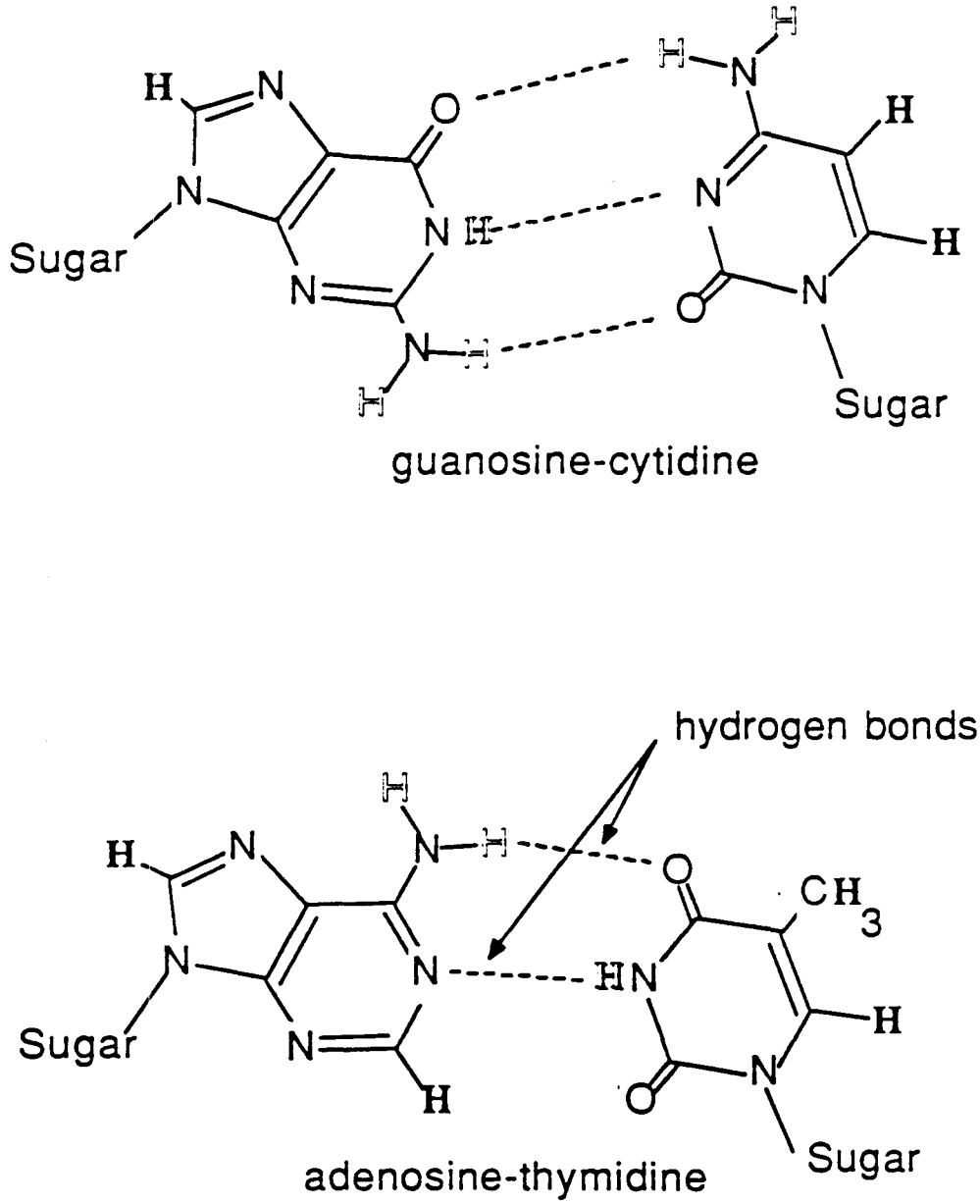


Figure 7

G-C and A-T base pairs occurring in DNA.

Exchangeable imino and amino protons (open), and

non-exchangeable aromatic protons (filled) are highlighted.

a single base pair between the two strands, at which point there is no stability to the structure and it may as quickly dissociate back to the random coil as go on to produce a full duplex. It would appear that at least a three base pair core is necessary before the remainder of the duplex forms in a spontaneous, cooperative manner. The stability of a duplex is reflected in the melting temperature or Tm of the double helix. Because the standard model used for nucleic structure is a two state model where the strands are either duplexed or random coils, the Tm of a particular duplex reflects the temperature at which half the strands are duplexed and the remaining half are random coils (Ornstein and Fresco, 1983a). The Tm of a duplex is dependent on several factors, the length of oligomer strands, the GC/AT ratio in the duplex, and the actual base sequence of the strands (Breslauer et al. 1986). In addition to these factors, the surrounding environment i.e. the ionic strength of the medium and the type of counter ions present will also have an effect on duplex stability (Cantor and Schimmel, 1980b).

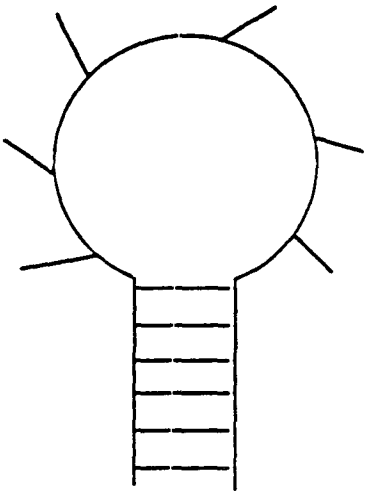
In the fully duplexed form, all of the base pairs may not remain hydrogen bonded to their complement but rather may exist in a rapid equilibrium between the hydrogen bonded base pair and an open state. This open and closed equilibrium is found particularily with the terminal base pairs in a duplex and is known as fraying (Patel and Hilbers, 1975a; Hilbers and Patel, 1975). A similar opening and closing of base pairs may also occur in the core of a duplex in regions containing long repeats of the weaker A-T base pair (Saenger, 1984b).

1.3.6 Imperfect Duplexes

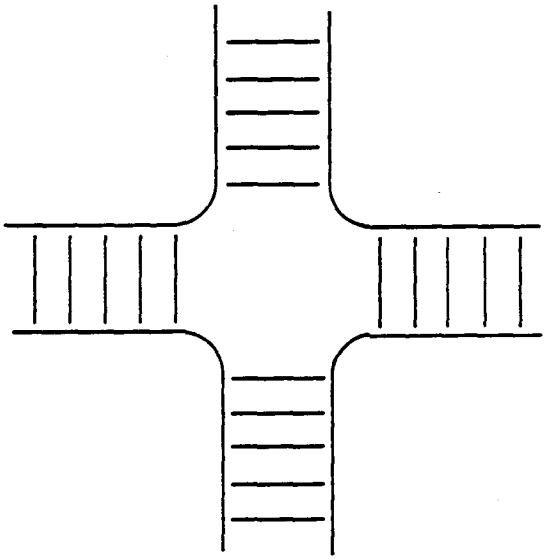
Other types of structures occur in imperfect duplexes when the two oligomers are not fully complementary. Duplexes may contain, bulged loops where regions of non-complementary bases loop out of the stacked structure, interior loops where a single base on one strand does not have a complement on the opposite strand, and hairpin loops which occur when a single strand turns back on itself to form a duplex leaving several bases at the turn structurally unable to participate in base pair formation, Figure 8 (Saenger, 1984c; Gralla and Crothers, 1973; Fink and Crothers, 1972). All of these aberrations in normal duplex structure will affect the overall stabilty of the duplex and will be reflected in the Tm of the double helix.

1.4 Nucleic Acid - Metal Ion Interaction

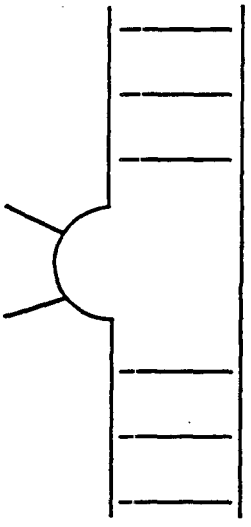
In their natural environment, nucleic acids are found to be complexed to many different metal ions. These, primarily ionic interactions, may occur with metal ions at several different sites on the DNA (Martin, 1985; Saenger, 1984d). Binding sites on the bases may be at either the oxygen or the nitrogen atoms, both of which are hard ligands, although not all of the hetero atoms available for binding are found to interact with equal propensity. The availabilty of a hetero atom to the metal ion may be affected by the electron density of the atom on the base, e.g. the lone pair of electrons on the N(6) amino group on adenine is delocalised into the ring system and makes this site a less favourable ligand site. Metal ion interaction may also be hindered structurally, e.g. the lone pair of electrons on N(3) of cytosine is not delocalised



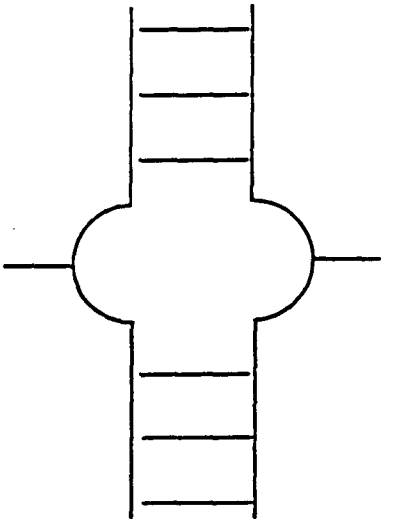
Hairpin Loop



Cruciform Structure



Internal Bulged Loop



Bulge Loop

Figure 8

Hairpin, cruciform and, internal and bulged loop formations found in duplex structures.

into the ring system but is sterically shielded by adjacent functional groups and again is less available for interaction with metal ions. The purine N(7) site is found to be a good ligand site for metal ions as this nitrogen atom is fully exposed even when the base is involved in a duplexed structure. Metal ion interaction is also found to occur at the negatively charged phosphate groups on the helix backbone with the metal ions surrounding the negatively charged phosphate oxygens helping to shield the electronic interactions between the adjacent charged groups.

1.5 Platinum-DNA Studies: Primary Mode of Reaction

1.5.1 Interstrand Cross-Linking

With the recognition of cellular DNA as the primary lesion site resulting from platinum treatment, many reseachers dedicated their work towards the understanding of the mechanism of interaction between cis-DDP and the nucleic acid. Comparisons have been drawn between the mechanism of activity of cis-DDP and classical alkylating agents because of the similarity in requirement for bi-functional reactivity and, biochemical effects (Roberts & Pera, 1983; Roberts et al. 1971). Many types of alkylating agents, i.e. the nitrogen mustards, are known to attack nucleic acids directly and form intermolecular cross-links between complementary strands. Once the strands are covalently linked to each other, further participation by either strand in a DNA replication would be impossible. This type of cross-linking in DNA by platinum does not appear to be biologically significant as this product represents only a small fraction, approximately one percent, of the isolated DNA-platinum adducts after treatment with the platinum compound (Eastman, 1982; Butour

& Maquet, 1981; Roberts & Friedlos, 1981; Shooter et al. 1972; Ball & Roberts, 1970). Studies involving both the cis and the trans-DDP isomers indicated that both isomers exhibited at least an equal propensity for binding to cellular components, i.e. DNA, RNA, and protein (Pascoe & Roberts 1974a, 1974b), with cis-DDP showing a greater ability for crosslinking cellular DNA (Roberts and Pera, 1983). The inability of the trans isomer to inactivate bacterial cell division suggests that the interstrand interaction is not biologically significant (Braddock et al. 1975; Shooter et al. 1972). The reaction of alkylating agents at the O(6) position of guanine is a known means of DNA mutation which leads to mispairing during replication (Loveless, 1969). There was suggestion that cis-DDP may interact with DNA in a similar fashion (Rosenberg, 1978; Millard et al. 1975; Gerchman & Ludlum, 1973). Although platinum reaction with the guanine O(6) site was never shown to occur in the same fashion as alkylation, the participation of the guanine O(6) position has always been a current topic and will be further discussed.

1.5.2 Intrastrand Cross-Linking

Another mode of platinum binding to DNA is intrastrand cross-linking, (see Figure 3). Many of the early studies on cis-DDP:DNA interactions suggested that it was the intrastrand binding which was the product of major biological importance (Filipski et al. 1980; Roos, 1977; Shooter et al. 1972). Investigations into the exact nature of the intrastrand binding led to several types of platinum chelates being proposed. Spectroscopic analysis, involving information obtained from UV, CD, and ORD studies, indicated that absorbances assigned to the sugar

and phosphate groups of the nucleic acid showed little change as a result of platinum binding (Tamburro et al. 1977; Munchausen & Rahn, 1975; Stone et al. 1974; Horacek & Drobnick, 1971). The major spectral changes, resulting from the formation of the cis-DDP:DNA complex, were observed in regions assigned to the nucleic acid bases. As a result, the primary site of platinum attack on the deoxyribonucleic acids was concluded to be on the nitrogenous bases (Mansy et al. 1973). This result is consistent with the chemical reactivity of the platinum(II) ion which is known to interact only weakly with oxygen, while forming stronger complexes with nitrogen containing ligands.

With the knowledge that the nucleic acid bases were the primary site of platinum attack, it was necessary to determine which, if any, of the bases were more active towards platinum attack, and what was the exact reactive site. Reactions were carried out between cis-DDP and several different natural DNAs, each characterized by their widely differing base composition. Spectroscopic examination of the platinum-DNA adducts revealed that the strongest spectral variations were observed in the DNA which contained abnormally high G-C content (Ganguli & Theophanides, 1979; Roos, 1977; Tamburro et al. 1977; Millard et al. 1975; Stone et al. 1974). It has also been shown that although there were products resulting from the platination of adenine and cytosine, the major reaction product involved a guanine-platinum adduct. Several groups have investigated the exact site of nucleobase platination using NMR and single crystal X-ray diffraction studies. These studies, on the formation of platinum-nucleobase, platinum-nucleoside, or platinum-nucleotide complexes, revealed that the major site of reaction

was on the bases which was in agreement with the conclusions reached from optical spectroscopic data. Under neutral pH conditions, the N(9) alkylated purine bases guanine and adenine react with cis-DDP at the N(7) site for both bases, (Lock et al. 1976b; Goodgame et al. 1975) as well as the N(1) position on adenine, Figure 9. Under the same neutral pH conditions the N(1) alkylated pyrimidines bases, thymine and cytosine, showed platination only at the N(3) site of cytosine (Lock et al. 1976a). Platinum binding to other nucleobase sites has been observed when reaction conditions were altered i.e. changing the pH of the medium causing protonation or deprotonation of different sites on the bases (Faggiani et al. 1981a, 1981b; Lippert & Schubert, 1981; Kistenmacher et al. 1979; Lock et al. 1978). These results led to further speculation as to the exact nature of the platinum intrastrand cross-link. The two proposals which gained primary attention were bidentate platinum binding to two different guanine bases on the same strand, and bidentate platinum binding to two sites on the same guanine base.

1.5.3 Intrastrand Cross-linking: The N(7),O(6)-Guanine Postulate

The proposed interaction between the platinum(II) ion and a single guanine base involves the initial attack on platinum by the N(7) site of the base followed by a second attack on platinum by the neighbouring O(6) site (Chu et al. 1978; Tamburro et al. 1977; Dehand & Jordanov, 1976). This type of interaction can only occur when the active sites on the platinum atom are cis to one another (Rosenberg, 1978; Macquet & Butour, 1978; Maquet & Theophanides, 1976; Goodgame et al. 1975). There were several arguments which supported this type of platinum binding. One is

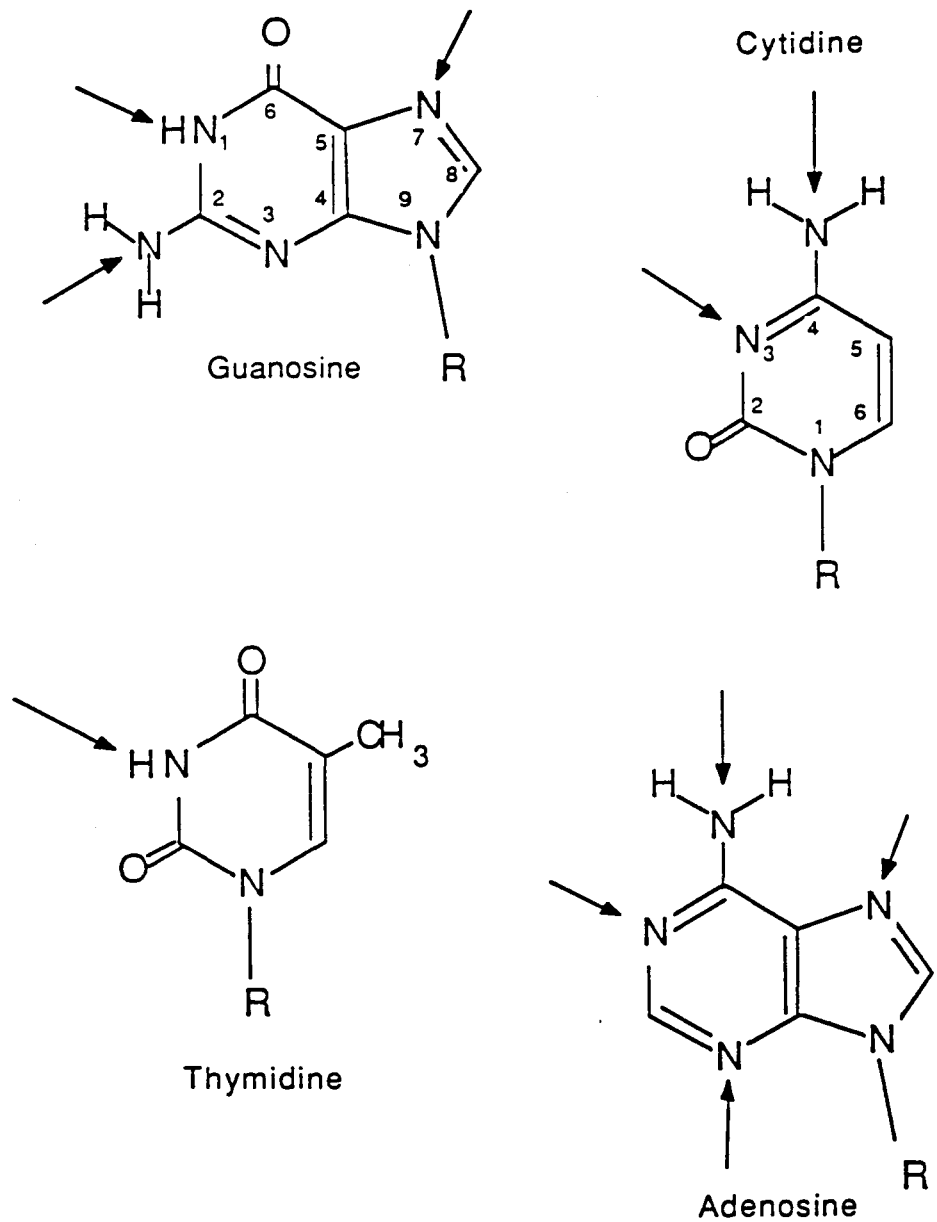


Figure 9

Sites of platination on the four nucleobases, reaction positions are indicated with arrows.

centred on the interaction of cis-DDP with the fully duplexed form of DNA. It has been suggested that any inter-base platinum binding, while the strands are involved in the duplexed structure, would meet with considerable steric hinderance. The conformational structure of Watson-Crick base pairs, i.e. twist angles, base pair rolls and twists, and individual base propeller twists, as well as the constrained square planar geometry about the platinum (II) ion would all contribute to the type of binding which would occur between the cis-DDP and DNA. All of these conformational restrictions led to the postulate that if the platinum reaction occurred with duplexed DNA, there was little chance of the reaction occurring between two separate bases and was most likely to occur solely on one guanine base. This thought in turn, led to the suggestion of a cis-DDP-[N(7),O(6)-guanine] complex and was supported, although not conclusively, by X-ray photoelectron and infra red spectroscopy results (Hadjiliadis & Theophanides, 1976; Millard et al. 1975; Macquet et al. 1975). The biological activity for this type of complex was thought to occur as a result of disruption of the Watson-Crick hydrogen bonding ability of the guanine base after reaction with platinum.

Upon the initial binding of platinum to the N(7) site there is an increase in the acidity of the N(1)-H proton of the guanine base, normally pK 9.8-10 (Martin, 1985; Saenger, 1984e), reflected in the observed decrease in the pKa of the N(1)-H proton by 1.5-2.0 pK units to a pK value of 8.5 (Dijt et al., 1984). This affect may allow for a shift in the keto-enol tautomerization equilibrium at the N(1)-O(6) amide site with a possible increase in concentration of the enolate structure which

may in turn attack the N(7) bound platinum forming the proposed N(7),O(6)-guanine bidentate chelate, Figure 10 (Rosenberg, 1978). Once formed, the chelated species decreases the ability of reforming the normal keto tautomer.

The overall effect of [N(7),O(6)-guanine]-platinum binding is the destruction of two hydrogen bonding sites on the guanine resulting in the loss of a G-C base pair. Involvement of the platinated base in strand replication, with the change in tautomeric form, causes the complexed site to more resemble the hydrogen bonding pattern of an adenine base rather than the correct guanine species, Figure 11 (Faggiani et al. 1980). This alteration would result in a sequence mutation. If several platinated guanine bases were present on the nucleic acid strand being replicated, there would be multiple coding mistakes (Rosenberg, 1978). The end result of the platinum-N(7),O(6)-guanine chelate being the destruction of the genetic information contained in the original sequence. Although there are many early publications in support of this type of platinum-guanine interaction, this mechanism as an explanation for the biological activity of cis-DDP is not favoured.

Recently, Wing and coworkers have reported some results which support the [N(7),O(6)-guanine]-cis-DDP chelate although not of the form discussed above (Wing et al. 1984). In their work, a crystal of the double helical "Dickerson dodecamer" was soaked in a solution of cis-DDP to allow interaction between the duplexed DNA and the platinum compound. Under the experimental conditions used to obtain the platinum bound duplex, X-ray data indicated binding of the platinum molecule to the N(7) site of guanine with a possible stablizing interaction between the

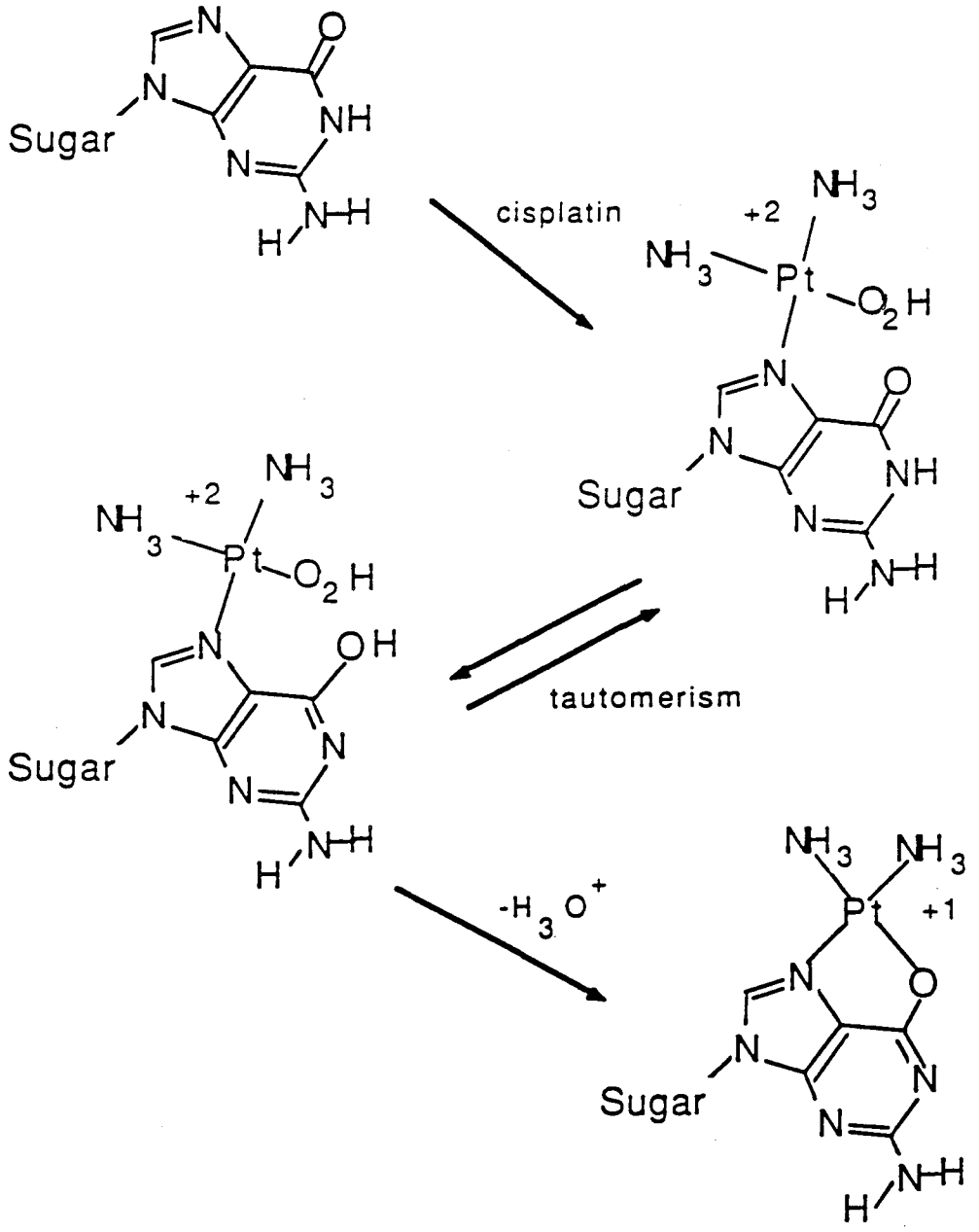


Figure 10

Possible mechanism of guanine-N(7),O(6)-cisplatin chelate formation via tautomerization after initial platinum binding to G-N(7) site.

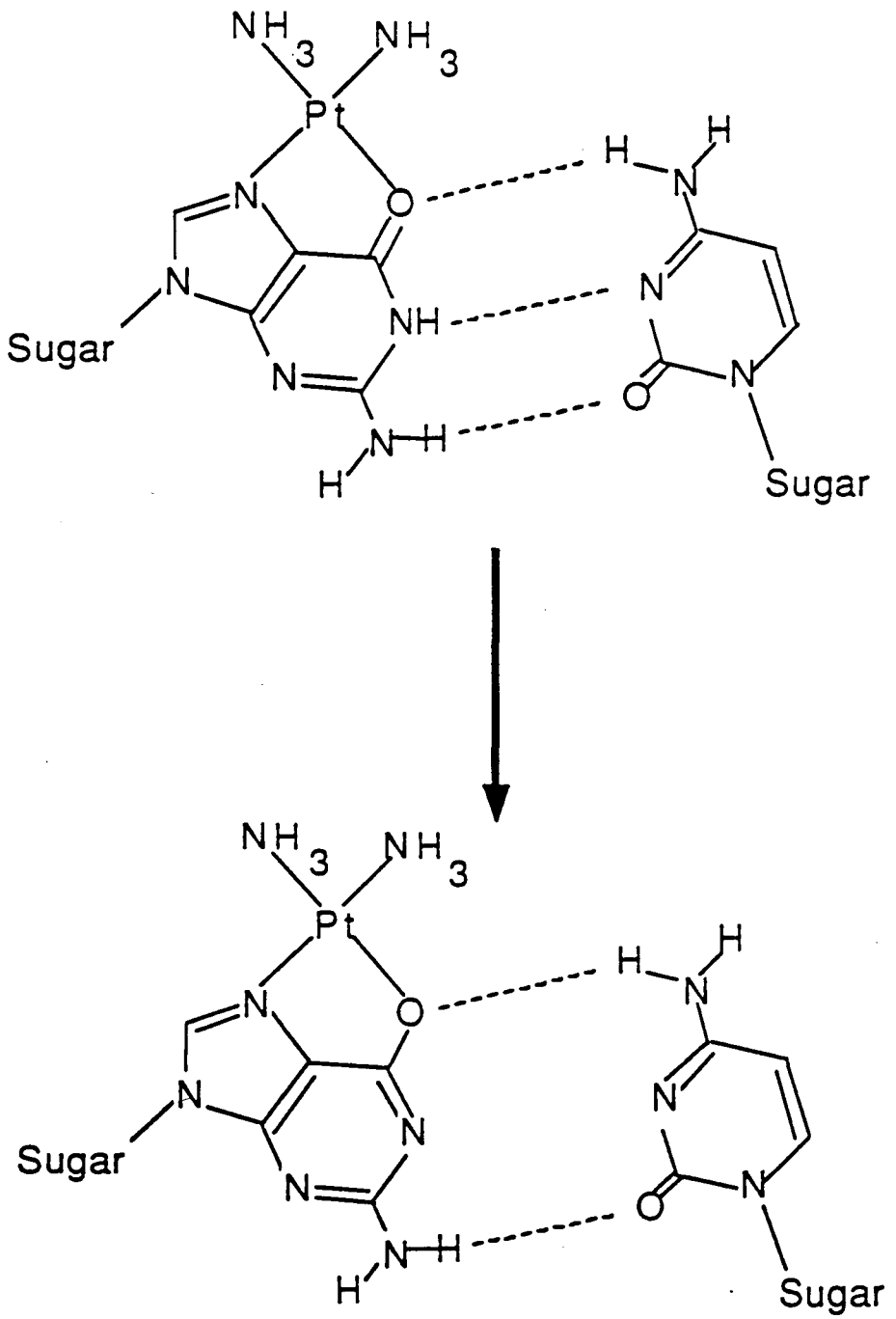


Figure 11

Disruption of G-C base pair hydrogen bonding scheme as a result of cisplatin-[N(7),O(6)-G] chelate formation.

platinum atom with the guanine O(6) position via a bridging water ligand. Wing notes, however, that the distance between the platinum and the O(6) atom is too long to be a direct bond and too short to include a molecule of water. The long distance between the platinum and O(6) negates the possibility of the mechanism previously discussed which included a direct platinum-oxygen bond. The distance between the platinum atom and guanine O(6) position is too short to directly accomodate a bridging water molecule, suggesting that the square planar platinum complex would need to be rotated out of the plane of the five membered ring to include the bridging ligand, Figure 12. This mode, and orientation of cis-DDP binding to guanine as an initial interaction between the antitumor compound and DNA has been previously suggested by Goodgame (Goodgame et al. 1975). Wing also suggests that the resultant movement of the complexed guanine base may in some way weaken, and lead to the eventual rupture of the glycosyl bond. The final result of platination may be the depurination of the complexed base which may, during DNA repair processes, lead to mutagenesis. This mechanism involving the cleavage of the glycosyl bond is similar to that put forth for the action of some alkylating agents (Foster et al. 1983), which cause depurination followed by sequence mutation upon strand replication (Brouwer et al. 1981). In contrast to this proposal, it has been shown that the platination of the N(7) site of guanosine does not weaken the base-sugar glycosidic linkage (Johnson et al. 1985, 1982).

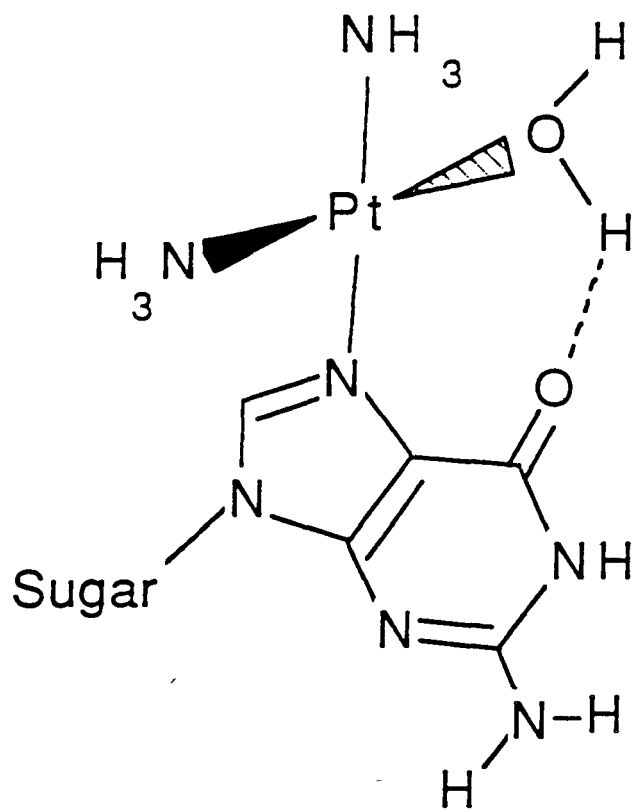


Figure 12 (a)

Proposed cisplatin-[N(7),O(6)-G] chelate via a solvent bridge.

(a) adapted from Wing et al. (1984).

1.5.4 Intrastrand Cross-Linking Between Two Guanines

The generally accepted pathway for cis-DDP activity is the formation of an intrastrand cis-$Pt(NH_3)_2$[d(-GpG) N(7),N(7)] chelate which was first proposed by P.J.Stone and coworkers in 1974 (Stone et al. 1974; Kelman et al. 1977). This mode of binding to DNA has gained support from enzymatic digestion of platinated DNA by use of either exonucleases or restriction endonucleases, as well as chemical digestion and examination of the adducts by various chromatographic techniques (Eastman, 1986, 1985, 1983; Fichtinger-Schepman et al. 1985; Johnson et al. 1985; Inagaki et al. 1984). Changes in enzymatic cleavage patterns between the normal and the platinated DNA indicated platinum binding to adjacent guanine bases, i.e. cis-Pt[dp(GpG)] structures as well as structures of the form cis-Pt[dp(GpNpG)] where N can be any of the four bases (Fichtinger-Schepman et al. 1982).

The intrastrand cross linked structures have received great attention from many research groups and as a result have produced many publications (den Hartog et al. 1985a, 1984a 1983; Sherman et al. 1985; Neumann et al. 1984). Studies on this type of drug-nucleic acid interaction lend themselves to the use of model systems involving short synthetic DNA oligomers.

In a study by den Hartog and co-workers, they have synthesized and isolated the cis-$Pt(NH_3)_2$[d(GpG) N(7),N(7)] complex (den Hartog et al. 1982). NMR analysis of this compound at 500 MHz produced the fully elucidated solution structure with several interesting features. The two guanine bases in the chelate maintain the normal anti geometry about their respective glycosyl bonds. The two 2'-deoxyribose rings exhibit

different conformations with respect to furanose pucker. The furanose associated with the 5′ base unit had taken up an unusual deoxyribose conformation, 100% N(C3′-endo). The 3′ terminal furanose was found to exist in the expected, normal S(C2′-endo) conformer. The authors stated that the resulting structure contained a noticeable kink in the backbone of the molecule which, if present in DNA, would be expected to lead to a local denaturation of the duplex.

In a continuation of this line of study involving longer synthetic oligomers containing the remaining three bases, thymidine, adenine, and cytosine as well as the adjacent guanine bases, results indicated that with all options available, the platinum compound reacted primarily with the adjacent guanine bases (Eastman, 1986; Fichtinger-Schepman et al. 1985; Inagaki & Kidani, 1985; Johnson et al. 1985; Cohen et al. 1980). Using oligomer strands which were self-complementary and containing adjacent guanine bases complexed to the platinum compound, several groups have shown that the presence of the chelate in the strand completely disrupts the possibility of duplex formation (Girault et al. 1984b; Caradonna et al. 1982). An apparent drawback in several of the experiments which examined the effects of platination on duplex stability was the self-complementary nature of the strands. Platination of the oligomer, and the subsequent attempt at duplex formation, would lead to a double helix containing two chelated regions both, because of the short sequence length, in close proximity to each other. In all cases examined where this type of arrangement was present, a total disruption of the dimerization process was observed. From these results came the suggestion that platination of the deoxy oligomers caused the loss of

normal hydrogen bonding ablity for the complexed bases, and consequently the denaturation of the platinated region of a double helix (Girault et al. 1984b; Caradonna et al. 1982).

Earlier work has indicated that the reaction of DNA with cis-DDP results in changes of duplex structure and stability. Platination of DNA has shown to result in an overall decrease in the length of the oligomer strand, this result suggesting a disruption of hydrogen bond formation and a localized unwinding of the duplex (Scovell & Collart, 1985; Cohen et al. 1980, 1979). The decrease in strand length was accompanied by displacement of bound water in the region of metal binding (Tamburro et al. 1977; Sinex & Stone, 1974). The stability of the double helical structure, reflected in the Tm of the duplex, exhibited a decrease in the transition temperature. Accompanying the drop in melting temperature was an unusual premelting behaviour observed while proceeding through the transition from the duplex to the coiled, single stranded structure (Tullius et al. 1983; Ganguli & Theophanides, 1981).

Recently, the examination of platinated synthetic oligomers has shown that duplex formation does indeed occur when the oligomers involved are not self-complementary but a mixture of complementary strands (den Hartog et al. 1984a, 1984b; Van Hemelryck et al. 1984). It was also clearly demonstrated in these studies that the platinated region of the duplex fully participates in base pair formation.

1.5.5 Single Crystal X-Ray Structure of cis-$Pt(NH_3)_2$[dpGpG]

In addition to the previously discussed results, the first single crystal X-ray diffraction study has been done involving cis-platinum and

a nucleic acid oligomer. The dimer dpGpG, has been studied as well, molecular mechanical calculations determining the effect of platination on duplex structure (Sherman et al. 1985; Kozelka et al. 1986, 1985). The X-ray structure, Figure 13, shows that the platinum atom remains in a square planar geometry after complexing to the deoxy dimer with the dihedral angle between the two guanine bases being approximately 80°. The extent to which the platinum chelate pulls the guanine bases together completely disrupts any normal intrastrand base stacking arrangement although both bases were still found to be in the normal anti conformation in a head-to-head orientation, that is with both O(6) oxygen atoms on the same side of the platinum coordination plane. The retained anti conformation about the glycosyl torsion angle allows for the hydrogen bonding regions of the two metalated guanine bases to be structurally available for base pair formation as previously observed by den Hartog (den Hartog et al. 1984b). The observed distance between the guanine O(6) oxygen atoms and the amine ligands exceeded 3.1 Å. The unit cell contained four crystallographically independent molecules, each with slighty different sugar conformations. In general, the 5′ terminal sugar was found to exist in the N(3′-endo) conformation. The 3′ terminal sugar, although exhibiting a greater pseudorotational range between the different structures, tended more towards the S(2′-endo) conformation. Of major interest, and with possible in vivo mechanistic implications, was the identification of a hydrogen bond between the 5′-terminal phosphate group and one of the amine ligands on the platinum atom. The presence of the hydrogen bond may be connected to the observation that

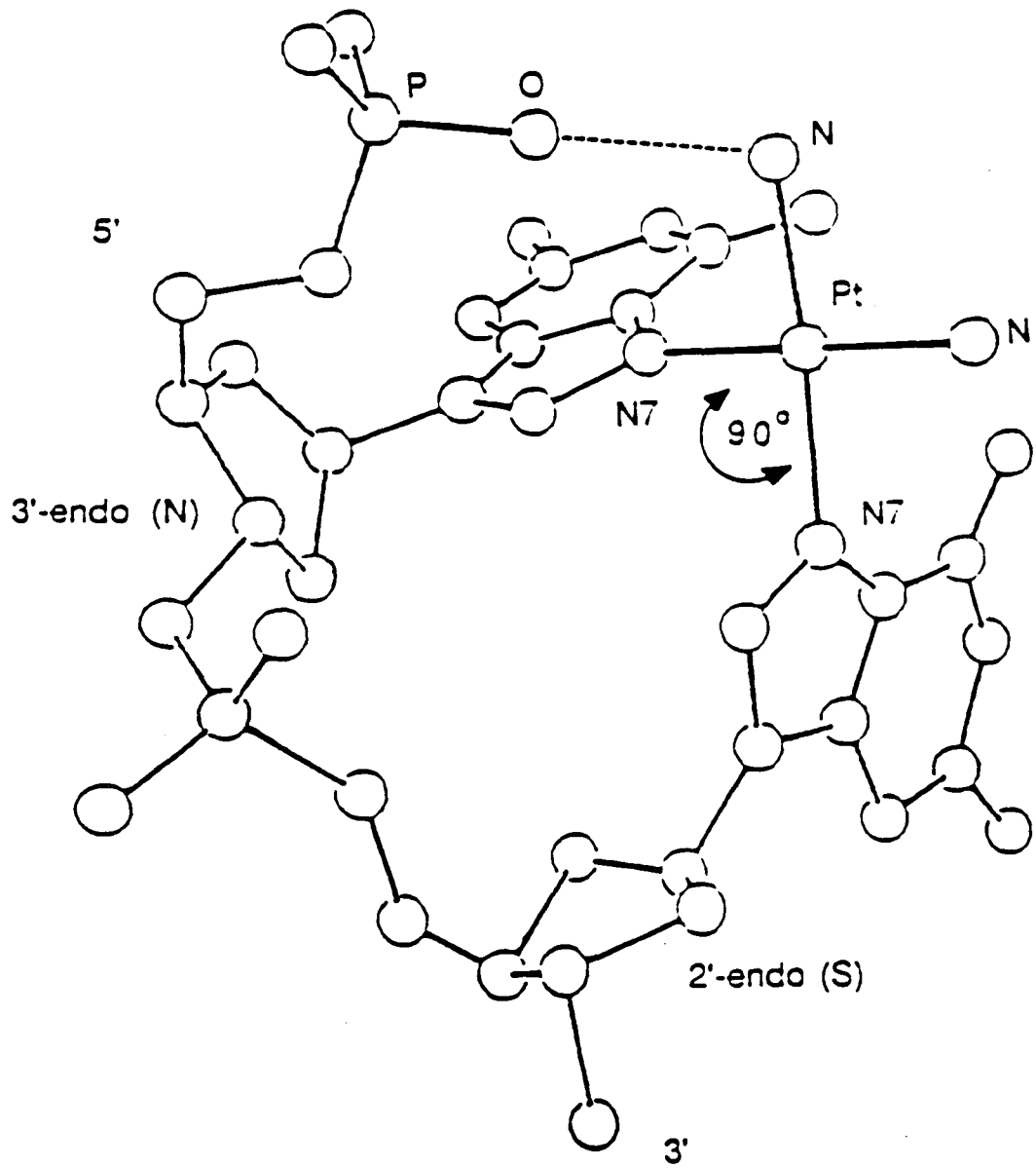


Figure 13 (a)

Crystal structure of cis-[Pt(NH_3)$_2${d(pGpG)}].

(a) reproduced with permission, Sherman et al. (1985).

guanosine monophosphate reacts faster with cis-DDP than does guanosine and that the 5'-GMP is preferred to the 3'-GMP (Eapen et al. 1985).

Second generation platinum drugs exhibit a marked variation in activity with amine substitution, with compounds containing secondary or tertiary amine ligands showing little or no biological activity (Braddock et al. 1975). Until the publication of this X-ray structure from Lippards group several possibilities for the observed changes in drug activity had been suggested, i.e. kinetic changes as a result of amine structure, affects on reactivity resulting from steric changes, and the possibility of solubility effects as a result of amine structure. There have been proposals as to the importance of hydrogen-bonding between the amine ligand on the platinum drug and the DNA receptor, as a means of stabilizing the complex, but there had been no firm experimental evidence for the existence of such a hydrogen-bonding interaction (Connors et al. 1972). The presence of the hydrogen-bond in the solid state structure may explain why platinum compounds containing unsubstituted amine ligands are more biologically active than those containing substituted amine groups. However, this hydrogen-bond has yet to be observed for similar structures in solution. The X-ray results are supportive of the solution structure obtained in the previously discussed NMR experiments on the cis-$Pt(NH_3)_2$[dGpG] by den Hartog and co-workers (den Hartog et al. 1982).

Of significance to the cis-DDP-[N(7),O(6)-guanine] discussions, the cis-DDP-[dpGpG] crystal structure shows that the distance between the amine ligands and the guanine O(6) oxygen is too long to accomodate any significant amount of hydrogen bonding and that there is no evidence for postulated guanine O(6)-platinum interaction via a bridging water

molecule (Wing et al. 1984). This result does not rule out such a structure existing along the reaction pathway between reaction of the platinum with the first guanine N(7) site and the subsequent reaction with the second guanine N(7) site.

The X-ray results and the molecular mechanics data support the NMR results in that both reveal that the formation of the cis-DDP-intrastrand crosslink between adjacent guanines does not greatly alter the natural structure of the DNA duplex. This may be significant in the understanding of biological activity and the fact that the cisplatin lesion is not completely removed by cellular repair mechanisms. The reason this may be so, as suggested by these results, may be that the three dimensional structure is not sufficiently altered for a repair enzyme to recognize the mutation.

1.5.6 Molecular Modelling Studies

Theoretical modelling on the mode of binding of cis-DDP to DNA by Miller and co-workers has shown some interesting results (Miller et al. 1985). In their work they have mathematically simulated the drug interacton with fully duplexed DNA. Under these structural constraints they have shown that the cis-DDP interaction with the N(7) sites of adjacent guanines is most favourable in the major groove of the duplex, a result observed in the crystal structure by Wing (Wing et al. 1984). Attempting a similar interaction in the minor groove indicated that the two guanine sites could not approach the necessary chelating distance (2.81) dictated by the square planar geometry of the platinum atom. Optimally, the binding of the platinum atom to the guanine base should be

along the axis of the nitrogen lone pair, but this is not possible if the double helical structure is to be maintained. Their results indicate that the adjacent bases need not be kinked to 90° to provide ideal ligand orientation for the square planar platinum (II) atom and that the complex could be formed without much loss of energy using strained bonds. Regarding complexes with sequences containing one intermediate base, i.e. dGpNpG, their results indicated that this type of chelate could not be formed while the sequence remained in the fully duplexed structure. The difference in bond energy between platinum bound to water and platinum bound to the N(7)-guanine site indicates that the platinum reaction with nucleic acids is exothermic. The excess energy provided by this reaction may be sufficient to cause local melting of the DNA and, as a result, binding of cisplatin would not be sequence limited to adjacent guanine pairs (Miller et al. 1985).

1.5.7 Kinetics and Reaction Intermediates of DNA:cisplatin Interactions

The results from kinetic studies examining the reaction of cisplatin compounds with DNA suggest that there are several reaction pathways which occur. The fastest reactions which have been observed are those which occur between the cisplatin diaquo species and adjacent guanine bases to form the side by side bis-guanine chelate (Fichtinger-Schepman et al. 1985; Johnson et al. 1985; Pinto & Lippard, 1985; Eastman 1983). When the platinum compound reacts with a guanine base which does not have an adjacent guanine either 3′ or 5′ to it, this monofunctionally bound platinum may exist for a long period of time with a half life of approximately fifteen hours (Kleinwachter et al. 1988). Examination of

aliquots of the reaction mixture at various times during the reaction reveals that the monofunctional adduct may comprise as much as ten to twenty percent of the nucleic acid bound platinum (Johnson et al. 1985; Eastman, 1983). The work also indicated that the amount of monofunctional platinum adduct was temperature dependent, with the concentration of monofunctionally bound platinum increasing with increased temperatures. The fourth ligand site on the platinum is occupied by a molecule of water. This monofunctionally bound platinum has several options as to where it may react. If there is an adjacent 5' adenine base, then a cis-Pt[d(ApG) N(7),N(7)] chelate may form (van der Veer et al. 1986a; Eastman, 1985; Fichtinger-Schepman et al. 1985). Also there is a possibilty of reaction with another guanine base which is not adjacent but which may have one or more intermediate bases separating it from the platinum bound guanine. The result from this reaction is the formation of the cis-Pt[dGpN$_n$pG] where N is any other base (Fitchtinger-Schepman et al. 1982). It would appear from the examination of molecular models that this type of adduct cannot be formed while the DNA maintains the duplexed form. It is, therefore, necessary for the duplex to undergo localized melting to allow this stucture to form. Another option available to the monfunctionally bound platinum is the formation of an interstrand cross-link to another guanine. In this situation it is necessary for the sequence in the reactive region to be either GC or CG so that the distance between the two guanine N(7) sites is not so far apart as to make the reaction impossible (Eastman, 1985). The results obtained from their product analysis showed that the concentrations of all of these different species increased with time while the

concentration of the most preferred complex, the intrastrand cross-link containing the adjacent guanine bases, remained constant.

1.5.8 dGpNpG Structures

Some work has been carried out which examines the structure of the cis-$Pt(NH_3)_2$[d(GpNpG)] complexes where N has been any of the four bases (Marcelis et al. 1983a; den Hartog et al. 1983). In the situation where the central base is another guanine there would be competition between the formation of the chelate involving adjacent guanine bases and chelate formation where binding is to the terminal guanine sites and the central base is not used. Under this competitive situation, results favour the chelate involving the adjacent guanine bases (Cohen et al. 1980). Under conditions of non-competitive platinum binding to the oligomers, i.e. where there are only two guanine bases present in the sequence, it has been possible to form the chelate with an intervening central base using cytosine, thymine, and adenine (van der Veer et al. 1986a, 1986b; Marcelis et al. 1982, Brouwer et al. 1981).

In work by den Hartog and co-workers, the complex cis-Pt[dGpCpG] has been isolated and examined by NMR (den Hartog et al. 1983). There are several significant structural differences between the dimer complex containing the adjacent guanine bases (den Hartog et al. 1982) and this structure which contains the extra central cytosine base. The platinum-dimer structure was shown to have both of the complexed guanine bases in the normal anti configuration. In this platinum-trimer complex, NOE results suggest that the metalated 3'-guanine tends towards a 50/50 syn-anti equilibrium while the chelated 5'-guanine exhibits a preference

for the abnormal (for B-DNA) syn conformation. Chemical shift as a function of variable temperature reveal other aspects of structural changes resulting from the platination of the trimer. The normal, uncomplexed trimer dGpCpG showed an increase in intrastrand stacking when the temperature of the system was decreased. This result is reflected in the behaviour of the aromatic base protons which will normally exhibit an upfield movement in chemical shift as adjacent aromatic bases stack on top of one another. In the complexed platinum-trimer structure there was no temperature effect on proton chemical shifts assigned to the aromatic bases. In the case of the central cytosine base, there was an overall decrease in diamagnetic shielding of the base protons when compared to the normal parent oligomer (Marcelis et al. 1983a). In this situation, the decrease in shielding at all temperatures indicated that the cytosine base was not spending much time stacked with either of its neighbouring guanine bases. Knowing this, as well as understanding the structural constraints placed on the terminal guanine bases as a result of platination, e.g. the platinum bridge pulling the two guanine bases close to each other, the structure of the chelated trimer was reported to be an oligomer strand containing a central "bulged base".

The effect of the bulged base as well as the conformational change in the guanine glycosidic torsion angle after reaction with cis-DDP presents several problems in duplex formation (den Hartog et al. 1983). The change in back-bone conformation necessary to accomodate the bulged base in the strand would presumably remove the central base out away from the normal hydrogen bonding region of the strand. The abnormal syn conformation of the one complexed guanine base would also effectively

remove this base from the hydrogen bonding region of a possible duplex structure. As a result of the formation of the cis-Pt[dGpNpG] structure, there is the possibility of the loss of at least two base pairs if this complex were to be involved in a duplexed structure, this is what was observed by den Hartog and co-workers. Two complementary strands were examined by NMR, one of the oligomers contained the cis-Pt[dGpTpG] complex while the second strand was normal (den Hartog et al. 1985c). NOE experiments were carried out on the longer platinated strand and the results indicated that the platinated region of the oligomer maintained the conformational structure previously described for the smaller complexed trimer.

1.6 Objectives

In order to gain a further understanding into the interaction of the anti-cancer compound cisplatin with DNA, its cellular target, model studies between cis-DDP and specific oligomer sequences have been carried out.

The first section of the work deals with the general synthetic procedures followed to produce the desired deoxy oligomers. A phosphotriester methodology was used, adapting some of the procedures developed in this laboratory for oligoribonucleotide synthesis.

The initial experiments concerned with cis-DDP : DNA interactions examines the formation of intrastrand crosslinks resulting in small internal loops in the oligomers. Internal loops of one, two, and three nucleotide units, a result of cis-DDP crosslinking between terminal guanines, have been examined.

The second section of cisplatin : DNA experiments was a continuation of internal loop formation discussed above. It was of interest to extend the oligomer chains to examine the effect of the cis-DDP internal loops on nucleotides external to the platinum-guanine crosslink.

The effect of the cisplatin : DNA interaction on the duplexing ability of DNA has been examined in an attempt to further understand the mechanism of action of the anti-cancer agent. Before the model systems containing the cis-DDP intrastrand crosslinks were studied, the same oligomer sequences were examined prior to platination. The non-complementary sequences were combined with fully, and partially complementary oligomers, and the formation and stabilities of the perfect and imperfect duplexes examined.

The final section of the study examines platinated oligomers containing internal nucleotide loops combined with fully and partially complementary sequences. Knowing the stability of the unplatinated oligomer duplexes would allow for a direct comparison with the same structures containing the cis-DDP crosslink. These experiments would give further indication to the effect of the cisplatin lesion and internal loop formation on duplex stability.

Chapter Two

Materials and Methods

2.1 Triester Synthesis of 2'-Deoxyribose Oligonucleotides:

General Outline of Synthetic Procedures

The synthesis of the oligodeoxyribonucleotides was carried out using a modified phosphotriester approach. The synthetic procedures for the deoxy oligomers are a combination of methods developed in this laboratory for oligoribonucleotide synthesis (Neilson & Werstiuk, 1971a, 1971b; Neilson, 1969) and methods used for deoxyribonucleotide work (Catlin & Cramer, 1973; Jones, 1984; Reese, 1978; Khorana, 1978; Visentin, 1984). The blocking of the different hydroxyl and amino functional groups on the individual monomers has been well documented (Khorana, 1978; Stawinski et al. 1977; Otter & Fox, 1973; Schaller et al. 1963). The general procedures have been outlined here, greater experimental detail may be obtained from the references.

Phosphorylation of the protected nucleosides was carried out utilizing the 2,2,2-trichloroethylphosphate monoester (England & Neilson, 1976) activated with 1-mesitylenesulphonly-1,2,4-triazole (MST) (Kataguri et al. 1974). Coupling of the 3'-phosphate diester nucleotide to the free 5'-hydroxyl group of the incoming nucleoside or nucleotide was achieved using 1-mesitylenesulphonyl-3- nitro-1,2,4-triazole (MSNT) (Jones et al. 1980; Reese et al. 1978).

Deblocking of the fully protected oligomer was carried out using a three step procedure. Initially, the acid labile 5'-monomethoxytrityl blocking group was removed by the dropwise addition of a 2-3% p-toluenesulphonic acid solution in methanol (Stawinski et al. 1977). A zinc-copper couple (Le Goff, 1964) in dimethylformamide (DMF) was used to reductively remove the 2,2,2-trichloroethyl protecting group from the phosphate triester. Finally, concentrated ammonia in methanol (1:1) was used to remove all base labile blocking groups (England & Neilson, 1976).

Throughout the various blocking and coupling procedures the reactions were monitored by silica gel thin layer chromatography (tlc). The glass backed silica plates were developed in a 10% methanol in methylene chloride solution (Solution A) unless otherwise stated. The results were visualised by spraying the plates with a 1% ceric sulphate in 10% sulphuric acid (Solution B) followed by charring on a hot plate at 150-200°C.

2.2 Amino Group Protection

The protection of the amino function on the cytosine and adenine bases was carried out using benzoyl chloride, and isobutyryl chloride was used for guanine (Narang et al. 1965; Schaller et al. 1963). The reaction procedure for the different bases was essentially the same. The unprotected nucleoside was dried by dissolving the starting material in anhydrous pyridine followed by the removal of the solvent under reduced pressure to azeotrope off any water. The dried material was then dissolved in anhydrous pyridine under nitrogen and the solution cooled to 0°C. The acid chloride of the desired protecting group was then added

dropwise to the stirred nucleoside solution. In the reaction, 3.5-4 equivalents of the acid chloride were used per equivalent of nucleoside resulting in the protection of not only the amino group but the free 3' and 5' hydroxyl groups as well, Figure 14.

The progress of the reaction was followed by silica gel thin layer chromatography (tlc). Development of the silica plates was carried out in Solution A and visualization of the tlc was done by spraying the plates with Solution B followed by charring at 100-150°C on a hot plate. Under these conditions the unprotected starting material remains at the baseline while the reacted nucleoside containing either one, two, or three protecting groups has an increasingly higher Rf value as the amino group and both of the hydroxyl groups become acylated.

When tlc indicated only one major product in the reaction mixture, the reaction was quenched with ice and the solution transferred into a separatory funnel containing methylene chloride. The organic layer was washed with saturated sodium bicarbonate followed by washing with distilled water. The organic layer was recovered and the solvent removed under reduced pressure. The resulting oil was dissolved in toluene which was used as an azeotrope to remove the last traces of pyridine. The impure product was dissolved in methylene chloride and purified on a silica gel column. Chromatography was carried out using a methanol in methylene chloride gradient (vol/vol) beginning with 1 % methanol and increasing the % methanol in 1-2 % increments. The final product, the triacylated nucleoside, was recovered in good yield, typically 80-90 %. The Rf value for the product in the 10 % methanol in methylene chloride solution was about 0.5.

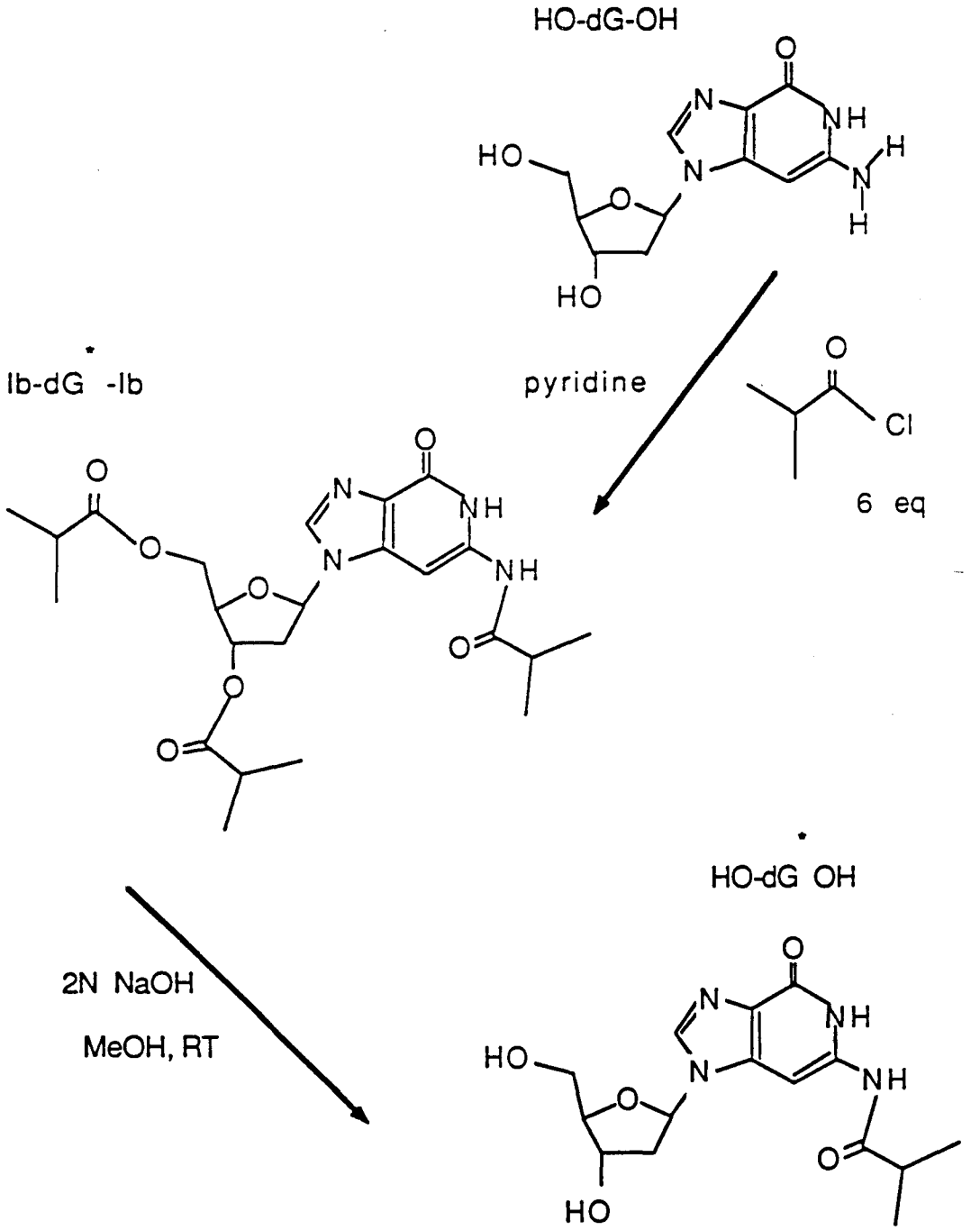


Figure 14

Peracylation of guanosine and selective deprotection of furanose hydroxyl groups to form amino protected nucleoside.

2.3 Protection of the Furanose Hydroxyl Groups

2.3.1 Deacylation of the 3′ and 5′-Hydroxyl Groups

Before the per-acylated nucleoside could be used in further synthetic reactions it was necessary to selectively remove the blocking groups from the 3′ and 5′ hydroxyl groups on the deoxy sugar moiety, Figure 14. Deblocking of the ester groups was carried out in a methanol solution of the fully acylated compound. To this mixture was added, dropwise, a 1:1 solution of 1N sodium hydroxide in methanol with the deblocking reaction being closely monitored by tlc. As the reaction progressed, the different de-acylation products were visible by tlc with the Rf value of the major spot decreasing as the two esters are hydrolysed. A compromise as to when the reaction is to be quenched was necessary as hydrolysis of the desired N-protected product occurs before complete deprotection of the two sugar hydroxyl groups has been achieved.

The reaction was quenched with the addition of chelex 50-X8 (pyridinium form) to the stirred reaction mixture until a neutral pH solution was aquired. The solution was filtered to remove the resin and the solute recovered. The solvent was removed under reduced pressure, with the final traces of pyridine being removed by azeotroping with toluene. The mixture of recovered products was dissolved in 1-2 % methanol in methylene chloride and applied to a silica gel column. Some methanol was necessary in the initial solvent mixture to promote the solution of the monoacylated product. The methanol gradient used to separate the reaction mixture could be increased rapidly as the desired product has a low Rf, typically 0.1-0.2. The earlier fractions, containing both the fully acylated nucleoside as well as the diacylated

product, were pooled and deblocked with later preparations. Yields of the N-protected nucleoside were normally on the order of 70-75 %.

2.3.2 Protection of the 5'-Hydroxyl Group

The free 5'-hydroxyl group of the N-derivatized nucleoside was protected with p-anisylchlorodiphenylmethane, (monomethoxytritylchloride), (mmTrCl) (Smith et al. 1962). The reaction was carried out under nitrogen in an anhydrous pyridine solution of the dried, amino protected nucleoside at 0°C, Figure 15. Drying of the starting material was carried out by dissolving the nucleoside in dry pyridine followed by the removal of the solvent under reduced pressure using a rotary evaporator attached to an oil pump, and finally sealing the vessel under dry nitrogen or argon. After the rapid addition of 1.1 equivalents of mmTrCl to the stirred reaction mixture, the vessel was sealed and allowed to slowly warm to room temperature. The reaction was left overnight to allow for complete reaction; normal reaction times were on the order of three to five hours.

Progress of the reaction was monitored using silica gel thin layer chromatography. The new spot on the tlc plate arising from the formation of the desired product was a yellow-orange colour after spraying with Solution B and development on a hot plate. The Rf value of the new product was approximately 0.8. The yellow colour is characteristic of a trityl containing compound and results from the formation of the trityl cation on the silica gel.

A minor side product arises from the reaction of both the 5' and the 3' hydroxyl groups with the mmTrCl. This product, also yellow in colour,

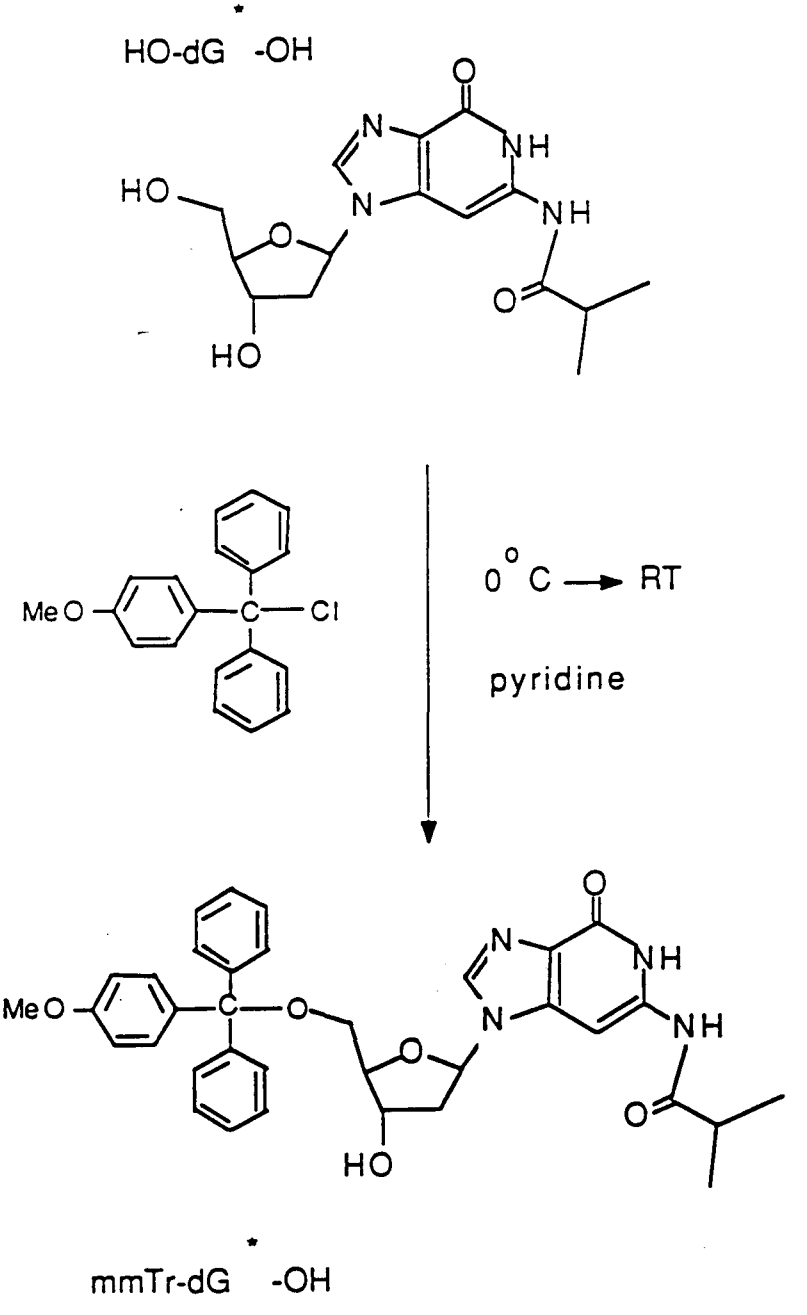


Figure 15

Selective protection of the 5'-hydroxyl group of the furanose ring.

is identified by tlc with an Rf value approaching 1.0. The starting material, which does not contain the trityl group, chars a black colour and has an Rf value of 0.1-0.2.

When no further reaction was observed by tlc, the mixture was quenched with ice and the resulting solution transferred to a separatory funnel. The mixture was dissolved in methylene chloride and the solution washed with saturated sodium bicarbonate and with water. The organic layer was recovered and the solvent removed under reduced pressure. The resulting oil was azeotroped several times with toluene to remove the final traces of pyridine. The impure product, often recovered as a foam, was dissolved in methylene chloride and applied to a silica gel column. With the large Rf difference between the desired reaction product and the unreacted starting material as well as any undesired side products, purification by silica gel column chromatography gave good yields. Chromatography was carried out using a methanol in methylene chloride gradient system, beginning with 0.5 % methanol and increasing the gradient slowly. Yields for the protection of the 5'-hyroxyl group with the monomethoxytrityl group were typically greater than 90 %.

2.3.3 Protection of the 3'-Hydroxyl Group

The type of derivatization carried out on the 3' hydroxyl group of the otherwise fully protected nucleoside depended on the future usage of the nucleoside in oligomer synthesis. If the nucleoside was to occupy a 3'-terminal position on the oligomer, no terminal phosphate group was necessary and the 3'-hydroxyl was protected using acetic anhydride in pyridine, Figure 16. If the nucleoside was to be used as the 5'-terminal

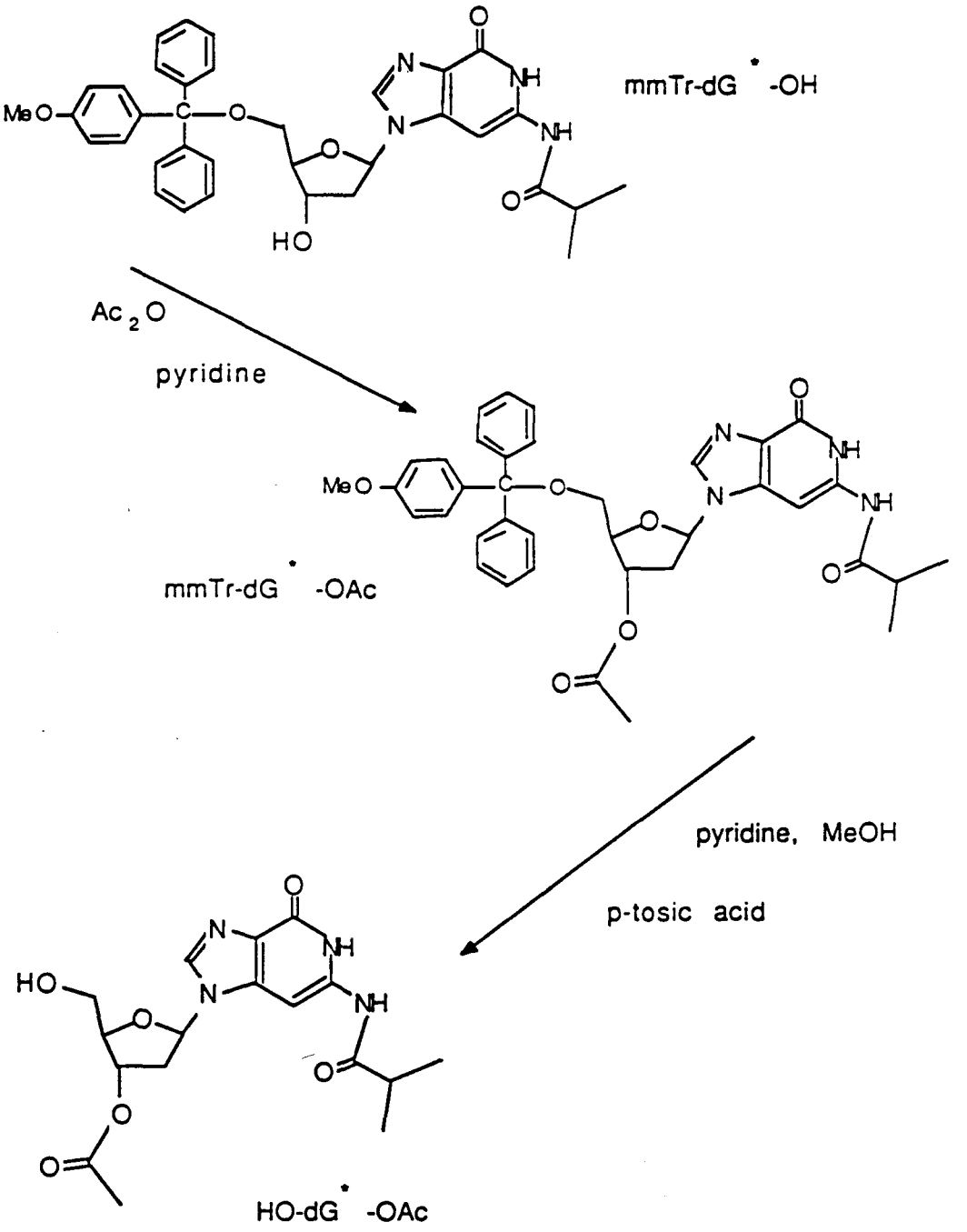


Figure 16

Selective acetylation of the 3'-hydroxyl group and deprotection of the 5'-hydroxyl group to form the incoming 3'-terminal nucleoside.

unit or in an intermediary position on the oligomer, i.e. the site of further chain extension, the 3'-hydroxyl group was phosphorylated (Catlin and Cramer, 1973).

2.3.4 Phosphorylation of the 3'-Hydroxyl Group

The phosphorylation of a protected nucleoside always took place at an open 3'-hydroxyl group as chain extension in the synthesis was from the 5'-terminus to the 3'-terminus. The phosphate group was introduced as the 2,2,2-trichloroethylphosphate monoester, Figure 17 (England and Neilson, 1977). Phosphorylation was initiated by treatment of the phosphate monoester with three equivalents of the activating agent 1-mesitylenesulfonyl-1,2,4-triazole (MST) in dry pyridine at 60°C for one hour. Initially this reaction mixture was found to be nonhomogeneous but, solution was attained after the one hour time period. This reaction results in the formation of a mixed anhydride species which was not isolated but mixed directly with the protected nucleoside containing the free 3'-hydroxyl group. The reaction mixture was sealed under nitrogen and stored in the dark. Progress of the reaction was followed by tlc with the formation of a cusp-shaped spot originating at the baseline of the plate in conjunction with the loss of the spot for the higher Rf starting material. When the reaction was complete, ice was added to the mixture and the resulting solution poured into methylene chloride. The solution was transferred to a separatory funnel and washed with saturated sodium bicarbonate and with water. The organic phase was isolated and the solvent removed under reduced pressure. The resulting oil was dried by azeotroping with anhydrous pyridine in preparation for the next

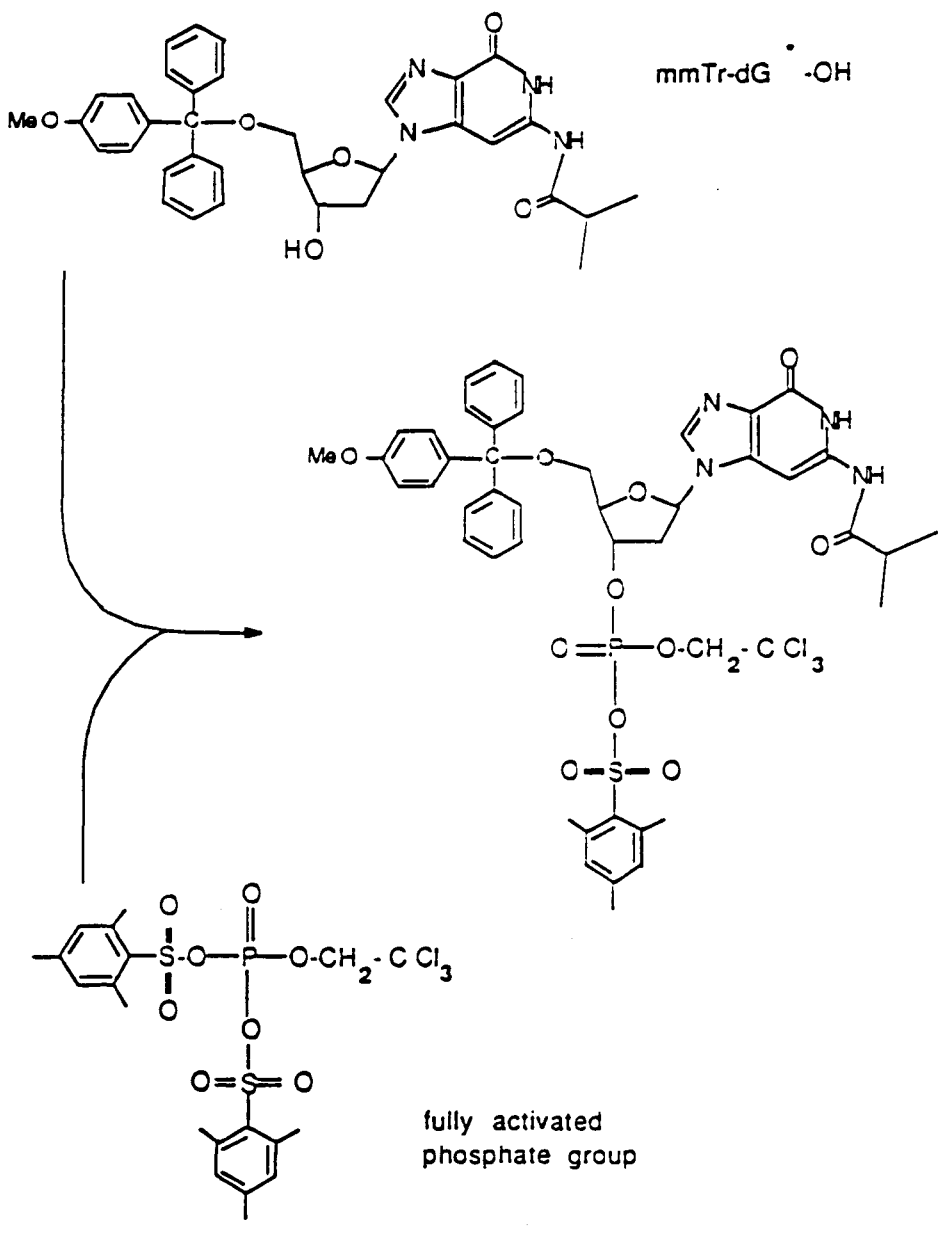


Figure 17

Reaction of the free 3'-hyroxyl group with fully activated phosphate monoester.

reaction. The phosphate diester product, which was used without further purification, was not isolated or stored but used directly.

The phosphorylated species can be used in one of two ways. If the nucleotide is to be the 5'-terminal unit in the oligomer, the phosphorylated compound can be reactivated and used directly for coupling to the next nucleotide moiety. If the nucleotide is to be used as an incoming unit, the phosphate diester function is then further protected with 2-cyanoethanol before deprotection of the 5'hydroxyl group on the deoxy sugar (Catlin & Cramer, 1973).

2.4 Full Protection of the 3'-Phosphate Diester

Formation of the fully protected nucleotide triester was carried out by reactivating the phosphate diester functionality with 1.1 equivalents of MST for approximately one hour at room temperature followed by the addition of five equivalents of 2-cyanoethanol, Figure 18 (Catlin and Cramer, 1973). The reaction is followed by tlc, with the loss of the cusped shaped spot of the diester compound from the baseline and the formation of the neutral phosphate triester showing an Rf value of approximately 0.8. The product maintains the yellow colour on tlc characteristic of the presence of the mmTr group.

When no further reaction was evident by tlc, the mixture was quenched with ice and the resulting solution mixed in methylene chloride and poured into a separatory funnel. The solution was washed with saturated sodium bicarbonate and with water. The organic layer was isolated and the solvent removed under reduced pressure. The resulting oil was azeotroped with toluene several times to remove the final traces

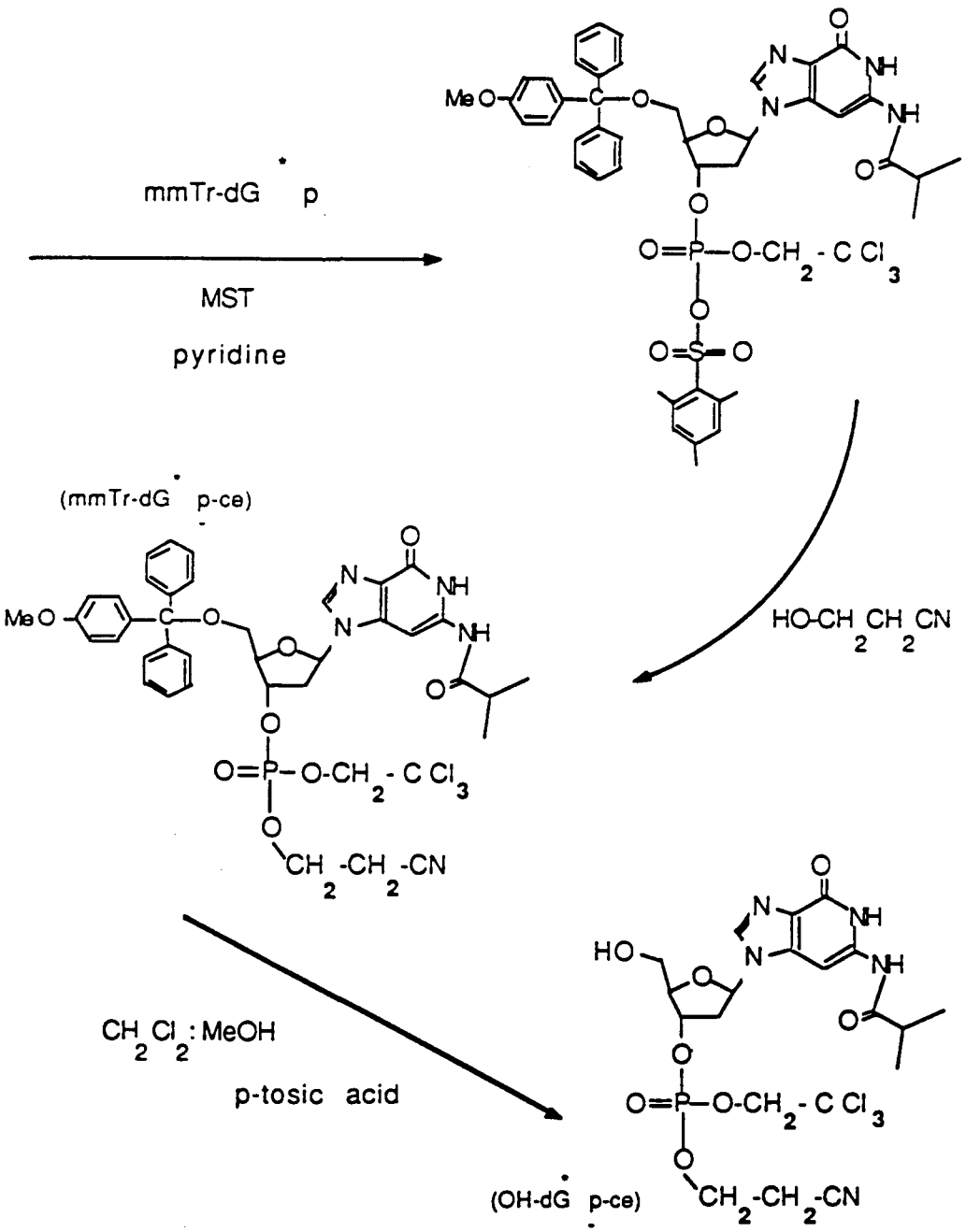


Figure 18

Formation of fully protected 3'-phosphate and selective deprotection of the 5'-hydroxyl group to form incoming 3'-monomer unit used in oligomer chain extension.

of pyridine. The impure mixture was dissolved in methlyene chloride and purified by silica gel chromatography.

2.5 Removal of the 5'-Monomethoxytrityl Group

For the now fully protected nucleotide monomer to be useful in oligomer synthesis the 5'-monomethoxytrityl group must be removed, Figure 18. The deprotection of the 5'-hydroxyl group was carried out using 2-3% p-toluenesulphonic acid in methanol. The monomer was dissolved in methylene chloride and the tosic acid solution added dropwise with stirring and constant monitoring of the reaction by tlc. The loss of the monomethoxytrityl group was shown by tlc with the formation of a yellow spot at the solvent front, resulting from the methylmonomethoxytrityl ether. The black spot at Rf=0.3 was the desired product, the 3'-phoshate triester nucleotide unit with a free 5'-hydroxyl group. This nucleotide could now be used as an incoming unit in a coupling reaction where further strand elongation in the 3' direction was desired.

2.6 Oligomer Elongation

Chain elongation was accomplished using a coupling reaction between the activated 3'-phosphodiester function of the 5'-terminal end of the oligomer with the free 5'-hydroxyl group of the incoming 3'-terminal unit, Figure 19. This reaction was carried out by combining the two components in a dry pyridine solution and adding 1.5 equivalents of 1-mesitylenesulphonyl-3-nitro-1,2,4-triazole (MSNT) to the mixture. Formation of the coupled product could be seen on tlc with the loss of both the cusp shaped spot of the activated phosphodiester at the

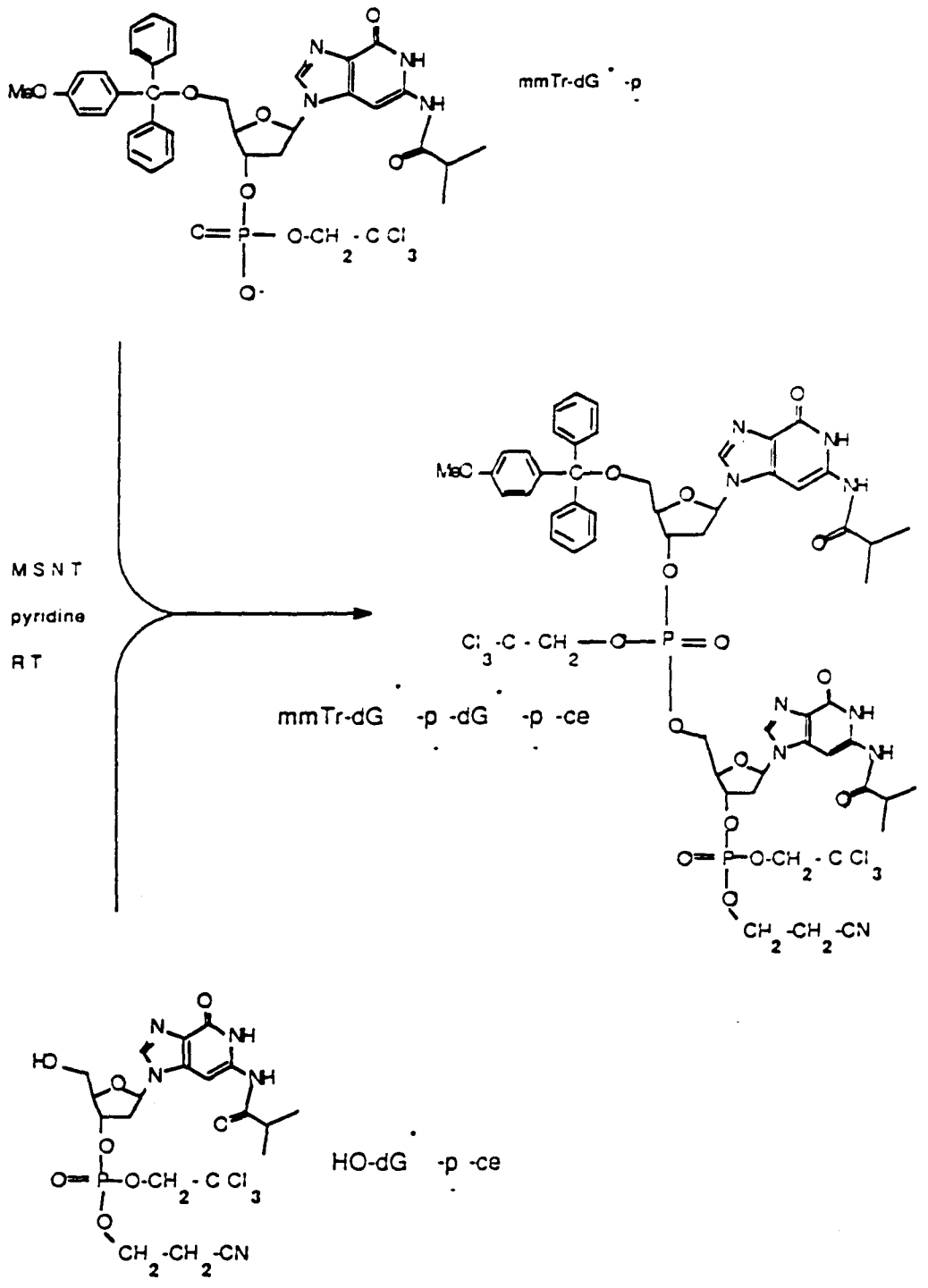


Figure 19

Formation of a fully protected d(GpG) dimer via coupling of free 5'-hydroxyl group with the MSNT activated phosphodiester.

baseline as well as the higher Rf black spot of the incoming unit. The appearance of a new, yellow coloured spot was evident as the coupling progressed and the desired product was formed.

When the coupling reaction was complete by tlc the reaction mixture was quenched with ice and the mixture dissolved in methylene chloride. The solution was washed with saturated sodium bicarbonate and with water. The organic layer was isolated and the solvent removed under reduced pressure followed by azeotroping of the resulting oil with toluene to remove the last traces of pyridine.

Further chain extension of this dimeric unit was possible by the selective deprotection of the terminal 3'-phosphotriester group (Sood & Narang, 1977; Adamiak et al. 1976). This was achieved by treatment of the deoxy oligomer with freshly distilled triethylamine (TEA) in pyridine. This reaction is selective to the cyanoethyl group and does not affect other base labile functions in the molecule. When the deprotection was complete, seen on tlc as the formation of a cusp-shaped spot at the baseline from the phosphodiester, the solution containing the oligomer was taken to an oil under reduced pressure and azeotroped with dry pyridine to remove the TEA. The oil was then redissolved in pyridine, combined with the incoming nucleotide and treated with the activating agent MSNT. The procedure for chain elongation was continued until the desired sequence was obtained.

After each coupling reaction, the reaction was quenched with ice and the mixture poured into methlyene chloride and transferred into a separatory funnel. The solution was washed with saturated sodium bicarbonate and with water. The organic layer was isolated and the

solvent removed under reduced pressure. The resulting oil was azeotroped with toluene to remove the final traces of pyridine. The impure product mixture was applied to a flash chromatography silica gel column and the desired product isolated. A small amount of product (5-10 mg) was deblocked, purified and checked by NMR after each coupling to ensure the integrity of the synthesis. For oligomers longer than three base units, dimers were used as building blocks rather than continuous monomer additions.

2.7 Deblocking of the Final Oligomer Sequence

The desired base sequence must be fully deprotected before the oligomer can be used for physical studies; this procedure is outlined on Figure 20. The 5'-terminal monomethoxytrityl group was removed first using tosic acid in methanol and methylene chloride. The reaction was quenched with ice and the mixture poured into methylene chloride and transferred to a separatory funnel. The organic phase was washed with saturated sodium bicarbonate and with water. The organic layer was isolated and the solvent removed under reduced pressure. The resulting oil was azeotroped with toluene to remove the final traces of pyridine and then dissolved in methylene chloride and applied to a flash silica gel column.

The remaining steps in the deblock procedure were carried out on oligomer samples of 10-15 mg. The sample was placed in a small screw topped vial along with approximately one ml of dry DMF and a small amount of zinc-copper couple. The reaction mixure was sealed and the solution heated to 50°C for two to three hours (England and Neilson, 1976). The

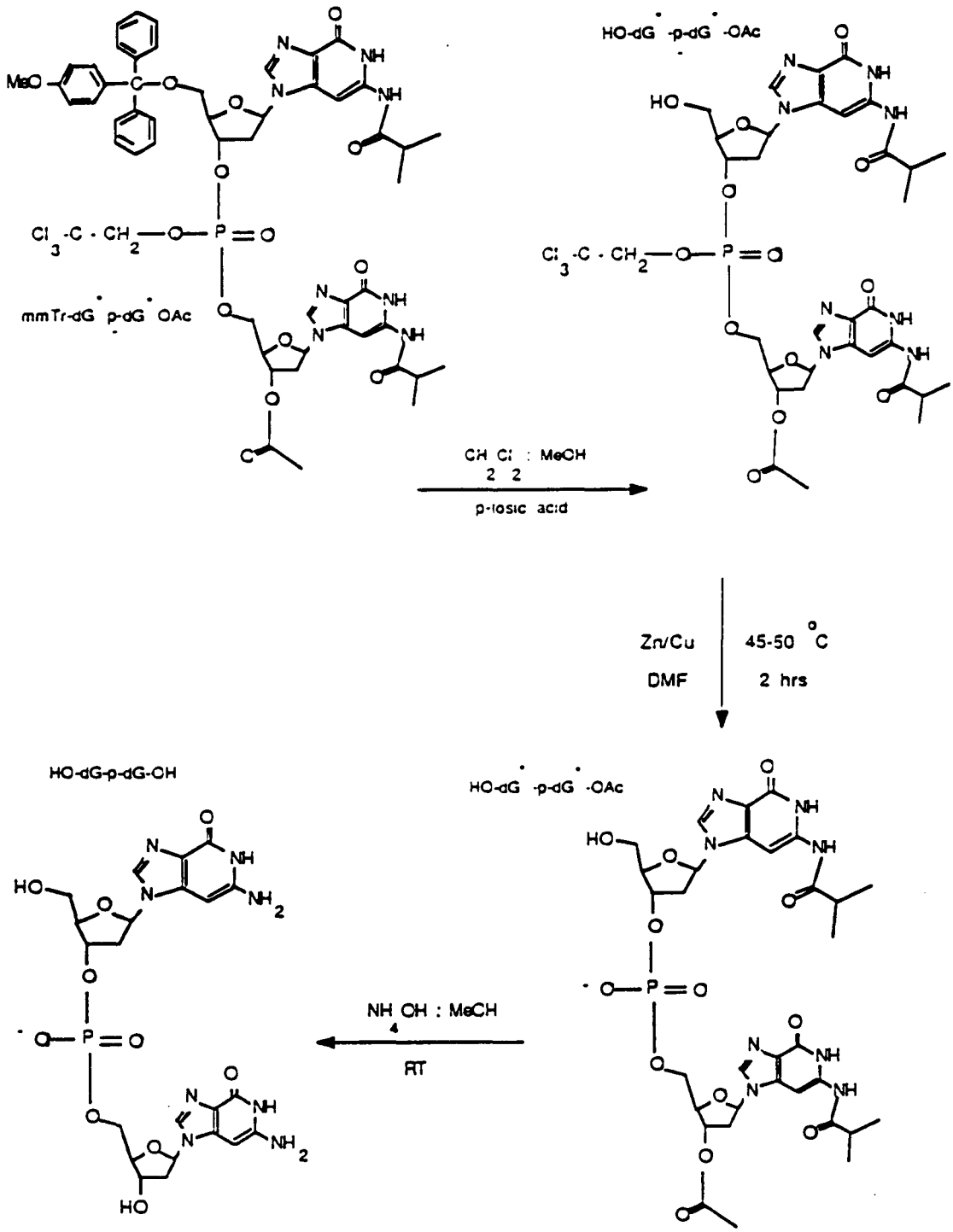


Figure 20

Outline of the complete deblock procedure to produce the free d(GpG) dimer.

result of this reaction is the reductive removal of the 2,2,2-trichloroethyl group from the phosphate triester linkage. After the designated time period the sample was removed from the heat and two ml of both methanol and concentrated ammonia were added to the mixture. The sample vial was resealed and allowed to react for two days at room temperature. This final deprotection step results in the removal of the base labile amino and hydroxyl protecting groups.

The mixture containing the fully deblocked sample was filtered through a sintered glass funnel to remove the zinc-copper couple and the filtrate then stirred for approximately one hour with chelex (NH_4^+ form) to remove any dissolved metal ions. The solution was again filtered to remove the chelex and the solvent removed under reduced pressure. The resulting product mixture was dissolved in a minimum amount of water and purified by descending paper chromatography on Whatman #1 paper using 1.0 M ammonium acetate and ethanol(abs) (1:1) as the mobile phase. The desire product, usually the lowest Rf band as seen under UV light (254 nm), was eluted wth HPLC grade water and the solution lyophylized. The solid material obtained was dissolved in 0.01 M ammonium acetate and loaded onto a Sep Pak which was subsequently washed with three x three ml aliquots of HPLC grade water. The deoxyoligomer was then eluted from the column using a solution of 30% acetonitrile in HPLC water. The resulting solution was lyophilized to dryness and then redissolved in water at which time reaction yields were determined by UV absorbance. Finally, the recovered product was taken up in D_2O and lyophilized to dryness before the sample was examined by ^1H NMR spectroscopy.

2.8 NMR Methodology

The NMR data in this work has been obtained from either a Bruker WM-250 or an AM-500 spectrometer. Both spectrometers were operating in Fourier transform (FT) mode and equipped with quadrature detection. In variable temperature experiments, probe temperatures were maintained with a Bruker variable temperature unit and calibrated with an external thermocouple unit. Temperatures are reliable to +/- 1°C.

NMR samples were prepared by lyophilization from D_2O, followed by dissolving the sample in 100% D_2O containing the appropriate buffer and salt. Samples contained t-butanol-OD as internal reference, 1.231 ppm, with chemical shift values recorded relative to sodium 2,2-dimethyl-2-silapentane-5-sulphonate (DSS).

In the variable pH studies, the pH titrations of the oligomers were carried out using solutions of either DCl or NaOD dissolved in D_2O. Additions of the acid or base were made directly into the NMR tube containing the sample and the pH was monitored using a pH electrode which could be inserted into the 5 mm NMR tube. The pH values quoted have not been corrected for deuterium effects.

The residual HDO peak observed for samples dissolved in D_2O was suppressed in the NMR spectrum using a standard Bruker program for solvent suppression. The observation of exchangeable imino protons in duplex structures required the sample be dissolved in a 90:10 H_2O:D_2O mixture. The water signal was eliminated using the 1:1 hard pulse technique, (Gronenborn and Clore, 1985; Clore *et al.* 1983).

Chapter Three

Results and Discussion

3.1 Introduction

The reaction of cis-$Pt(NH_3)_2Cl_2$ with DNA has been widely studied. Examination of the platinum compound's interaction with both short deoxy oligomers and intact DNA has led to a better understanding of the mechanism of action of the antitumor drug (Scovell et al. 1985, Marcelis et al. 1983b, Lippard 1983, Johnson et al. 1980). It is generally believed that the primary site of attack on DNA by cis-$Pt(NH_3)_2Cl_2$ is in G-C rich regions and a preference has been shown for adjacent guanosine bases (Fichtinger-Schepman et al. 1985, Inagaki et al. 1985, Stone et al. 1974). Enzymatic digestion of calf thymus DNA, which had been previously treated with cis-$Pt(NH_3)_2Cl_2$, revealed the presence of several different platinum containing adducts (Fichtinger-Schepman et al. 1982). The major product was identified as the cis-$Pt(NH_3)_2$[d(GpG)-N7(1),N7(2)] complex. Minor products from the digestion suggested complexes of the cis-$Pt(NH_3)_2(dGpX_npG)$, where X is one or more intermediate bases ie. A, T, or C (Eastman 1986, 1985, 1983, Fichtinger-Schepman et al. 1982). It was these types of structures, which form internal bulges or loops, which have been the focus in this section of our research.

The sequences studied were; d(GpTpG), d(GpTpTpG), d(GpTpTpTpG), and their platinated analogs cis-$Pt(NH_3)_2$[d(GpTpG)-N7(1),N7(3)],

cis-$Pt(NH_3)_2$[d(GpTpTpG)-N7(1),N7(4)], and cis-$Pt(NH_3)_2$[d(GpTpTpTpG)-N7(1),N7(5)]. The reaction of cis-$Pt(NH_3)_2(Cl)_2$ with adjacent guanine bases has been thoroughly examined by many techniques and the structure of the adduct has been theoretically modelled (Kozelka et al. 1986, 1985, Miller et al. 1985) as well as having been experimentally determined by NMR (den Hartog et al. 1982) and the x-ray structure of cis-$Pt(NH_3)_2$[d(pGpG)] has been solved (Sherman et al. 1985). Further, the effects of the cis-Pt[d(GpG)] chelate on adjacent bases within a single stranded oligomers have been examined (den Hartog et al. 1985b, Neumann et al. 1984, Marcelis et al. 1983a). It was of interest in our research to study, not the effects of the cis-$Pt(NH_3)_2Cl_2$ on nucleic acid bases external to the complex, but rather to examine the possible formation of bulged loop adducts. At the time this work was started, research had not been published on these types of structures, although their existence had been suggested (Alix et al. 1981, Brouwer et al. 1981). Since that time, several reports have been published concerning loop formation, these have confined themselves, however, to the cis-$Pt(NH_3)_2$[d(GpX_npG)] complex where n=1 (den Hartog et al. 1983, Marcelis et al. 1982). In this work we have examined sequences of the form d(GpX_nG), where X=Tp and n=1,2, and 3, Figure 21. Thymine was chosen as the internal base in an effort to avoid possible side reactions with either adenine or cytidine, both of which have the possibility of reaction with cis-$Pt(NH_3)_2Cl_2$.

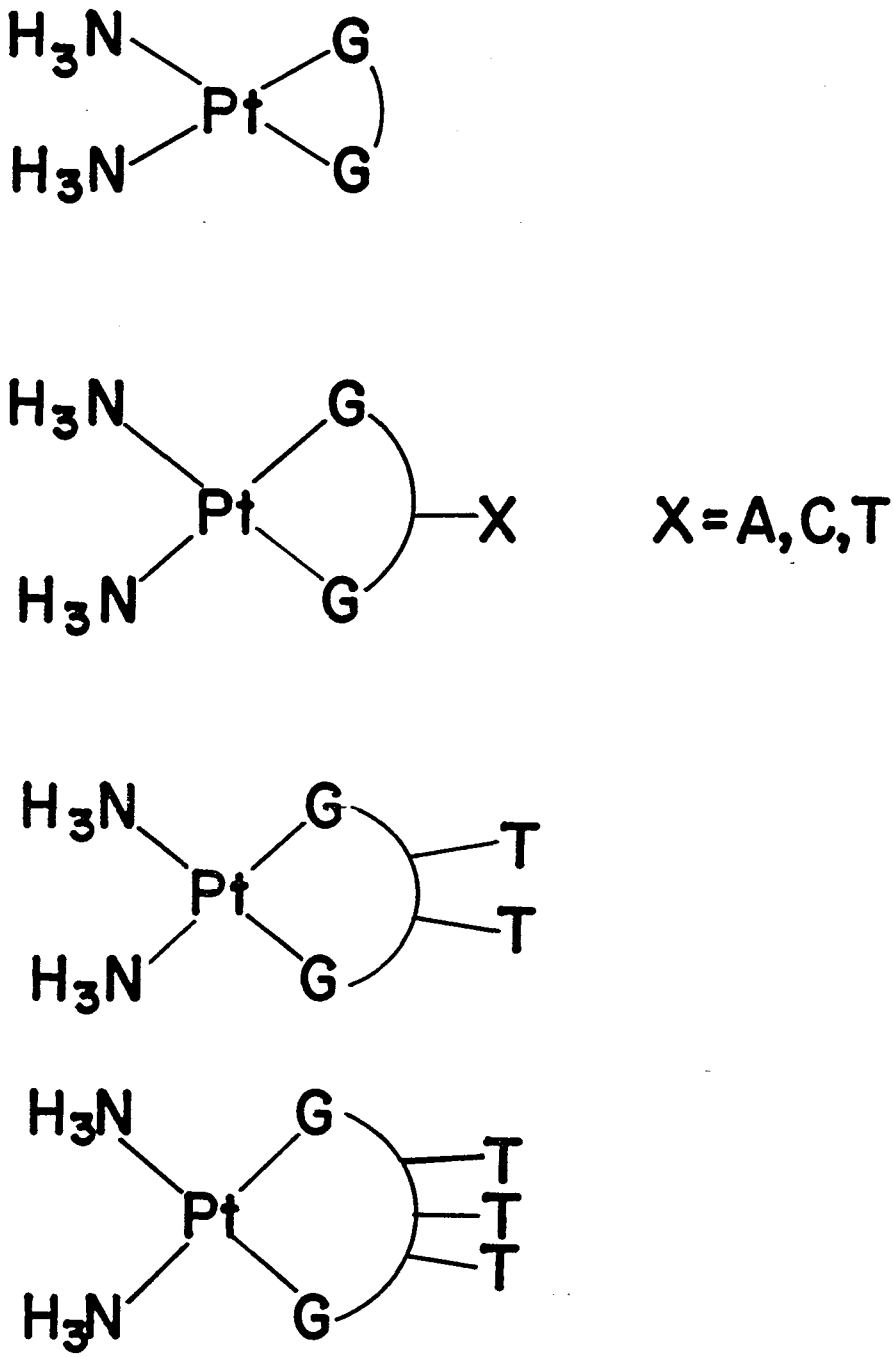


Figure 21

Schematic structures of cisplatin derivatives formed through interaction with short DNA oligomers.

3.2 Formation of cis-$Pt(NH_3)_2$-Oligomer Complexes

The sequences involved were synthesized using a modified phosphotriester technique as outlined in Chapter 2.

The formation of the platinum-oligomer adducts was carried out at concentrations of 10^{-3}-10^{-5} M in oligomer in distilled deionized water. This low concentration was used to lessen the possible formation of polymeric species i.e. intermolecular end to end reactions. The cis-$Pt(NH_3)_2Cl_2$ was synthesized as outlined in Dhara (1970) and a freshly prepared aqueous platinum solution was used for reaction with the oligomers. Initially, one equivalent of cis-$Pt(NH_3)_2Cl_2$ per strand of oligomer was added to the reaction mixture, the solution was then sealed and stored in the dark at room temperature for two days. After this time, the pH of the reaction mixture was found to have dropped to between four and two. The pH of the solution was adjusted to seven using a dilute ammonium hydroxide solution and the reaction mixture was again sealed and stored in the dark at room temperature.

The progress of the platination reaction could be followed by UV spectroscopy and by reverse phase HPLC. Examination of the UV spectrum over the course of the reaction showed a shift in both the λ_{max} and λ_{min} to longer wavelengths. This was a general result seen for all oligomers examined here and has also been documented elsewhere (Girault et al. 1982a). The UV data for the three oligomers d(GpTpG), d(GpTpTpG), d(GpTpTpTpG) as well as their platinated adducts is given in Table 1.

HPLC was most useful in following the progress of the reaction. The reverse phase column used was a Waters C_{18} Nova Pak. The mobile phases were A) 0.1 M ammonium acetate, pH 7 and B) methanol, run over a gradient

from 0-40% methanol at a flow rate of 0.8 ml/min. As observed by HPLC, the oligomer-platinum reaction was rapid for the first 24-48 hours after which time the reaction rate decreased markedly. After seven days all reaction had stopped and if results indicated the reaction not to be complete, another 0.5 equivalents of cis-$Pt(NH_3)_2Cl_2$ was added to the mixture. The solution was again sealed and stored in the dark. At this point, only one or two more days were necessary to allow the reaction to go to completion. HPLC was not only helpful in following the progress of the reaction but also illustrated that for each platination carried out, only one major product was formed. In each case, under the conditions used, the platinated oligomer had a slightly shorter retention time than the starting material.

The reaction mixture was purified by loading the aqueous solution onto a Waters C_{18} Sep Pak, washing the mini-column several times with pure water to remove unreacted platinum salts, and then eluting the desired product from the C_{18} column using a solution of 30% acetonitrile in water. The product, cis-$Pt(NH_3)_2[d(GX_nG)]$ was lyophylized to dryness and isolated as the ammonium salt.

The purified platinum-oligomer complexes were examined by HPLC, ^1H NMR, and platinum analysis by ICP ([1]). The ICP analysis confirmed a platinum:oligomer ratio of one to one. The HPLC results showed that only one major product was formed in each case, with the platinated material having only a slightly shorter retention time than the normal oligomer. Analysis by UV spectroscopy revealed that in each reaction, greater than 95% of the initial UV absorbing material was recovered as the platinated

---

[1]. Thanks to Johnson Mathey for the platinum analysis.

Table 1

Wavelength (nm) of absorbance maxima and minima of short DNA oligomers before and after reaction with cis-$Pt(NH_3)_2Cl_2$.

	λ_{max}	λ_{min}
d(GTG)	254	228
cis-$Pt(NH_3)_2$[d(GTG)-N7(1),N7(3)	260.5	234.5
d(GTTG)	255.5	228.5
cis-$Pt(NH_3)_2$[d(GTTG)-N7(1),N7(4)	261.5	234.5
d(GTTTG)	257	229.5
cis-$Pt(NH_3)_2$[d(GTTTG)-N7(1),N7(5)	262	234

product. These results, along with the NMR data which are to follow, led to the conclusion that the products formed in each reaction were intrastrand cis-$Pt(NH_3)_2[N(7)G]_2$ crosslinks with internal bulges of one, two, and three thymine bases respectively.

Both the product and starting material were checked by ^1H NMR at approximately 70°C. The assignments of observed proton resonances to specific nucleotides in the oligomer sequence have been based on calculated chemical shift values according to the work of Bell et al. 1985, Table 2. As has been observed in previous studies (Girault et al. 1982a, Chottard et al. 1980), platination of the guanine bases in the oligomers resulted in the downfield shift of the G-H8 resonances. This effect has been illustrated in Figure 22 for the three strands used in this study, and the following sequences have been included for comparison, cis-Pt[d(GpCpG)] (den Hartog et al. 1983) and cis-Pt[d(GpG)] (den Hartog et al. 1982).

3.3.1 pH Titration Studies

The primary method which has been used to determine the site of cis-$Pt(NH_3)_2Cl_2$ reaction with DNA oligomers is a pH titration of the complexes as monitored by ^1H NMR (Neumann et al. 1984, Marcelis et al. 1983a, Caradonna et al. 1982, Chottard et al. 1980). The major reaction sites which have been observed for the various bases are N(7), N(1) and possibly O(6) of guanine (Hitchcock et al. 1983, Maquet et al. 1983, Chottard et al. 1980), N(1) and N(7) of adenine, N(3) and N(4) of cytosine, and O(2), O(4) and N(3) of deprotonated thymine, with the strongest preference being shown for N(7) of guanine. By carrying out a

Table 2

Assignment of proton chemical shifts (ppm) for short oligomers based on calculated values at 70°C.

		dGpTpG 1-2-3		dGpTpTpG 1-2-3-4		dGpTpTpTpG 1-2-3-4-5	
		calc.	obs.	calc.	obs.	calc.	obs.
5′							
G	H8	7.981	7.879	7.943	7.888	7.943	7.901
	H1′	6.232	6.160	6.261	6.195	6.259	
T	H6	7.540	7.473	7.580	7.567	7.607	7.588
	H1′	6.240	6.168	6.275	6.210	6.294	
	CH3	1.807		1.804		1.837	
T	H6	-----	-----	7.534	7.508	7.573	7.577
	H1′	-----	-----	6.249	6.141	6.284	
	CH3	-----	-----	1.836		1.833	
T	H6	-----	-----	-----	-----	7.555	7.520
	H1′	-----	-----	-----	-----	6.264	
	CH3	-----	-----	-----	-----	1.867	
G	H8	7.965	7.956	7.992	7.958	7.989	7.962
	H1′	6.254	6.207	6.278	6.223	6.274	
3′							

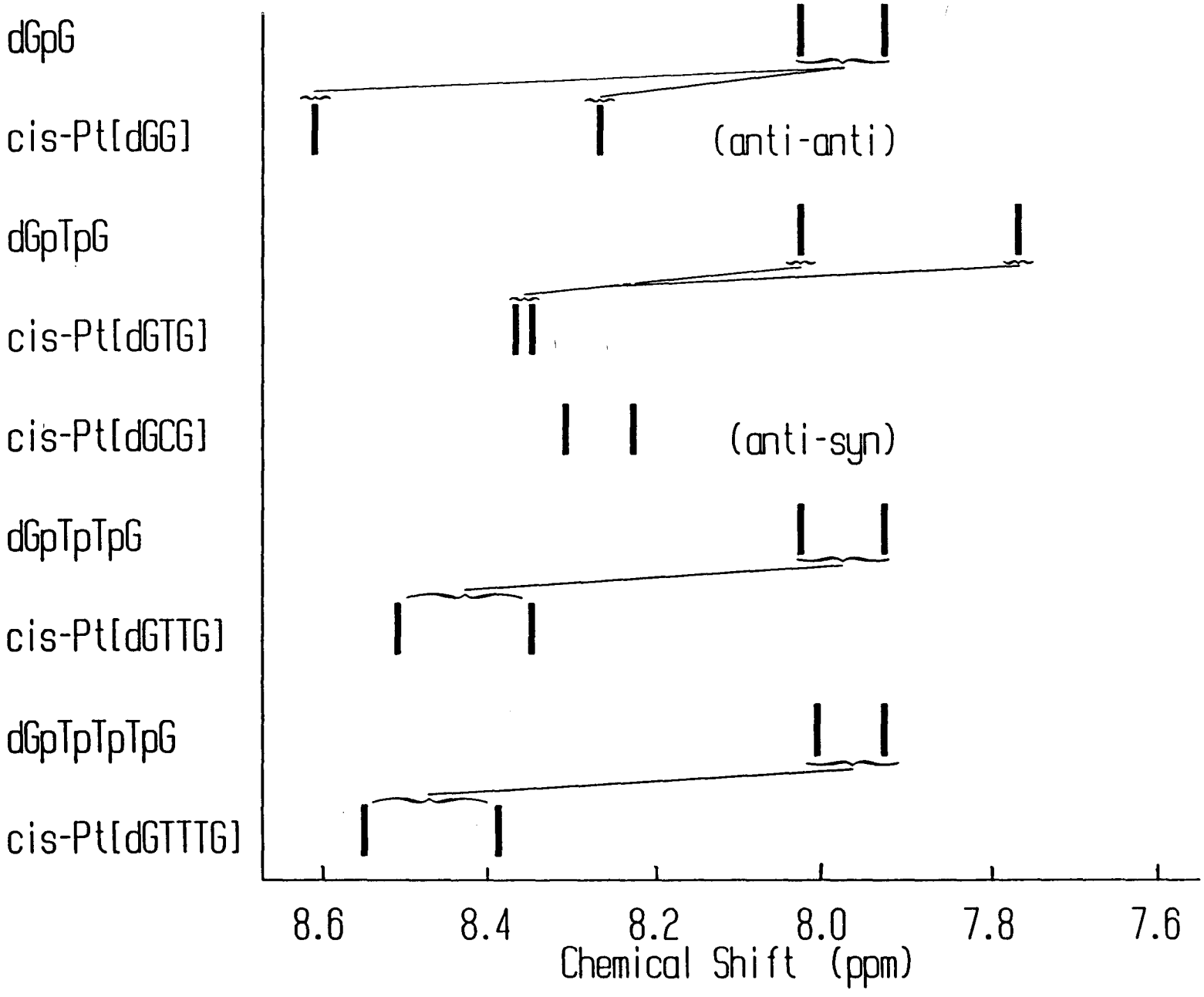


Figure 22 Effect of cisplatin reaction at guanine N(7) site on chemical shift of G-H8.

pH titration on the DNA oligomer, both before and after reaction with cis-$Pt(NH_3)_2Cl_2$, the sites of platinum binding can be determined. This is possible because the sites in the molecule open to attack by platinum are also the sites which can be protonated i.e. N(7) of guanine, or deprotonated i.e. N(3) of thymine. Whether the reaction is by protonation or deprotonation of the nitrogens on the heterocyclic bases or reaction by cis-$Pt(NH_3)_2^{2+}$ at similar sites, each reaction results in a change in the electron density in the heterobase ring system. The overall effect, pertinent to reaction site determination, is an observed change in the chemical shifts of the non-exchangeable protons on the bases involved.

The three deoxyoligonucleotides d(GpTpG), d(GpTpTpG), d(GpTpTpTpG), and their cis-$Pt(NH_3)_2$ analogs have been examined by ^1H NMR over a wide pH range, Figures 23-25 and Figures 26-28 respectively. The chemical shift vs. pH data for the non-exchangeable base protons and the H1' sugar protons, where discernable, are given in Tables 3-8.

3.3.2 Titration Studies of Parent Oligomers

Examination of the G-H8 signals for the normal strands, Figure 23, 24, and 25, indicates little change in the chemical shift for these resonance signals over the pH range four to nine. For pH values which are less than four, the G-H8 resonance begins to move downfield, which indicates the protonation of the N(7) site on guanine. The pKa for this site has been reported to be 2.4 (Martin 1985, Izatt et al. 1971). When the pH was taken above nine there was a small shift in the G-H8 signal to

higher field, which has been attributed to the deprotonation of the N(1) of guanine, pKa = 9.5.

The T-H6 resonance for the different unplatinated oligomers exhibit little movement over most of the pH range examined. It was not until the pH was taken above ten that a small shift of the H6 signal to higher field was observed, the result of deprotonation at N(3), pKa = 9.9 (Martin, 1985). The chemical shift of the T-CH_3 group appears insensitive to N(3) deprotonation and therefore has not been given.

3.3.3 Titration Studies of Oligomers Complexed with Cisplatin

Examining the results obtained for the platinated species, as shown in Figure 26, 27, and 28, several changes are readily apparent when compared to results for their respective non-platinated parent oligomers. Focussing on the G-H8 resonances, over the pH range 1.5-7, there is no effect on chemical shift with the change in pH. It is important to note that the protonation of guanine N(7), observed in the normal strands at pH values below four, has not occurred. This result indicates that the N(7) site on guanine base is no longer open to protonation and is an indication that the purine N(7) site is occupied by a platinum atom. The presence of the platinum at the guanine N(7) site is further suggested by the overall shift of the G-H8 curve to lower field. The formation of the Pt-N coordinate covalent bond, which uses the lone pair of electrons on the ring nitrogen, decreases the electron density in the heterobase ring system resulting in the observed downfield shift of the G-H8 resonance. Examining the basic pH range, the G-H8 curves show a shift to higher field with the deprotonation of N(1). In

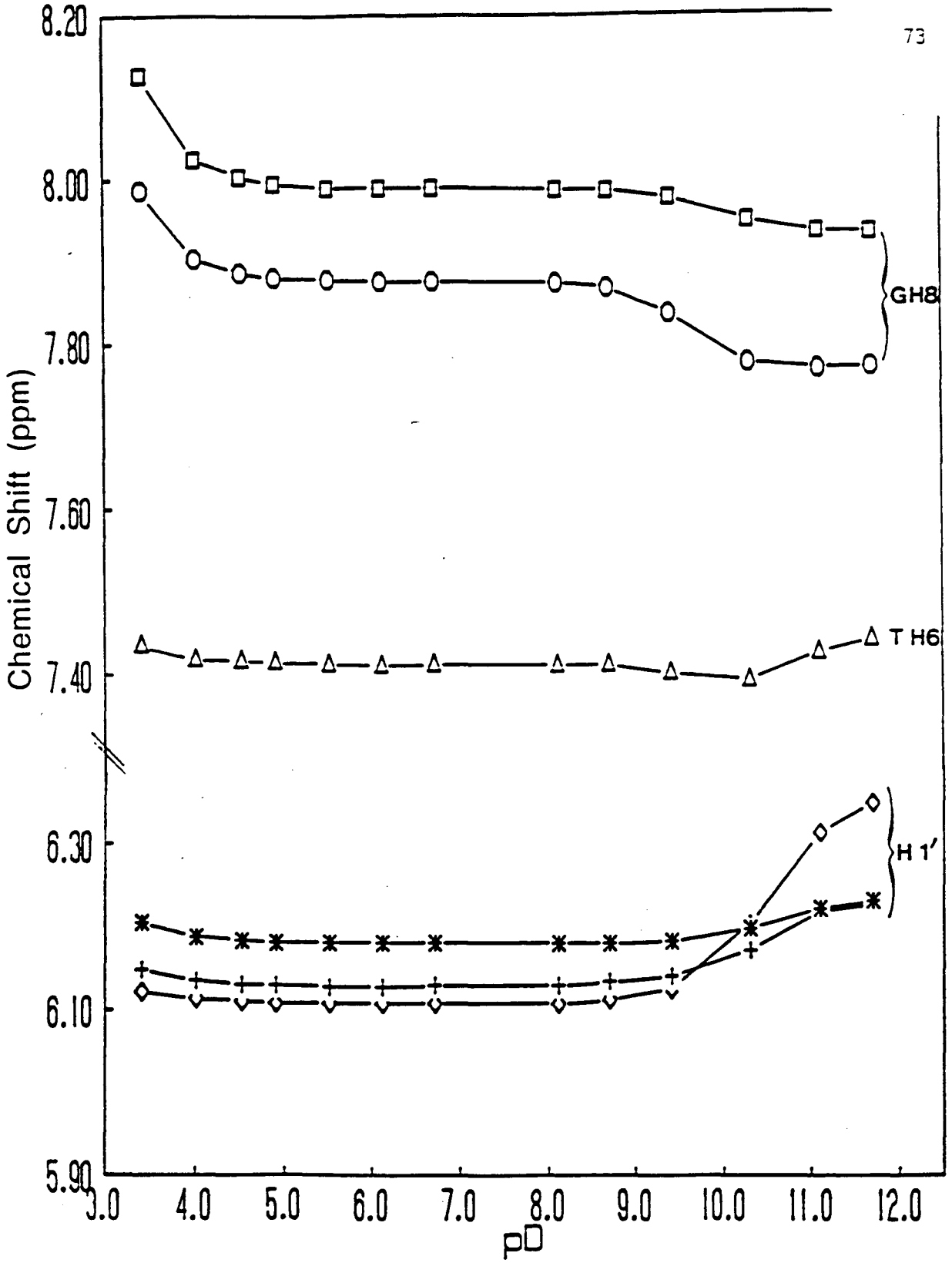


Figure 23

Chemical shift of H1'-anomeric and aromatic protons as a function of pD for d(GTG).

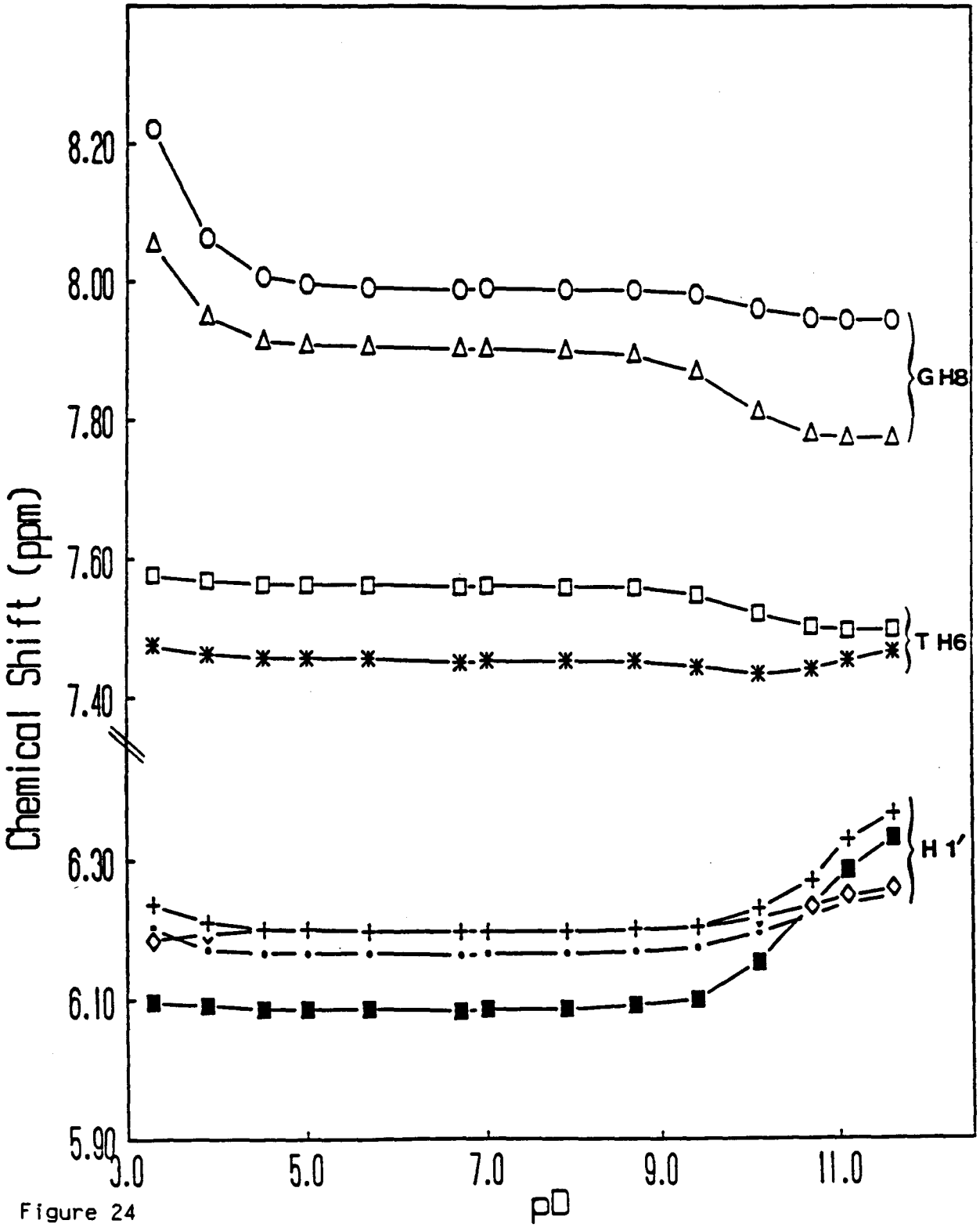


Figure 24

Chemical shift of H1'-anomeric and aromatic protons as a function of pD for d(GTTG).

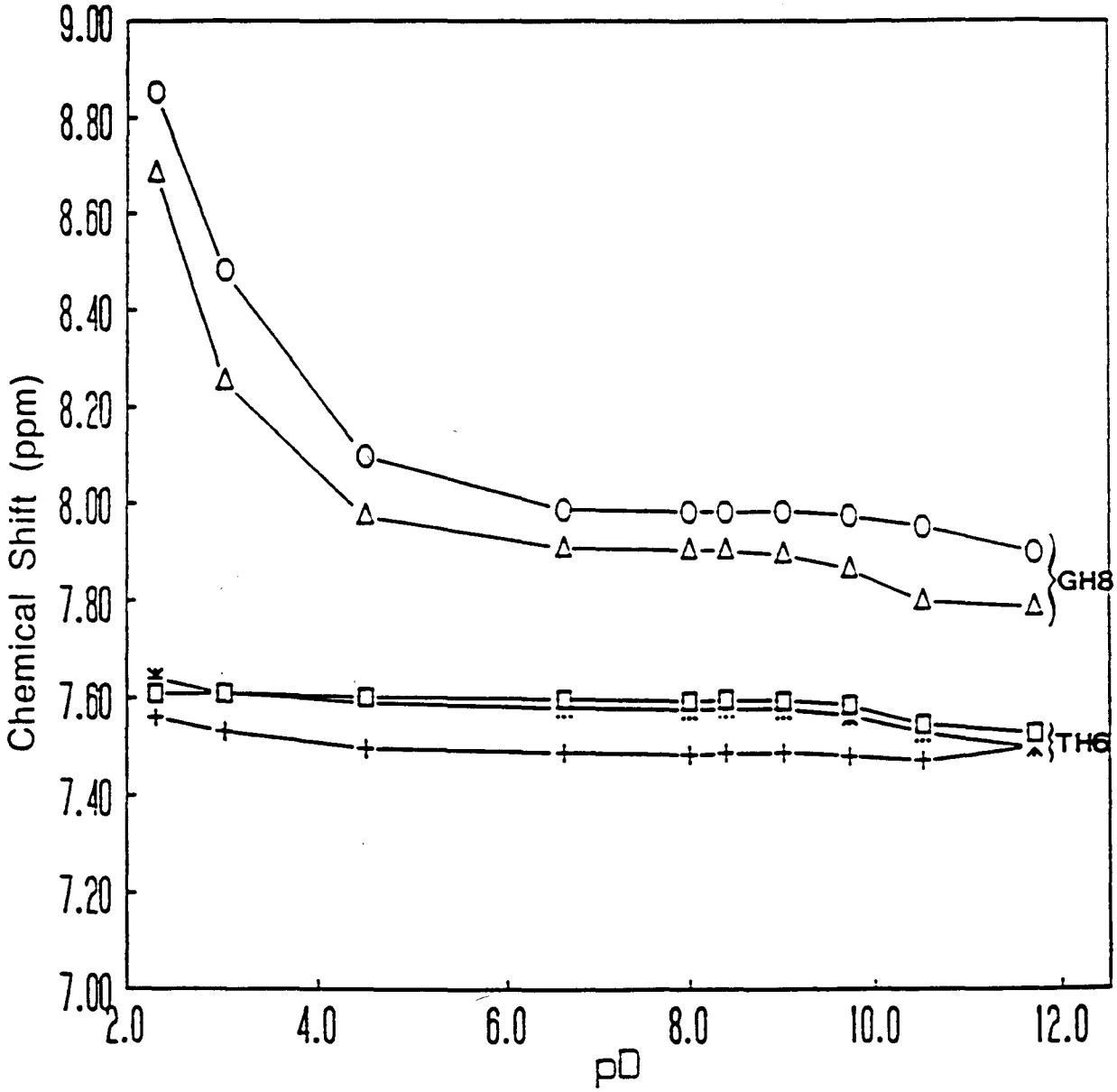


Figure 25

Chemical shift of aromatic protons as a function of pD for d(GTTTG).

TABLE 3

Chemical shift as a function of pH;
d(GpTpG), 3.95 mM, 22.4°C.
1-2-3

Chemical Shift vs pH

Proton	pH 3.4	4.0	4.5	4.9	5.5
G(1)-H8	8.126	8.025	8.002	7.995	7.990
G(3)-H8	7.987	7.904	7.886	7.880	7.876
T(2)-H6	7.434	7.417	7.414	7.413	7.411
G(3)-H1′	6.206	6.188	6.183	6.182	6.181
T(2)-H1′	6.147	6.134	6.131	6.130	6.128
G(1)-H1′	6.122	6.112	6.110	6.109	6.108

Proton	pH 6.1	6.7	8.1	8.7	9.4
G(1)-H8	7.990	7.989	7.988	7.986	7.977
G(3)-H8	7.875	7.750	7.872	7.865	7.836
T(2)-H6	7.409	7.411	7.409	7.408	7.397
G(3)-H1′	6.181	6.181	6.182	6.181	6.183
T(2)-H1′	6.128	6.129	6.130	6.181	6.142
G(1)-H1′	6.107	6.109	6.109	6.112	6.126

Proton	pH 10.3	11.1	11.7
G(1)-H8	7.949	7.935	7.933
G(3)-H8	7.776	7.767	7.768
T(2)-H6	7.389	7.421	7.436
G(3)-H1′	6.198	6.223	6.232
T(2)-H1′	6.173	6.218	6.227
G(1)-H1′	6.203	6.314	6.350

TABLE 4

Chemical shift as a function of pH;
d(GpTpTpG), 3.95 mM, 22.4°C.
1-2-3-4

Chemical Shift vs pH

Proton	pH 3.3	3.9	4.5	5.0	5.7
G(4)-H8	8.222	8.062	8.007	7.997	7.991
G(1)-H8	8.056	7.949	7.915	7.909	7.905
T(2)-H6	7.577	7.568	7.565	7.564	7.564
T(3)-H6	7.476	7.463	7.458	7.458	7.457
G(4)-H1'	6.236	6.210	6.200	6.199	6.198
T(2)-H1'	6.185	6.196	6.200	6.199	6.198
G(1)-H1'	6.202	6.170	6.166	6.166	6.165
T(3)-H1'	6.097	6.090	6.087	6.087	6.086

Proton	pH 6.7	7.0	7.9	8.7	9.4
G(4)-H8	7.989	7.990	7.988	7.988	7.982
G(1)-H8	7.903	7.904	7.901	7.895	7.872
T(2)-H6	7.560	7.564	7.561	7.561	7.550
T(3)-H6	7.453	7.456	7.455	7.455	7.448
G(4)-H1'	6.197	6.198	6.198	6.199	6.202
T(2)-H1'	6.197	6.198	6.198	6.199	6.202
G(1)-H1'	6.163	6.165	6.165	6.168	6.174
T(3)-H1'	6.084	6.086	6.086	6.090	6.100

Proton	pH 10.1	10.7	11.1	11.6
G(4)-H8	7.963	7.949	7.946	7.946
G(1)-H8	7.813	7.782	7.777	7.777
T(2)-H6	7.524	7.506	7.501	7.502
T(3)-H6	7.437	7.443	7.457	7.470
G(4)-H1'	6.230	6.268	6.328	6.364
T(2)-H1'	6.215	6.232	6.248	6.258
G(1)-H1'	6.194	6.219	6.236	6.248
T(3)-H1'	6.151	6.232	6.286	6.329

TABLE 5

Chemical shift as a function of pH;
d(GpTpTpTpG). 3.95 mM, 26.5°C.
1-2-3-4-5

Chemical Shift vs pH

Proton	pH 2.3	3.0	4.5	6.6	8.0
G(5)-H8	8.854	8.481	8.099	7.985	7.984
G(1)-H8	8.687	8.253	7.973	7.908	7.903
T(2)-H6	7.612	7.609	7.601	7.598	7.595
T(3)-H6	7.643	7.609	7.589	7.581	7.577
T(4)-H6	7.560	7.529	7.497	7.488	7.484

Proton	pH 8.4	9.0	9.7	10.5	11.7
G(5)-H8	7.984	7.983	7.975	7.953	7.900
G(1)-H8	7.903	7.896	7.862	7.795	7.784
T(2)-H6	7.597	7.595	7.583	7.544	7.528
T(3)-H6	7.579	7.577	7.564	7.526	7.494
T(4)-H6	7.487	7.486	7.479	7.469	7.500

the platinated species, the proton at N(1) of guanine is slightly more acidic, a generally observed result (Chottard et al. 1980b).

A comparison of the thymine H-6 signals between the platinated species and their non-platinated parent molecules shows little change in chemical shift as a result of reaction with cis-$Pt(NH_3)_2Cl_2$. Most noteworthy is the shift to higher field, both before and after reaction with cis-$Pt(NH_3)_2Cl_2$, by the T-H6 signals for pH values greater than eight. This result indicates that the N(3)-H site is unaffected and that platination has not occurred on any thymine bases in the molecules studied.

3.3.4 Conclusion to Cisplatin-Oligomer Product Analysis

Combining the results from the pH titrations along with those from HPLC and ICP data, it was concluded that the reaction of d(GpTpG), d(GpTpTpG), and d(GpTpTpTpG) with cis-$Pt(NH_3)_2(Cl)_2$ formed one to one platinum-oligomer complexes. The nature of the binding was an intrastrand crosslink between the platinum and the two N(7) positions of the terminal guanine bases. The intervening bases, consisting of one, two, and three thymine bases respectively, have been forced out of the stack by the platinum clipped guanine bases forming a bulged loop structure.

3.4.1 Variable Temperature Studies

The use of variable temperature (VT) experiments have been a vital tool in the examination of nucleic acids (Alkema et al. 1982, 1981, Romaniuk et al. 1979). Whether it is used in conjuction with UV, CD, NMR

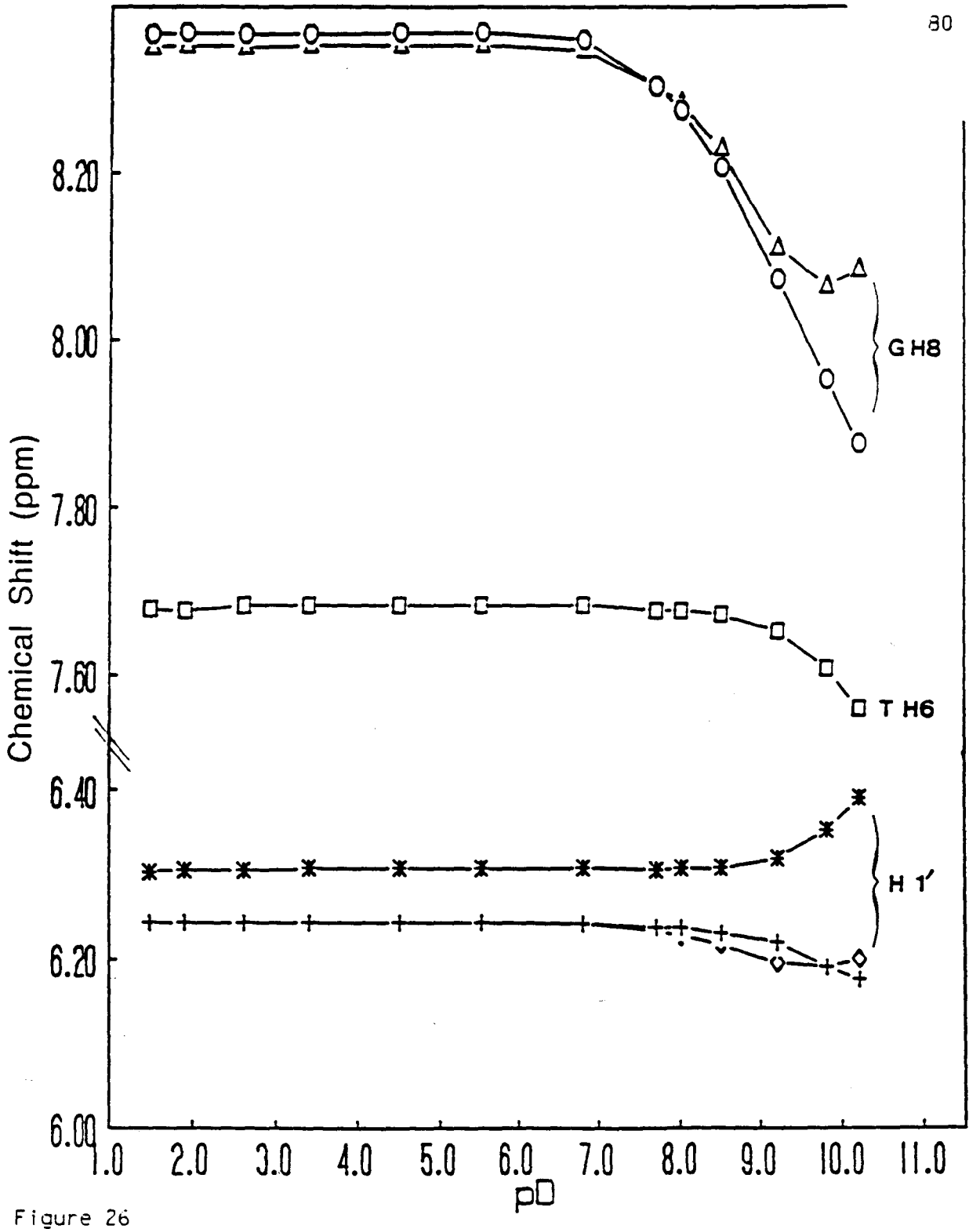


Figure 26

Chemical shift of H1'-anomeric and aromatic protons as a function of pD for cis-$Pt(NH_3)_2$[d(GTG)-N7(1),N7(3)].

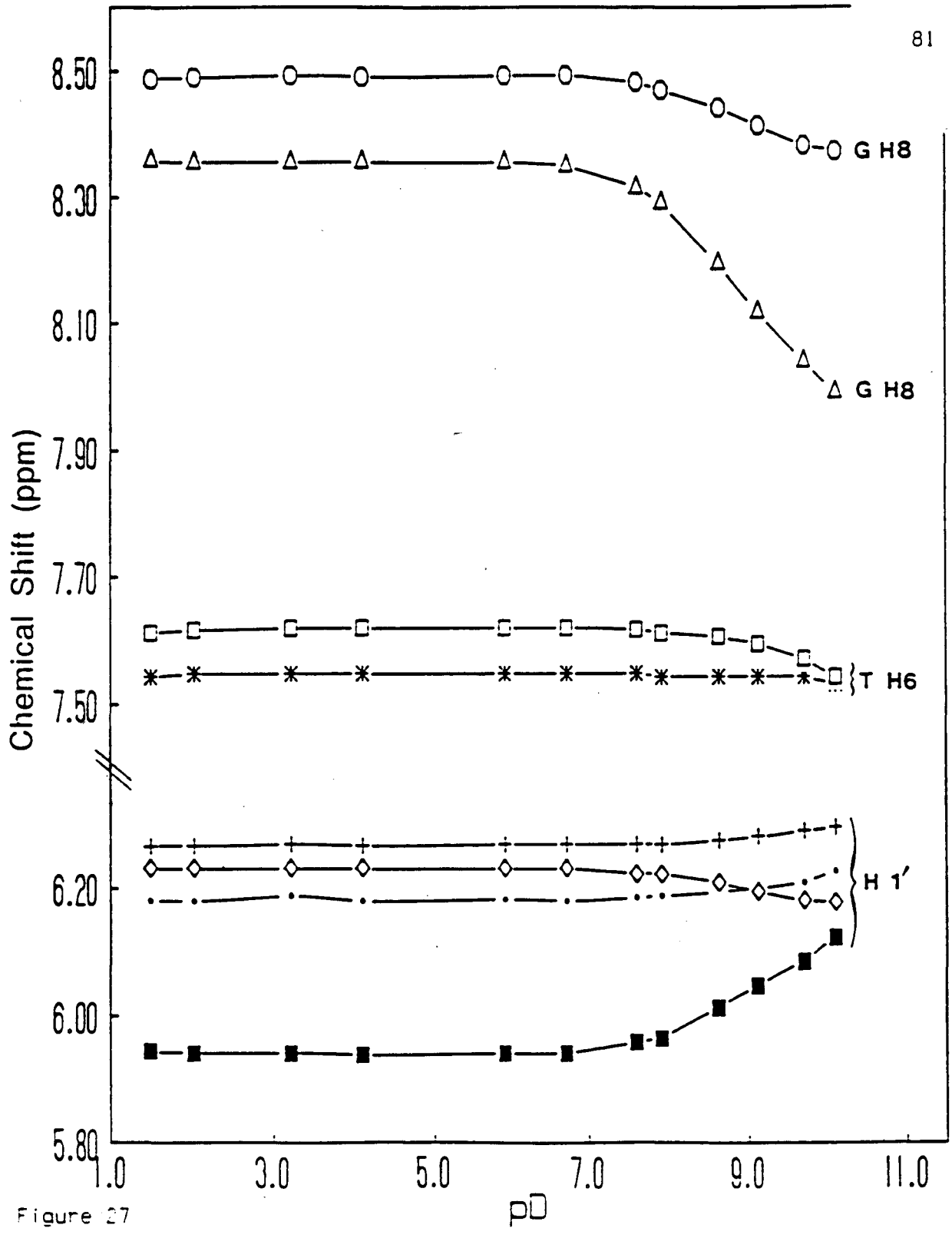


Figure 27

Chemical shift of H1'-anomeric and aromatic protons as a function of pD for cis-$Pt(NH_3)_2$[d(GTTG)-N7(1),N7(4)].

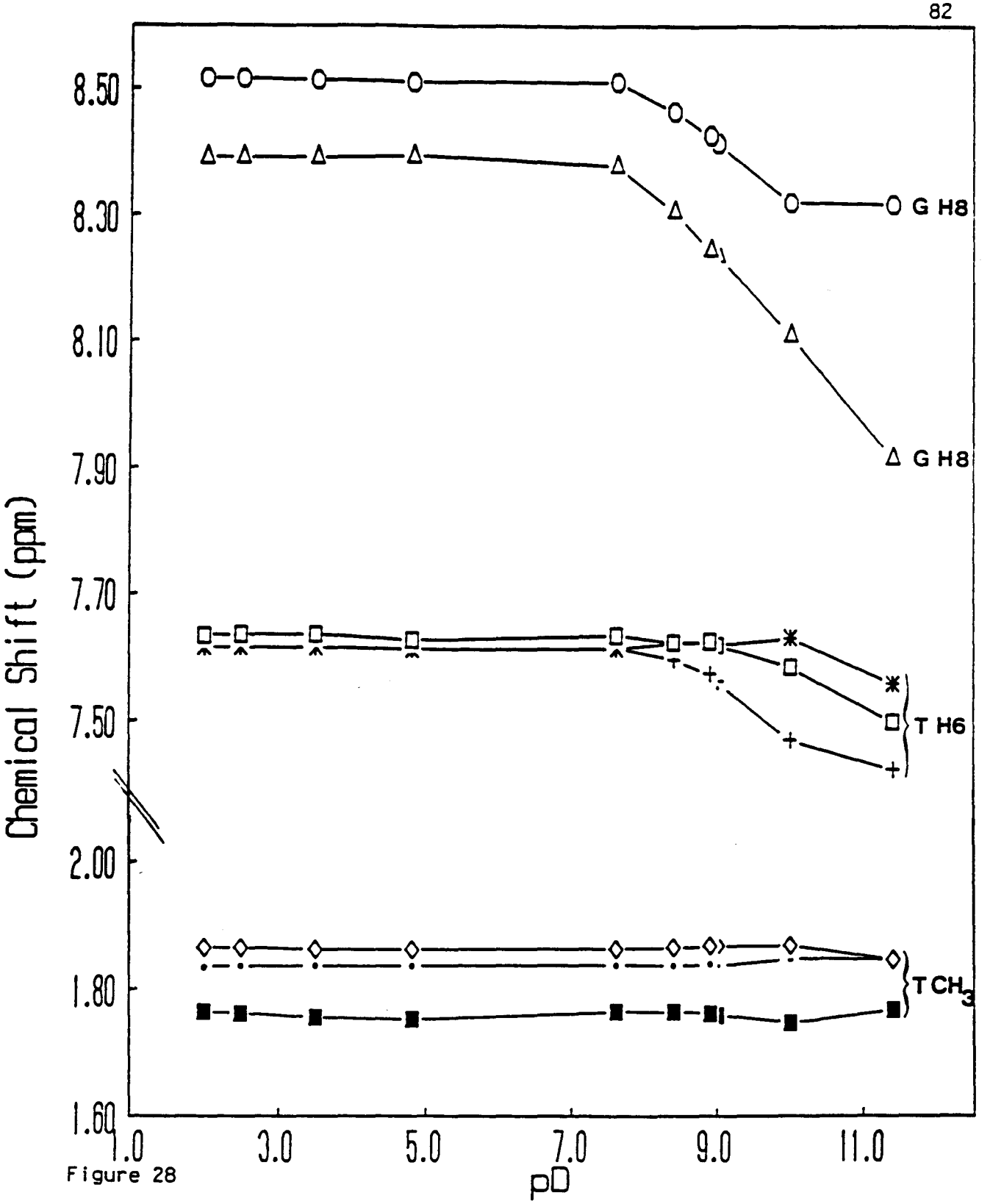


Figure 28

Chemical shift of aromatic and methyl protons as a function of pD for cis-$Pt(NH_3)_2$[d(GTTTG)-N7(1),N7(5)].

TABLE 6

Chemical shift as a function of pH;
cis-$Pt(NH_3)_2$[d(GpTpG)-N7(1),N7(3)], 3.90 mM, 23.4°C.
1-2-3

Chemical Shift vs pH

Proton	pH 1.5	1.9	2.6	3.4	4.5
G-H8	8.366	8.369	8.367	8.367	8.370
G-H8	8.352	8.353	8.352	8.353	8.353
T-H6	7.679	7.677	7.683	7.683	7.684
H1'	6.303	6.305	6.306	6.307	6.307
H1'	6.244	6.244	6.243	6.243	6.243
H1'	6.244	6.244	6.243	6.243	6.243

Proton	pH 5.5	6.8	7.7	8.0	8.5
G-H8	8.370	6.361	8.304	8.277	8.208
G-H8	8.353	8.347	8.304	8.284	8.229
T-H6	7.684	7.683	7.677	7.677	7.672
H1'	6.308	6.307	6.305	6.307	6.308
H1'	6.243	6.241	6.237	6.236	6.231
H1'	6.243	6.241	6.232	6.228	6.217

Proton	pH 9.2	9.8	10.2
G-H8	8.072	7.953	7.876
G-H8	8.111	8.064	8.084
T-H6	7.652	7.607	7.559
H1'	6.318	6.352	6.390
H1'	6.218	6.191	6.175
H1'	6.194	6.191	6.200

TABLE 7

Chemical shift as a function of pH;
cis-$Pt(NH_3)_2$[d(GpTpTpG)-N7(1),N7(4)]. 3.95 mM, 23.4°C.
1-2-3-4

Chemical Shift vs pH

Proton	pH 1.5	2.0	3.2	4.1	5.9
G-H8	8.487	8.491	8.493	8.491	8.493
G-H8	8.357	8.356	8.356	8.356	8.355
T-H6	7.611	7.616	7.619	7.618	7.618
T-H6	7.543	7.546	7.548	7.546	7.547
H1′	6.267	6.267	6.268	6.267	6.268
H1′	6.232	6.231	6.231	6.231	6.231
H1′	6.180	6.181	6.187	6.181	6.182
H1′	5.944	5.942	5.942	5.938	6.940

Proton	pH 6.7	7.6	7.9	8.6	9.1
G-H8	8.493	8.483	8.472	8.443	8.413
G-H8	8.351	8.316	8.293	8.195	8.118
T-H6	7.618	7.616	7.612	7.605	7.593
T-H6	7.547	7.547	7.542	7.542	7.542
H1′	6.268	6.269	6.268	6.275	6.281
H1′	6.231	6.224	6.222	6.207	6.193
H1′	6.181	6.185	6.188	6.193	6.199
H1′	5.942	5.958	5.965	6.014	6.048

Proton	pH 9.7	10.1
G-H8	8.382	8.372
G-H8	8.039	7.989
T-H6	7.570	7.542
T-H6	7.541	7.531
H1′	6.289	6.294
H1′	6.180	6.178
H1′	6.209	6.226
H1′	6.085	6.122

TABLE 8

Chemical shift as a function of pH;
cis-$Pt(NH_3)_2$[d(GpTpTpTpG)-N7(1),N7(5)], 3.95 mM, 21.5°C.
1-2-3-4-5

Chemical Shift vs pH

Proton	pH 2.0	2.5	3.5	4.8	7.6
G-H8	8.516	8.517	8.514	8.511	8.510
G-H8	8.394	8.393	8.394	8.395	8.378
T-H6	7.634	7.637	7.637	7.629	7.636
T-H6	7.615	7.617	7.617	7.614	7.617
T-H6	7.615	7.617	7.617	7.614	7.617
T-CH3	1.864	1.864	1.863	1.862	1.863
T-CH3	1.834	1.836	1.837	1.836	1.836
T-CH3	1.763	1.762	1.754	1.752	1.765

Chemical Shift vs pH

Proton	pH 8.4	8.9	9.0	10.0	11.4
G-H8	8.466	8.426	8.417	8.321	8.319
G-H8	8.306	8.247	8.239	8.110	7.917
T-H6	7.626	7.627	7.623	7.587	7.500
T-H6	7.626	7.627	7.623	7.634	7.562
T-H6	7.599	7.575	7.564	7.474	7.426
T-CH3	1.866	1.867	1.867	1.867	1.845
T-CH3	1.836	1.838	1.837	1.846	1.845
T-CH3	1.764	1.761	1.757	1.748	1.767

or other techniques, VT work allows the study of intra and intermolecular interactions as a function of temperature. For oligomers which are non-complementary, i.e. are unable to form a base-paired, double helix-like structure, VT results can reveal intrastrand base stacking as well as the accompanying changes in the sugar-phosphate backbone structure. When base-pairing occurs between complementary or partially complementary oligomers and a double helix type structure is attained, the formation can be monitored using variable temperature conditions.

UV, CD, and other spectroscopic methods of this type are useful in that they allow the observation of helical structure formation in an overall sense. Results from these techniques, however, are unable to detect the movement of the individual bases and various backbone components. To this extent, the use of NMR has been most valuable and widely employed. All of the different types of nuclei found in nucleic acids are observable by NMR - ^{1}H, ^{13}C, ^{31}P, ^{15}N, and ^{17}O - and have been utilized by many researchers. The mainstay of the work has utilized ^{1}H NMR.

In this research, variable temperature ^{1}H NMR has been used to study the effects of platination on the structure of short, non-complementary deoxyoligomers. The sequences studied were d(GpTpG), d(GpTpTpG), d(GpTpTpTpG) and their cis-$Pt(NH_3)_2(Cl)_2$ analogs. The results from the VT work for the normal oligomers have been listed in Tables 9-11, and the chemical shift versus temperature data plotted in Figures 29-31.

The non-complementary base sequence of the deoxy oligomers, and the presence of the poor stacking thymine bases results in few changes in the chemical shift with temperature variation. Examination of the plots of

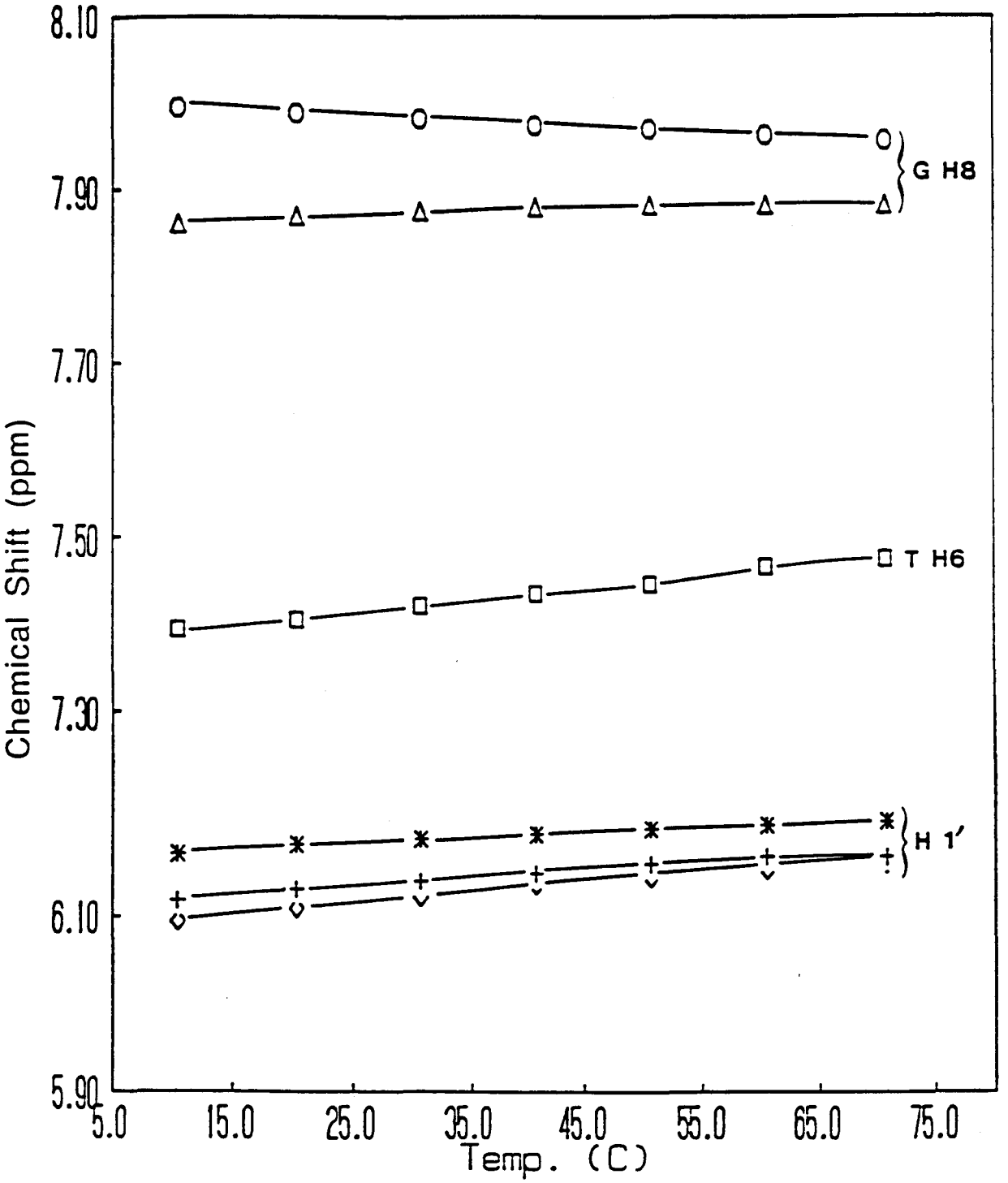


Figure 29

Chemical shift of H1'-anomeric and aromatic protons as a function of temperature for d(GTG).

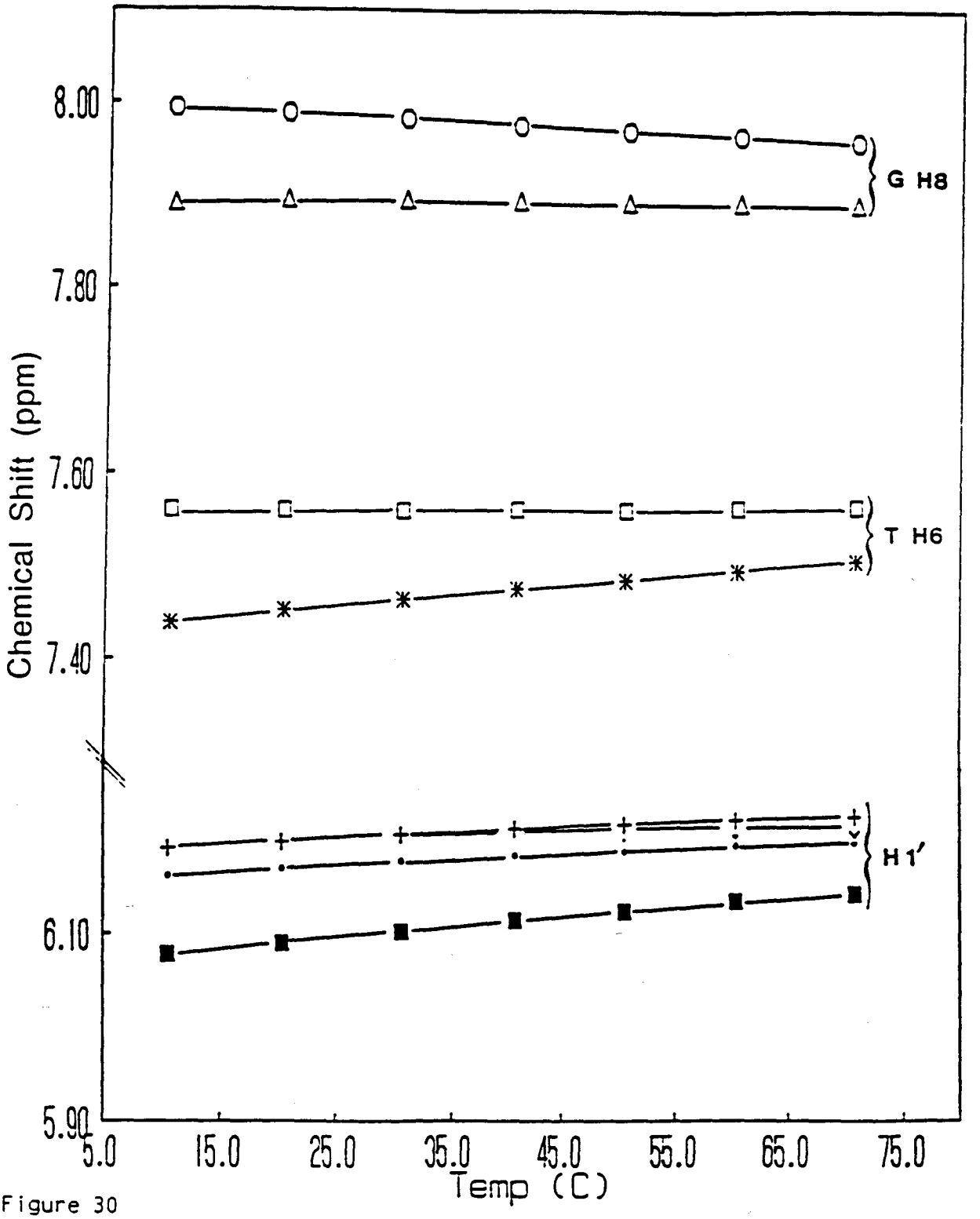


Figure 30

Chemical shift of H1'-anomeric and aromatic protons as a function of temperature for d(GTTG).

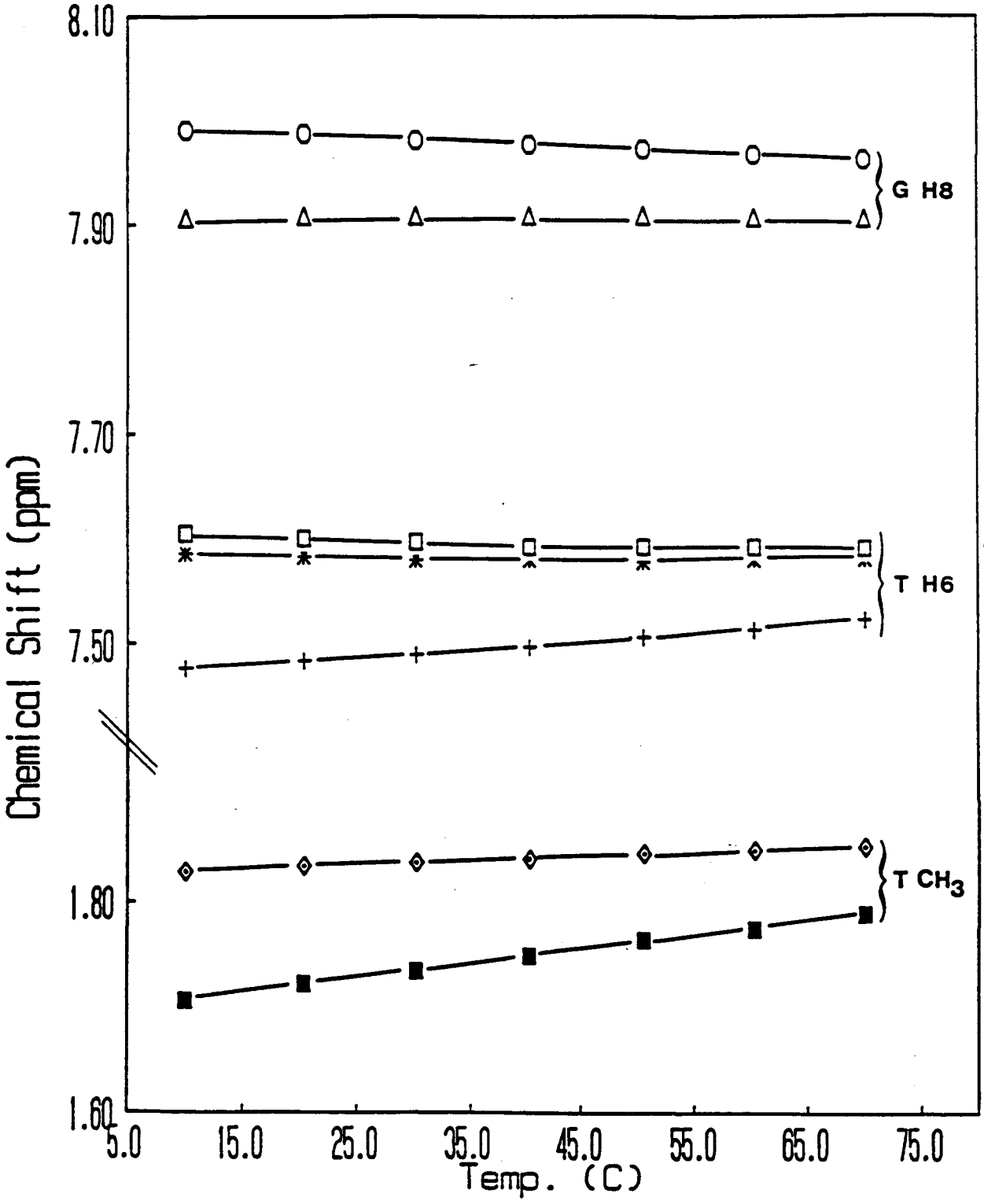


Figure 31

Chemical shift of aromatic and methyl protons as a function of temperature for d(GTTTG).

TABLE 9

Chemical shift as a function of temperature;
d(GpTpG), 3.5 mM, no salt, no buffer.
1-2-3

Chemical Shift vs Temperature (C)

Proton	T(C)	70.7	60.3	50.6	40.8
G(1)-H8		7.956	7.962	7.969	7.975
G(3)-H8		7.879	7.879	7.878	7.876
T(2)-H6		7.473	7.464	7.446	7.433
G(3)-H1'		6.207	6.203	6.199	6.193
T(2)-H1'		6.168	6.167	6.159	6.148
G(1)-H1'		6.160	6.152	6.144	6.134

Proton	T(C)	30.7	20.3	10.6
G(1)-H8		7.982	7.990	7.997
G(3)-H8		7.872	7.867	7.859
T(2)-H6		7.420	7.406	7.394
G(3)-H1'		6.187	6.180	6.171
T(2)-H1'		6.139	6.129	6.116
G(1)-H1'		6.122	6.108	6.093

TABLE 10

Chemical shift as a function of temperature;
d(GpTpTpG), 3.5 mM, no salt, no buffer.
1-2-3-4

Chemical Shift vs Temperature (C)

Proton	T(C)	70.7	60.3	50.6	40.8
G(4)-H8		7.958	7.964	7.970	7.976
G(1)-H8		7.888	7.890	7.891	7.892
T(2)-H6		7.567	7.565	7.563	7.562
T(3)-H6		7.508	7.497	7.487	7.476
G(4)-H1'		6.223	6.219	6.214	6.210
T(2)-H1'		6.210	6.210	6.208	6.206
G(1)-H1'		6.195	6.192	6.187	6.182
T(3)-H1'		6.141	6.133	6.123	6.113

Proton	T(C)	30.7	20.3	10.6
G(4)-H8		7.982	7.989	7.995
G(1)-H8		7.892	7.892	7.889
T(2)-H6		7.560	7.560	7.561
T(3)-H6		7.464	7.452	7.439
G(4)-H1'		6.205	6.196	6.190
T(2)-H1'		6.201	6.196	6.190
G(1)-H1'		6.176	6.168	6.160
T(3)-H1'		6.101	6.089	6.077

TABLE 11

Chemical shift as a function of temperature;
d(GpTpTpTpG), 3.0 mM, no salt, no buffer.
1-2-3-4-5

Chemical Shift vs Temperature (C)

Proton	T(C)	70.0	60.2	50.5	40.3
G(5)-H8		7.962	7.968	7.973	7.978
G(1)-H8		7.901	7.903	7.905	7.905
T(2)-H6		7.588	7.590	7.590	7.591
T(3)-H6		7.577	7.577	7.576	7.577
T(4)-H6		7.520	7.511	7.503	7.495
T-CH3		1.853	1.849	1.846	1.840
T-CH3		1.853	1.849	1.846	1.840
T-CH3		1.790	1.776	1.764	1.749

Proton	T(C)	30.1	20.2	10.0
G(5)-H8		7.982	7.988	7.992
G(1)-H8		7.905	7.905	7.904
T(2)-H6		7.595	7.599	7.605
T(3)-H6		7.578	7.581	7.585
T(4)-H6		7.487	7.482	7.476
T-CH3		1.837	1.834	1.828
T-CH3		1.837	1.834	1.828
T-CH3		1.735	1.722	1.707

chemical shift vs. temperature for the normal, noncomplexed strands shows little significant change in chemical shift over the entire temperature range studied. These results indicate that there has been no increase in intrastrand base stacking with decrease in temperature in these oligomers.

The results for the cis-$Pt(NH_3)_2$ analogues of the above sequences are listed in Tables 12-14, and the chemical shift vs. temperature plots are given in Figures 32-34. These data reveal similar results to those observed for the non- platinated species. Comparing d(GpTpG) with cis-$Pt(NH_3)_2$[d(GpTpG)-N7(1),N7(3)], Figures 29 and 32 respectively, the curves for the G-H8 resonances in the platinated adduct have been shifted downfield over the entire temperature range studied, a result which has been previously discussed in the pH titration section. The T-H6 signal in the platinated material has also been shifted downfield, approximately 0.2 ppm, although no platination has occurred on the thymine base. This change in chemical shift is the result of gua-gua intrastrand crosslink formation which pushes the internal thymine base out of any intrastrand base stacking arrangement which may have existed in the normal parent oligomer. Although it has been shown that the base stacking effects in the normal strand were minimal, the presence of the 3' and 5' purine bases adjacent to the internal pyrimidine would be expected to have some shielding effect on the protons of the thymine base. The formation of the internal bulge upon platination removes the influence of the flanking bases ie. their diamagnetic shielding effect is removed and as a consequence the T-H6 resonance is shifted downfield.

Comparing the tetramer and the pentamer with their platinated analogues, the results again show little change in chemical shift as a result of temperature variation. In both cases, none of the internal thymine bases are dually flanked by purine bases, as in the case of the trimer. As a result, it would be expected that the thymine with the 3′ guanine neighbour would be slightly more shielded than that with the purine as the 5′ neighbour (Ornstein et al. 1983b). The least shielded thymine in the pentamer would be the central base which has pyrimidine bases as 3′ and 5′ nearest neighbours (Giessner-Prettre and Pullman, 1975a,b; Patel and Tonelli, 1975b). Because the shielding effects of the purine bases are expected to be less apparent when compared with the trimer, it would also be expected that platination of the tetramer and the pentamer would have a lesser effect on the shielding of the internal thymine bases. This was, in fact, what was observed. In both cases, the VT curves for the T-H6 protons were only slightly deshielded upon platination of the guanine. This result is again a consequence of bulged loop formation which forces the internal bases out of any intrastrand base stacking arrangement present within the parent strand. In the platinated material, the chemical shifts for the H6 protons of the different thymine bases have become nearly equivalent reflecting the fact that these bases now exist in nearly similar environments. This observation is discussed further in the next section of this work.

3.4.2 Conclusion to Variable Temperature Studies

The following conclusions can be taken from the variable temperature studies: In the normal strands there has been little effect on molecular

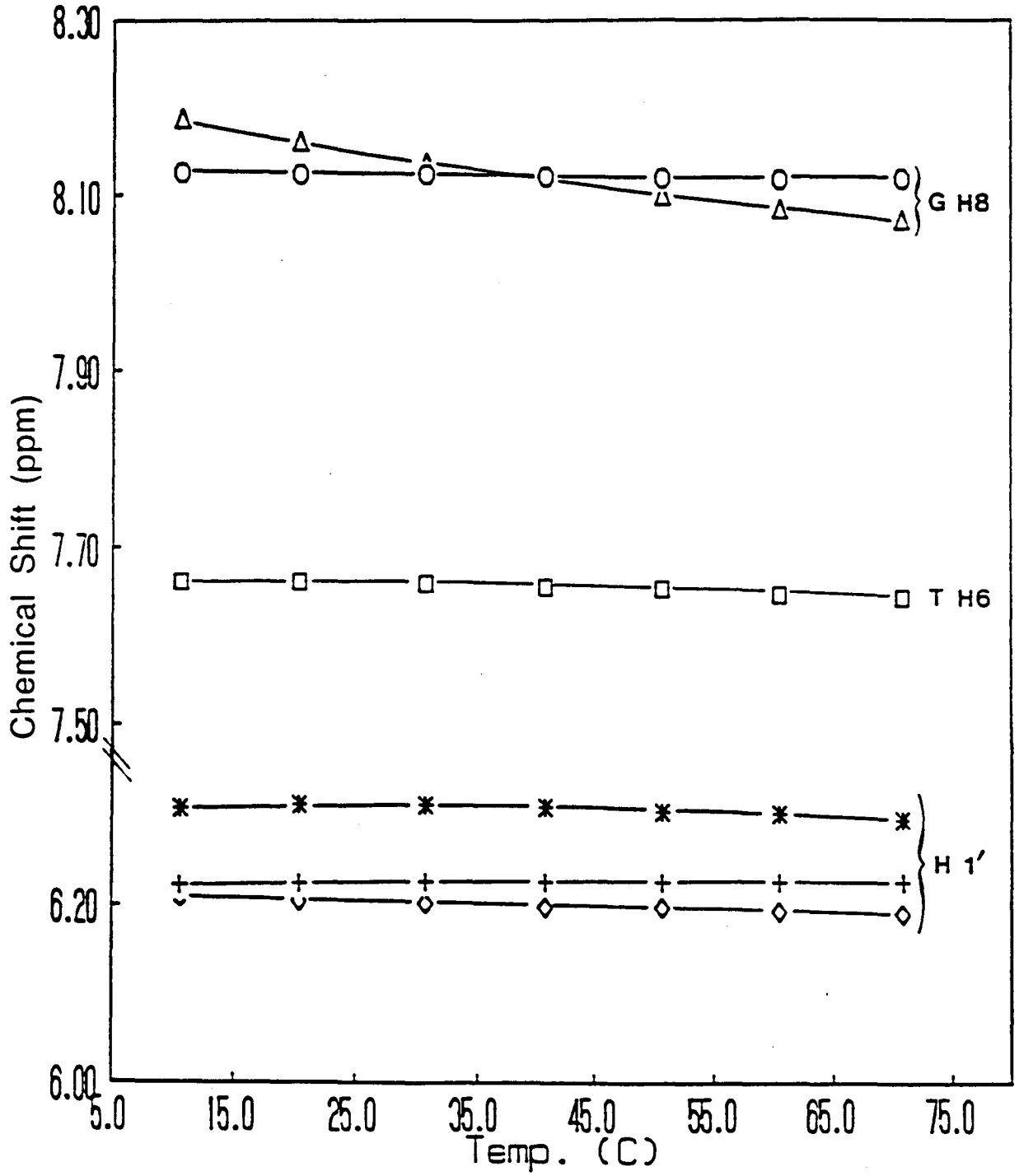


Figure 32

Chemical shift of H1'-anomeric and aromatic protons as a function of temperature for cis-$Pt(NH_3)_2$[d(GTG)-N7(1),N7(3)].

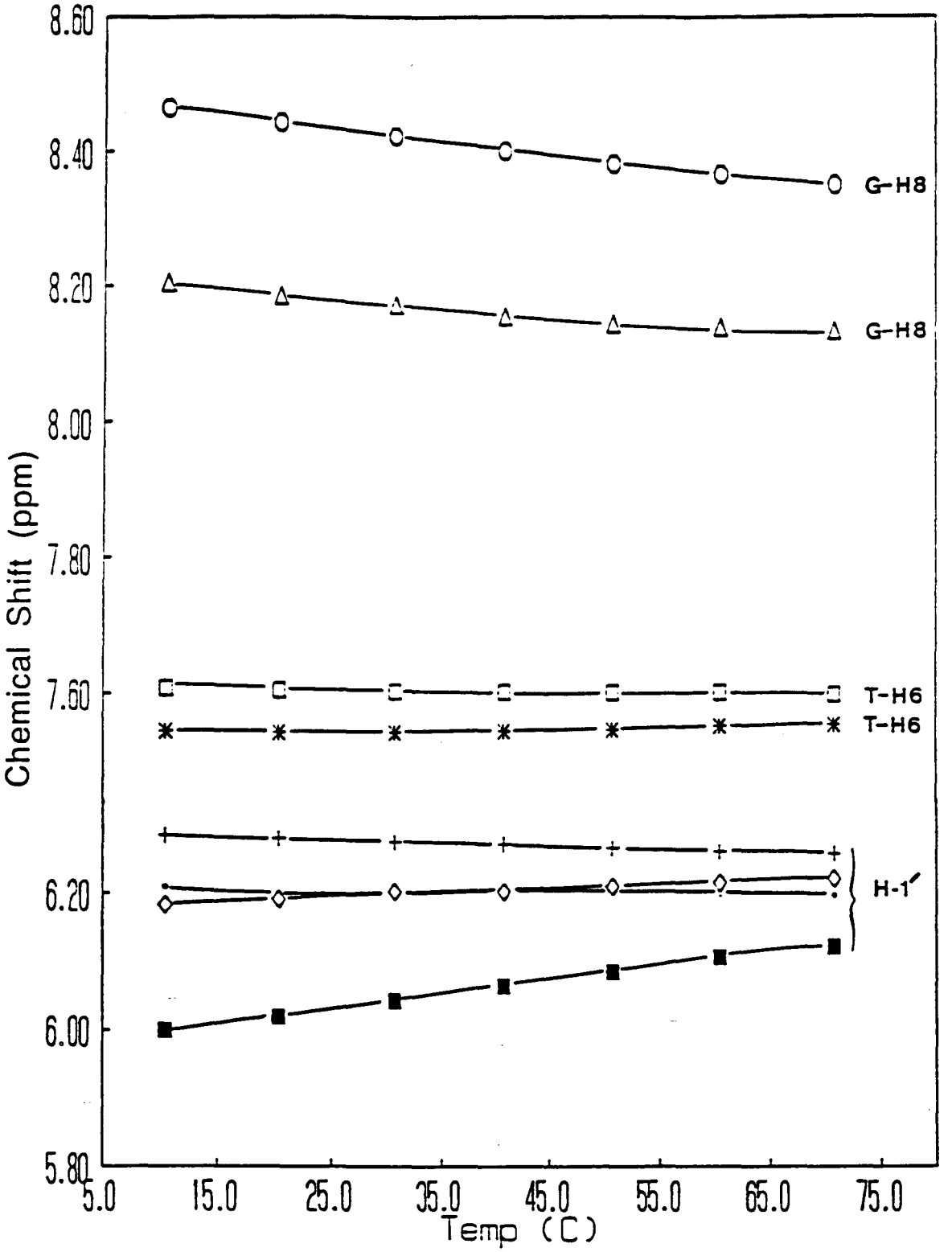


Figure 33

Chemical shift of H1'-anomeric and aromatic protons as a function of temperature for <u>cis</u>-$Pt(NH_3)_2$[d(GTTG)-N7(1),N7(4)].

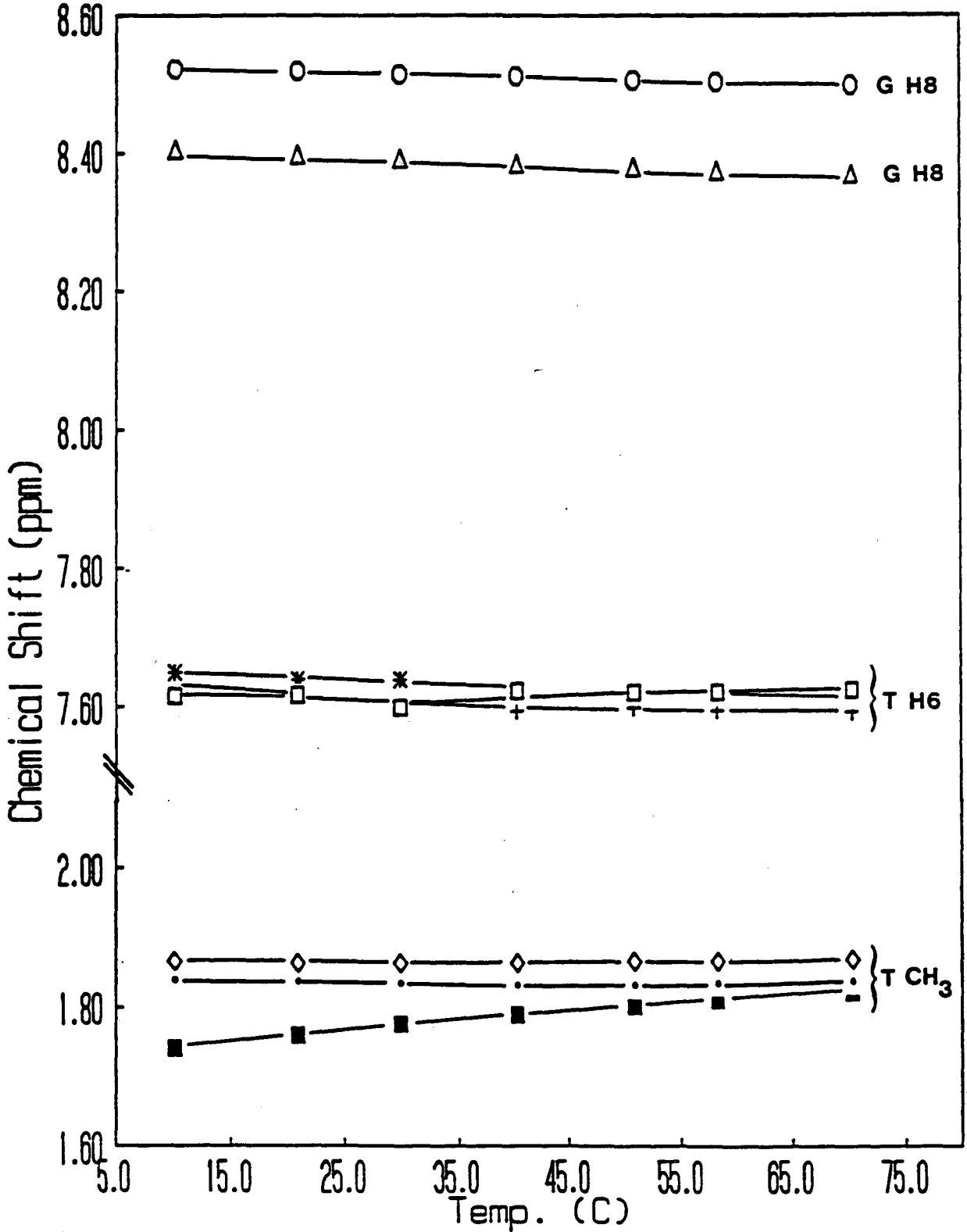


Figure 34

Chemical shift of aromatic and methyl protons as a function of temperature for cis-$Pt(NH_3)_2$[d(GTTTG)-N7(1),N7(5)].

TABLE 12

Chemical shift as a function of temperature;
cis-$Pt(NH_3)_2$[d(GpTpG)-N7(1),N7(3)], 3.5 mM,
1-2-3
no salt, no buffer.

	Chemical Shift vs Temperature (C)			
Proton	T(C) 70.7	60.3	50.6	40.8
G-H8	8.116	8.117	8.119	8.120
G-H8	8.068	8.082	8.097	8.114
T-H6	7.641	7.646	7.651	7.655
H1'	6.297	6.302	6.306	6.309
H1'	6.226	6.226	6.226	6.225
H1'	6.191	6.193	6.196	6.198

Proton	T(C) 30.7	20.3	10.6
G-H8	8.123	8.123	8.125
G-H8	8.135	8.159	8.184
T-H6	7.658	7.661	7.660
H1'	6.311	6.311	6.308
H1'	6.226	6.223	6.221
H1'	6.201	6.204	6.207

TABLE 13

Chemical shift as a function of temperature;
cis-$Pt(NH_3)_2$[d(GpTpTpG)-N7(1),N7(4)], 3.5 mM,
1-2-3-4
no salt, no buffer.

Chemical Shift vs Temperature (C)

Proton	T(C)	70.7	60.3	50.6	40.8
G-H8		8.350	8.366	8.383	8.401
G-H8		8.129	8.136	8.141	8.152
T-H6		7.600	7.601	7.602	7.602
T-H6		7.554	7.550	7.547	7.544
H1'		6.256	6.260	6.265	6.269
H1'		6.219	6.214	6.209	6.202
H1'		6.196	6.197	6.198	6.202
H1'		6.122	6.104	6.084	6.063

Proton	T(C)	30.7	20.3	10.6
G-H8		8.421	8.443	8.464
G-H8		8.166	8.183	8.201
T-H6		7.603	7.604	7.606
T-H6		7.542	7.542	7.544
H1'		6.273	6.277	6.282
H1'		6.201	6.190	6.183
H1'		6.201	6.201	6.210
H1'		6.041	6.018	5.999

TABLE 14

Chemical shift as a function of temperature;
cis-$Pt(NH_3)_2$[d(GpTpTpTpG)-N7(1),N7(5)], 3.0mM,
1-2-3-4-5
no salt, no buffer.

Chemical Shift vs Temperature (C)

Proton	T(C)	70.3	58.2	50.8	40.2
G-H8		8.500	8.504	8.506	8.512
G-H8		8.366	8.372	8.376	8.383
T-H6		7.623	7.620	7.620	7.623
T-H6		7.614	7.620	7.620	7.623
T-H6		7.592	7.595	7.598	7.594
T-CH3		1.868	1.866	1.865	1.864
T-CH3		1.835	1.833	1.832	1.832
T-CH3		1.821	1.811	1.802	1.788

Proton	T(C)	29.8	20.8	10.2
G-H8		8.516	8.520	8.524
G-H8		8.390	8.396	8.404
T-H6		7.600	7.618	7.615
T-H6		7.640	7.639	7.649
T-H6		7.602	7.618	7.628
T-CH3		1.864	1.863	1.865
T-CH3		1.834	1.836	1.840
T-CH3		1.774	1.760	1.742

conformation as a result of temperature change. There was no significant increase in the intrastrand base stacking with decrease in temperature. Formation of the platinum chelate with the terminal guanine bases, which in turn forced the internal thymine base(s) out of any existing stacking interaction, resulted in a slight deshielding of the thymine protons. This result is similar to the observations made for cis-$Pt(NH_3)_2$[d(GCG)-N7(1), N7(3)], (den Hartog et al., 1983). Within the bulged loop region of the tetramer and the pentamer, where there is the possiblity of interaction between the bases in the bulge, no evidence of base-stacking in the loop was observed. In general the structure of the platinated oligomers would appear to be static, with little conformational change as a function of temperature. This result is not unexpected because of the conformationally restrictive nature of the platinum chelate.

3.5 Exchangeable Imino Proton Study

Examination of the exchangeable imino protons on the heterocyclic bases in the study of nucleic acids is most useful in the observation of double helix formation (Weiss et al. 1984, Patel et al. 1982a, Hilbers et al. 1975). When Watson-Crick, or other types of base pairs are formed, it is possible to detect the imino protons involved in the inter base hydrogen bonds by ^1H NMR as their exchange rates are greatly decreased upon base-pair formation. Imino protons have also been observed where access to these protons by water molecules has been conformationally restricted, again resulting in a decrease in their rate of exchange with solvent molecules. The formation of hairpin loop structures in nucleic acids results in the restricted movement of water to the sites of

exchangeable protons on the bases involved in the loop structure (Haasnoot et al. 1983).

The formation of the intrastrand cis-$Pt(NH_3)_2$[N(7)G, N(7)G] complex is similar to the point of nucleation for the beginning of a hairpin loop. For a normal hairpin it has been suggested that the minimum number of bases needed in the loop is three to seven (Haasnoot et al. 1980, Tinoco et al. 1971). However, when the base of the hairpin is covalently held together, a smaller number of bases in the loop may be sufficient. In the formation of the bulged loops with the three sequences examined in this section by the reaction with cis-$Pt(NH_3)_2Cl_2$, loops of one, two, and three bases in length are found. As has been observed in larger hairpin loops, there is restricted access to the exchangeable imino protons on the thymine bases by water molecules allowing for the observation of their N(3)-H proton signals in NMR.

The three platinated oligomers were initially examined in a 90:10 H_2O:D_2O solution buffered at pH 7.0. The fact that any signal at all was observed for the exchangeable protons implies that the rate of exchange for these protons with the solvent molecules has been decreased. For both cis-$Pt(NH_3)_2$[d(GpTpTpG)-N7(1),N7(4)] and cis-$Pt(NH_3)_2$[d(GpTpTpTpG)-N7(1),N7(5)], a resonance peak at approximately 11.2 ppm is visible at 0°C. This signal has been assigned to the N(3)-H of the thymine bases in the internal bulge. The signal broadened as the temperature was increased, with the peak broadening out beyond recognition at 25°C. These results have been interpreted in a similar manner to signals assigned to bases found in the loop region of a hairpin structure (Haasnoot et al. 1980). In the case of the cis-$Pt(NH_3)_2$[d(GpTpG)-

N7(1),N7(3)] complex there was no indication of the peak at 11.2 ppm, even at 0°C. For the Pt-trimer molecule, where only one base is looped out, the absence of the signal at 11.2 ppm suggests that there is unhindered access to the N(3) site on the lone thymine base for solvent molecules. When the loop is made larger, as was the case with the tetramer and the pentamer, the internal loops must orient themselves in such a way as to hinder the exchange of the N(3)-H of thymine with the surrounding water molecules. As the temperature is increased, an increase in motion within the loop occurs and the rate of exchange increases to the point where the signal broadens out completely.

The same experiments were repeated with the system buffered at pH 5.5. Similar results were obtained with minor exceptions. For the cis-$Pt(NH_3)_2$[d(GpTpG)-N7(1),N7(3)], a very broad signal centred about 11.2 ppm was visible at 0°C. This signal was broadened out by 5°C, again indicating with the trimer complex that the lone thymine base in the bulge has little steric hinderance to exchange of its imino proton with water. For the larger loop systems, the decrease in pH helped to sharpen the resonance signal at low temperature. Again the signal broadened as the temperature was increased, completely disappearing by 35°C. An example of the NMR spectral results for cis-$Pt(NH_3)_2$[d(GTTTG)-N7(1),N7(5)] is given in Figure 35.

A signal of this type has been observed in several different nucleic acid structures. As stated earlier, a resonance peak has been observed for the bases found in the loop of a hairpin structure (Haasnoot et al. 1983). Also, in the examination of poly-dT, a signal was observed which has been assigned to the N(3)-H of the thymine bases, resonating at 11.0

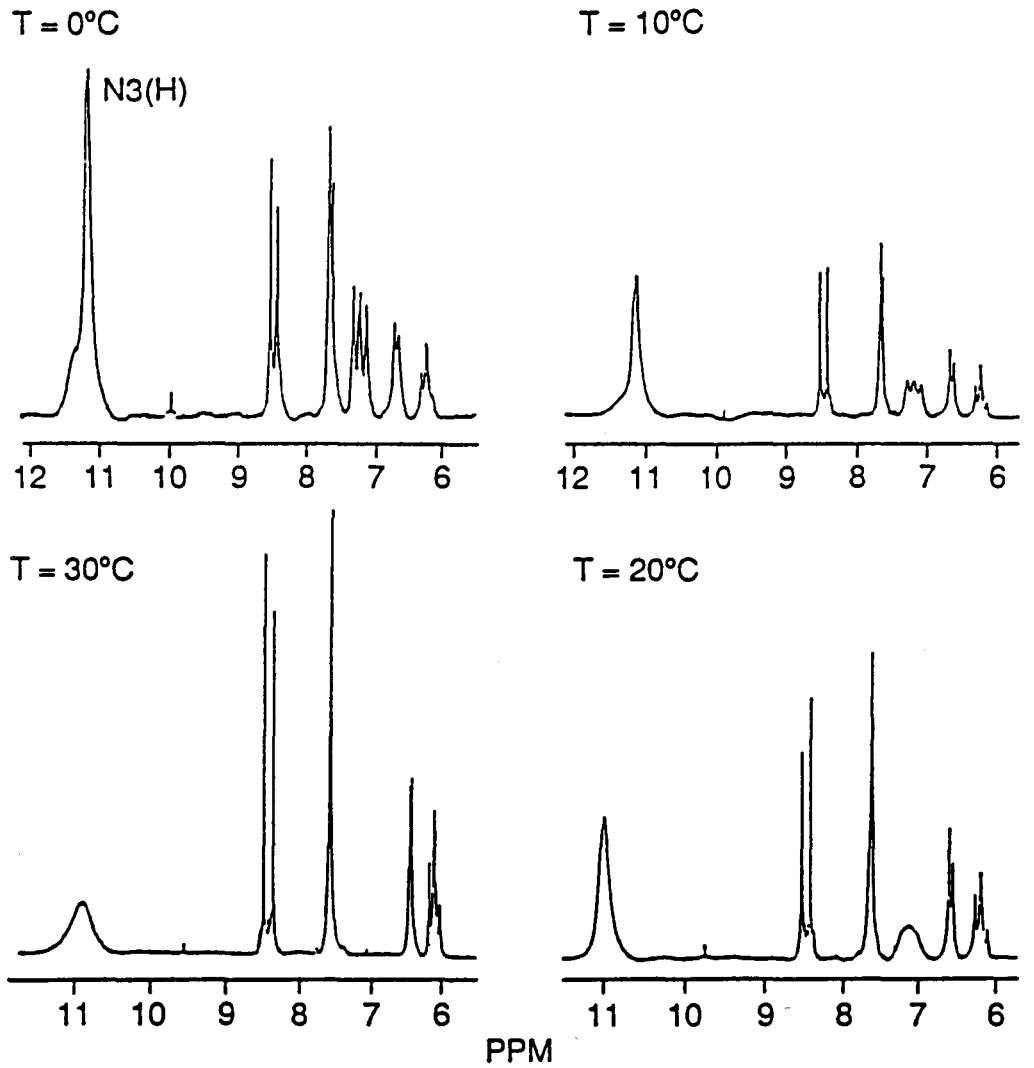


Figure 35

Observation of thymine N(3)-H exchangeable imino proton as a function of temperature for <u>cis</u>-$Pt(NH_3)_2$[d(GTTTG)-N7(1),N7(5)], pD 5.5, H_2O:D_2O 9:1.

ppm (Haasnoot et al. 1983, 1979). This result has the closest chemical shift to that observed in the platinated species and, in the case of poly-dT, has been interpreted as the formation of a random coil conformation rather than a helical structure. A similar interpretation would likely apply in the cisplatin-oligomer complexes, where some form of structurally crowded grouping is present in the loop region of the complex, not likely containing any helical nature.

3.6 Conclusions

In the study of the oligomers d(GpTpG), d(GpTpTpG) and d(GpTpTpTpG), all were found to react with cis-$Pt(NH_3)_2(Cl)_2$ to form one major reaction product. The site of the platinum binding has been determined by pH titration as followed by ^1H NMR, with reaction occurring only at the N(7) site of the guanine bases. The nature of cis-$Pt(NH_3)_2Cl_2$ binding to the three different oligomers was the formation of an intrastrand platinum-$[N(7)G]_2$ chelate between the terminal guanine bases in the oligomers. The internal thymine bases in all three sequences have been forced out of a single strand stacking conformation to form bulged loops, with the intramolecular movement within the whole complex being somewhat restricted. Although there was no indication for the interaction between bases within the loop, there was evidence for the restricted interaction of water molecules with this loop section of the molecule. This decrease in interaction between the solvent and the loop region of the complex allowed the observation of the exchangeable thymine imino protons at low temperature. The loop region, as a result, is believed to take up a

random coil-like conformation which sterically hinders the access of water molecules to this section of the platinum-oligomer complex.

Chapter Four

4.1 Introduction

4.1.1 cis-$Pt(NH_3)_2Cl_2$ interaction with longer deoxyribonucleic acid oligomers.

The interaction of the anti-tumor compound cis-$Pt(NH_3)_2(Cl)_2$ with DNA has been widely studied but the mode of action of the drug has not yet been completely elucidated. There have been several mechanisms proposed for the platinum compound's anti-neoplastic activity, many of which have fallen out of favour as research on the drug has progressed. As recently as 1983, it was thought that the formation of the cis-$Pt(NH_3)_2$[d(GpG)] complex within a strand of DNA resulted in the complete disruption of hydrogen bonding ability in the complexed region of the molecule (Caradonna et al. 1982, Girault et al. 1984b, Marcelis et al. 1983b). Closer scrutiny of the short oligomer strands used in the earlier work which led to this conclusion, however, reveals shortcomings in the choice of sequence. In several instances self-complementary strands were studied, ie. d(AGGCCT) (Caradonna et al. 1982), which result in two platinum chelates per double helix with only terminal A-T base pairs to secure the duplex. Results have shown that structural modifications of the duplex to this extent did not allow the formation of the double helix. More recent work using two complementary sequences ie. (1) d(CATCGGCATC) + (2) d(GATGCCGATG), where only strand (1) contained the cis-$Pt(NH_3)_2$[d(GpG)] complex, has resulted in the formation

of a double helix (Kozelka et al. 1986). Not only was the formation of the duplex observed, but the core G-C base pairs were shown to be stable to 35°C under the conditions used. Several other publications using complementary sequences have arrived at similar results (den Hartog et al. 1985a, 1984a, 1984b, van Hemelryck et al. 1984). With the understanding that it was possible to have duplex formation which included deoxyoligomers modified with cis-$Pt(NH_3)_2(Cl)_2$, our work using internal loops was extended to longer oligomer strands.

Focussing on the metal atom in the platinum-DNA adduct, the square planar geometry about the platinum(II) restricts the distances and angles within the region of the chelate. The bond angle between the two dG-N(7) sites centred on the platinum atom would be approximately 90° and the distance between the two N(7) atoms would also be fixed (Figure 36). Applying these parameters to the structure of any of the platinated oligomers, regardless of the type of chelate ie. d(GpG) or $d(GpX_npG)$, the geometry about the guanine bases, i.e. distances and angles, would be fixed by the platinum atom. Results from den Hartog et al.(1983, 1982) have shown an important structural difference between the d(GpG) and the d(GpCpG) oligomers after they have been reacted with cis-$Pt(NH_3)_2(Cl)_2$. Their work has shown that in the cisplatin-dimer complex, both of the guanine bases remain in the anti conformation with respect to their sugar moieties (den Hartog et al. 1982). In the case of the cisplatin-trimer adduct it was shown that one guanine retains the original anti conformation while the second guanine, perhaps as a means for relief of strain in the chelate, has rotated about its glycosyl bond into a more syn-like conformation (den Hartog et al. 1983). The

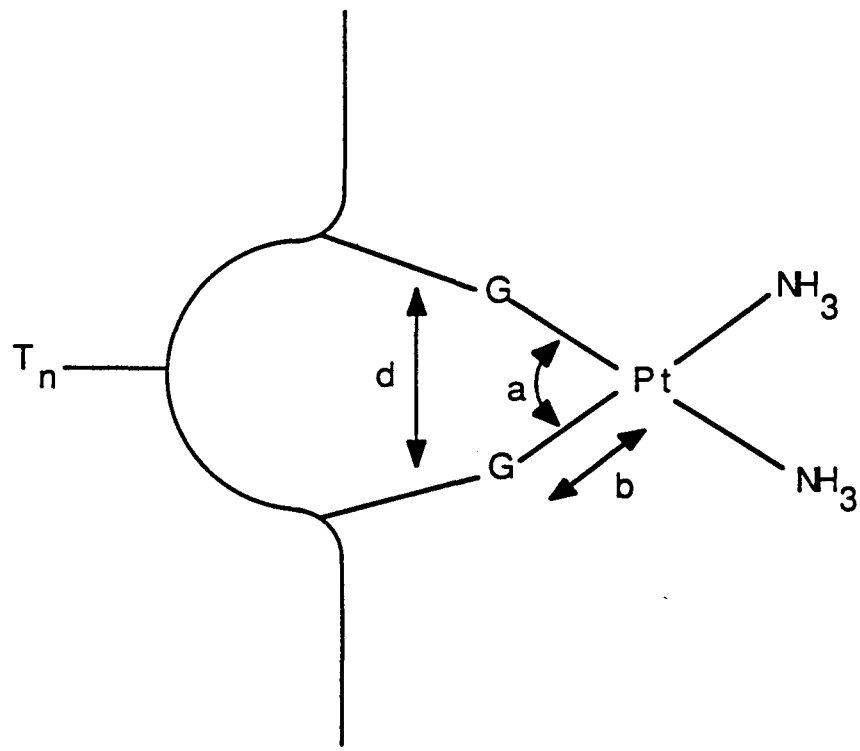


Figure 36

Proposed structural conformation about the platinum atom in a cisplatin-DNA adduct. Illustration shows the guanine-guanine distance (d), the Pt-guanine bond length (b), and the G(N7)-Pt-G(N7) bond angle (a) in the complex.

importance of this alteration in orientation is the loss of the guanine base in the syn conformation from participation in hydrogen bond formation. This result is an important factor in the loop structures formed in the present study and consequently, their ability to form base pairs.

4.1.2 Outline

To continue the work described in Chapter 3.1, it was desirable to incorporate the internal bulged loops formed by reaction of cis-$Pt(NH_3)_2(Cl)_2$ with deoxy oligomers into longer oligomer strands to determine their ability to form duplex structures. This section of the reseach has been divided into four sections A) The characterization of the non-complementary deoxyoligomers; (i) d(CTGGTC), (ii) d(CTGTGTC), and (iii) d(CTGTTGTC) as well as their cisplatin adducts, B) The investigation of normal duplex formation by (i), (ii), and (iii) with their complements, (iv) dGACCAG, (v) dGACACAG, and (vi) d(GACAACAG) respectively, C) The study of duplex formation between the non-complementary sequences (ii) + (iv), and (iii) + (v), both of which contain an extra internal thymine base on one strand, and (iii) + (iv) where (iii) contains two extra internal thymine bases and D) The investigation into duplex stability for the mixtures (i-Pt) + (iv), (ii-Pt) + (v), (iii-Pt) + (vi), which are all fully complementary mixtures, (ii-Pt) + (iv) and (iii-Pt) + (v), both of which are missing the complement to the looped out thymine, and (iii-Pt) + (iv) where the complements to both thymine bases involved in the internal bulge are missing. This has been outlined on Table 15.

Table 15

Proposed experiments with normal and platinated deoxy oligomers.

A) Incorporation of loops into longer strands.

1. (i) dCTGGTC
2. (i-Pt) cis-$Pt(NH_3)_2$[d(CTGGTC)-N7(3),N7(4)]
3. (ii) dCTGTGTC
4. (ii-Pt) cis-$Pt(NH_3)_2$[d(CTGTGTC)-N7(3),N7(5)]
5. (iii) dCTGTTGTC
6. (iii-Pt) cis-$Pt(NH_3)_2$[d(CTGTTGTC)-N7(3),N7(6)]

B) Investigation into normal duplex stability.

7. (i) dCTGGTC + (iv) dGACCAG
8. (ii) dCTGTGTC + (v) dGACACAG
9. (iii) dCTGTTGTC + (vi) dGACAACAG

C) Formation of imperfect duplexes.

10. (ii) dCTGTGTC + (iv) dGACCAG
    Presence of one extra thymine base.

11. (iii) dCTGTTGTC + (v) dGACACAG
    Presence of one extra thymine base.

12. (iii) dCTGTTGTC + (iv) dGACCAG
    Presence of two extra thymine bases.

D) Duplex formation with platinated sequences.

13. (i-Pt) cis-$Pt(NH_3)_2$[d(CTGGTC)-N7(3),N7(4)]
    + (iv) dGACCAG.

14. (ii-Pt) cis-$Pt(NH_3)_2$[d(CTGTGTC)-N7(3),N7(5)]
    + (v) dGACACAG.

15. (ii-Pt) cis-$Pt(NH_3)_2$[d(CTGTGTC)-N7(3),N7(5)]
    + (iv) dGACCAG.

16. (iii-Pt) cis-$Pt(NH_3)_2$[d(CTGTTGTC)-N7(3),N7(6)]
    + (vi) dGACAACAG.

17. (iii-Pt) cis-$Pt(NH_3)_2$[d(CTGTTGTC)-N7(3),N7(6)]
    + (v) dGACACAG.

18. (iii-Pt) cis-$Pt(NH_3)_2$[d(CTGTTGTC)-N7(3),N7(6)]
    + (iv) dGACCAG.

4.2.1 Internal loops in longer strands

The sequences investigated were (i) d(CTGGTC), (ii) d(CTGTGTC) and (iii) d(CTGTTGTC) as well as their cis-$Pt(NH_3)_2(Cl)_2$ adducts, (i-Pt), (ii-Pt), and (iii-Pt) respectively. The normal deoxy oligomers were prepared by a phosphotriester method, as previously outlined in Chapter 2. The formation of the cis-$Pt(NH_3)_2(Cl)_2$ adducts was performed as given in the previous section and the reactions were again monitored by UV and reverse phase HPLC. As with the platinum reactions in the previous study, platination of the deoxy oligomers was accompanied by a shift in the UV to longer wavelengths for both the λ_{max} and the λ_{min}, (Table 16). HPLC and ^1H NMR results indicated only one major product was recovered from the three platinum-oligomer reactions and in each case UV analysis showed this product to contain greater than 95% of the original UV absorbing material. Assignment of the proton resonances of the parent oligomers to a specific nucleotide has been carried out using calculated chemical shift values obtained by the method of Bell et al. 1981, Table 17.

4.2.2 pH Titration Studies

The reactive sites on the deoxyribonucleic acid oligomers towards cis-$Pt(NH3)_2(Cl)_2$ were determined by the comparison of pH titration results for the normal and platinated oligomer strands as obtained by proton NMR. The experimental data have been listed in Tables 18-23 and plots showing the change in chemical shift as a function of pH from this work are given in Figures 37-42.

Table 16

Wavelength (nm) of absorbance maxima and minima of DNA oligomers before and after reaction with cis-$Pt(NH_3)_2Cl_2$.

	λ_{max}	λ_{min}
d(CTGGTC)	257	230
cis-$Pt(NH_3)_2$[d(CTGGTC)-N7(3),N7(4)	265	236
d(CTGTGTC)	258	231
cis-$Pt(NH_3)_2$[d(CTGTGTC)-N7(3),N7(5)	265	236
d(CTGTTGTC)	262	233
cis-$Pt(NH_3)_2$[d(CTGTTGTC)-N7(1),N7(5)	265	235

Table 17

Assignment of aromatic proton chemical shifts (ppm)
for longer oligomers based on calculated values at 70°C.

		dCpTpGpGpTpC 1-2-3-4-5-6		dCpTpGpTpGpTpC 1-2-3-4-5-6-7		dCpTpGpTpTpGpTpC 1-2-3-4-5-6-7-8	
		calc.	obs.	calc.	obs.	calc.	obs.
5′							
C	H6	7.744	7.728	7.753	7.733	7.753	7.767
	H5	5.999	5.985	5.999	5.987	5.999	6.037
T	H6	7.561	7.468	7.575	7.398	7.584	7.479
	CH3	1.826	1.853	1.858	1.856	1.859	1.847
G	H8	7.920	7.813	7.946	7.913	7.971	7.927
T	H6	-----	-----	7.580	7.490	7.599	7.494
	CH3	-----	-----	1.821	1.736	1.812	1.747
T	H6	-----	-----	-----	-----	7.583	7.467
	CH3	-----	-----	-----	-----	1.855	1.817
G	H8	7.961	7.907	7.961	7.919	7.991	7.932
T	H6	7.582	7.500	7.587	7.512	7.613	7.502
	CH3	1.805	1.757	1.825	1.759	1.844	1.754
C	H6	7.802	7.819	7.808	7.828	7.808	7.837
	H5	6.040	6.031	6.068	6.040	6.068	6.069
3′							

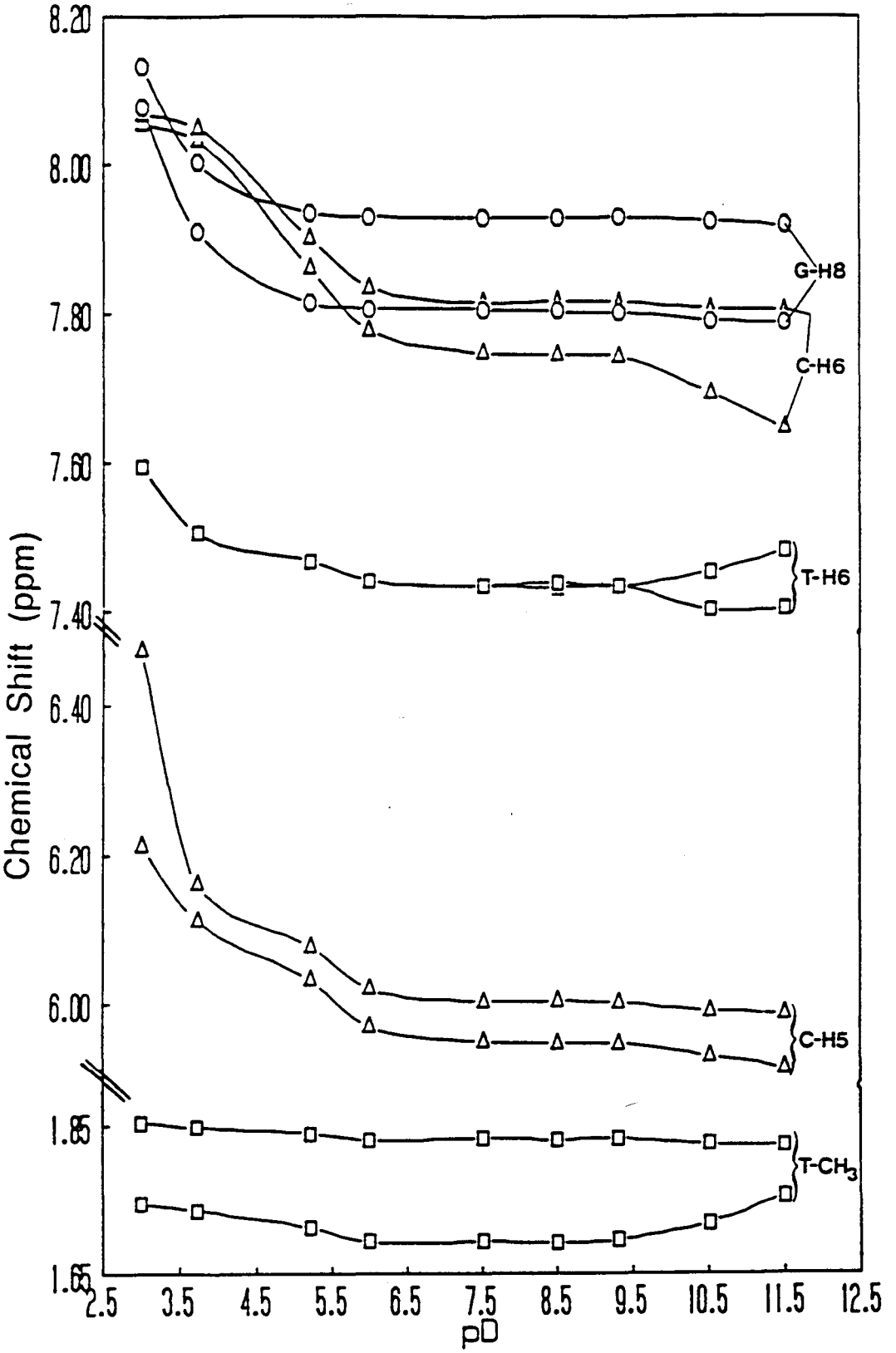


Figure 37

Chemical shift of aromatic and methyl protons as a function of pD for d(CTGGTC).

TABLE 18

Chemical shift as a function of pH;
d(CpTpGpGpTpC), 1.5 mM, 25°C, no salt, no buffer.
1-2-3-4-5-6

Chemical Shift vs pH

Proton	pH 3.0	3.7	5.2	6.0	7.5
G(4)-H8	8.131	8.002	7.935	7.930	7.927
G(3)-H8	8.077	7.910	7.814	7.807	7.805
C(6)-H6	8.067	8.049	7.902	7.836	7.814
C(1)-H6	8.054	8.032	7.862	7.778	7.746
T(2)-H6	7.593	7.505	7.468	7.442	7.435
T(5)-H6	7.593	7.505	7.468	7.442	7.435
C(6)-H5	6.475	6.162	6.079	6.023	6.006
C(1)-H5	6.213	6.112	6.034	5.972	5.952
T(2)-CH3	1.854	1.847	1.839	1.830	1.832
T(5)-CH3	1.745	1.735	1.712	1.694	1.694

Proton	pH 8.5	9.3	10.5	11.5
G(4)-H8	7.928	7.929	7.924	7.919
G(3)-H8	7.803	7.802	7.791	7.789
C(6)-H6	7.818	7.816	7.807	7.805
C(1)-H6	7.745	7.742	7.694	7.646
T(2)-H6	7.440	7.435	7.455	7.483
T(5)-H6	7.433	7.435	7.403	7.405
C(6)-H5	6.007	6.005	5.995	5.993
C(1)-H5	5.950	5.950	5.935	5.920
T(2)-CH3	1.830	1.831	1.824	1.822
T(5)-CH3	1.692	1.696	1.719	1.755

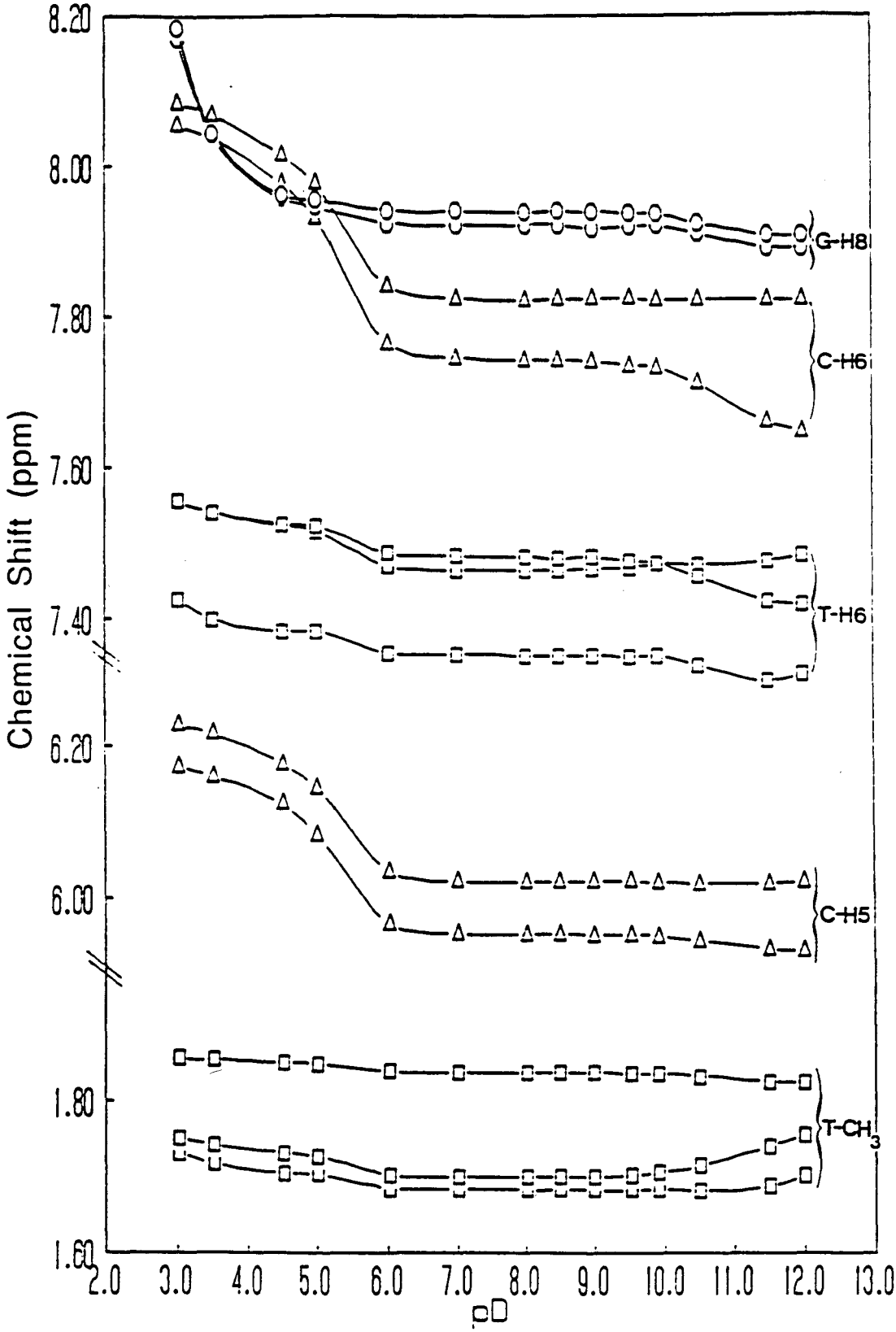


Figure 38

Chemical shift of aromatic and methyl protons as a function of pD for d(CTGTGTC).

TABLE 19

Chemical shift as a function of pH;
d(CpTpGpTpGpTpC), 1.5 mM, 25°C, no salt, no buffer.
1-2-3-4-5-6-7

Chemical Shift vs pH

Proton	pH 3.0	3.5	4.5	5.0	6.0
G(5)-H8	8.184	8.044	7.960	7.954	7.939
G(3)-H8	8.168	8.038	7.956	7.945	7.922
C(7)-H6	8.082	8.067	8.014	7.977	7.839
C(1)-H6	8.053	8.036	7.976	7.931	7.763
T(4)-H6	7.553	7.539	7.523	7.521	7.485
T(6)-H6	7.553	7.539	7.523	7.514	7.468
T(2)-H6	7.424	7.398	7.381	7.382	7.353
C(7)-H5	6.227	6.215	6.174	6.144	6.032
C(1)-H5	6.172	6.159	6.122	6.082	5.964
T(2)-CH3	1.856	1.854	1.850	1.848	1.838
T(6)-CH3	1.751	1.742	1.731	1.727	1.702
T(4)-CH3	1.731	1.717	1.705	1.703	1.685

Proton	pH 7.0	8.0	8.5	9.0	9.5
G(5)-H8	7.938	7.937	7.938	7.937	7.934
G(3)-H8	7.920	7.920	7.920	7.915	7.917
C(7)-H6	7.822	7.820	7.821	7.821	7.821
C(1)-H6	7.743	7.740	7.739	7.738	7.732
T(4)-H6	7.480	7.479	7.477	7.478	7.473
T(6)-H6	7.461	7.461	7.462	7.463	7.464
T(2)-H6	7.350	7.349	7.348	7.348	7.346
C(7)-H5	6.018	6.017	6.017	6.017	6.016
C(1)-H5	5.950	5.948	5.948	5.947	5.946
T(2)-CH3	1.837	1.837	1.836	1.836	1.835
T(6)-CH3	1.699	1.699	1.699	1.700	1.702
T(4)-CH3	1.684	1.683	1.683	1.683	1.683

Proton	pH 9.9	10.5	11.5	12.0
G(5)-H8	7.934	7.921	7.905	7.905
G(3)-H8	7.918	7.907	7.888	7.887
C(7)-H6	7.820	7.819	7.819	7.820
C(1)-H6	7.729	7.708	7.657	7.644
T(4)-H6	7.470	7.452	7.420	7.415
T(6)-H6	7.470	7.467	7.473	7.480
T(2)-H6	7.349	7.335	7.315	7.324
C(7)-H5	6.015	6.012	6.013	6.015
C(1)-H5	5.944	5.938	5.926	5.924
T(2)-CH3	1.835	1.831	1.824	1.824
T(6)-CH3	1.706	1.714	1.740	1.754
T(4)-CH3	1.685	1.681	1.688	1.702

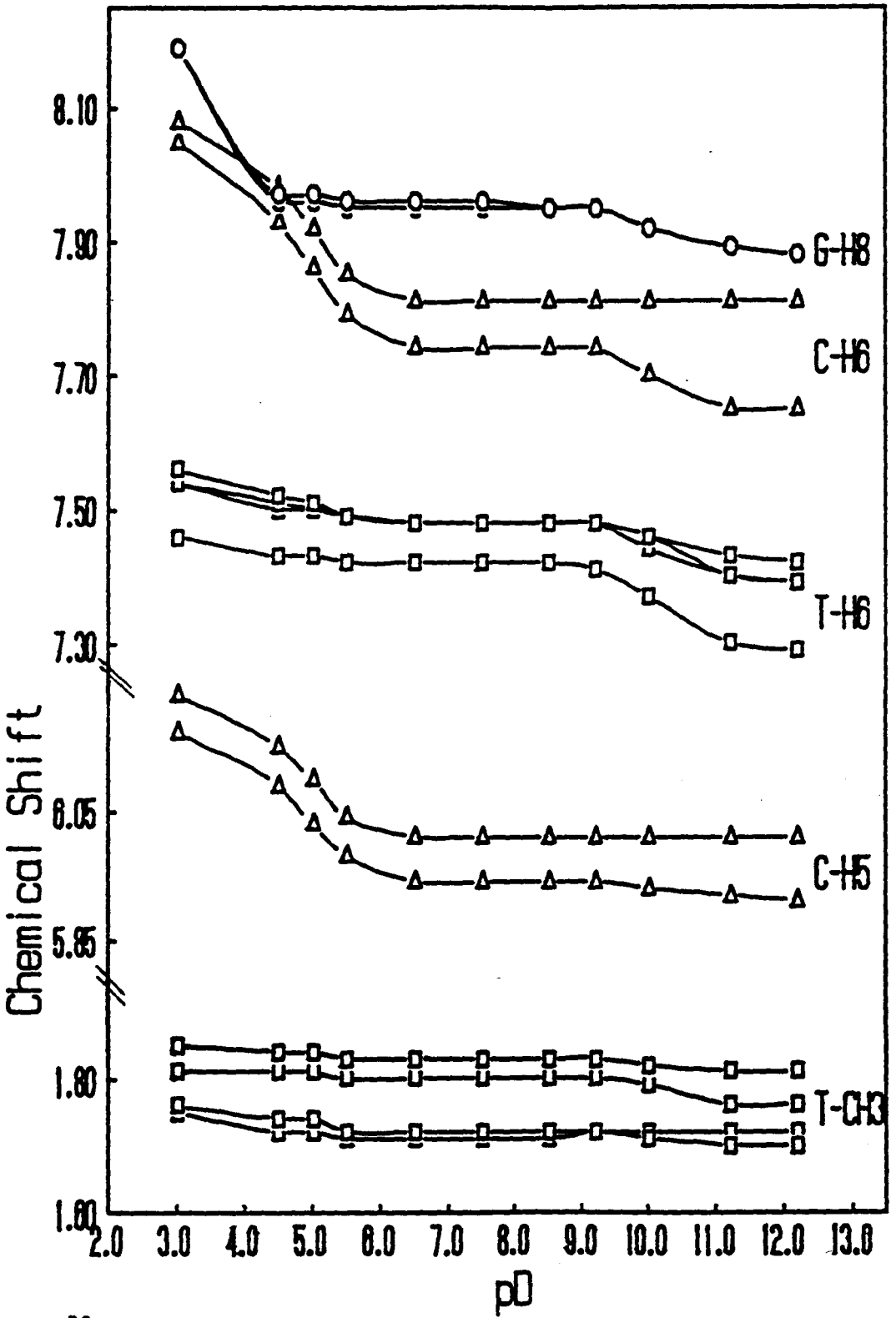


Figure 39

Chemical shift of aromatic and methyl protons as a function of pD for d(CTGTTGTC).

TABLE 20

Chemical shift as a function of pH;
d(CpTpGpTpTpGpTpC), 1.5 mM, 25°C, no salt, no buffer
1-2-3-4-5-6-7-8

Chemical Shift vs pH

Proton	pH 3.0	4.5	5.0	5.5	6.5	7.5
G(6)-H8	8.191	7.973	7.968	7.960	7.959	7.959
G(3)-H8	8.191	7.964	7.959	7.952	7.950	7.951
C(8)-H6	8.080	7.977	7.916	7.851	7.815	7.811
C(1)-H6	8.050	7.927	7.859	7.794	7.744	7.740
T(7)-H6	7.562	7.522	7.512	7.488	7.482	7.483
T(4)-H6	7.536	7.509	7.499	7.488	7.482	7.483
T(2)-H6	7.536	7.504	7.499	7.488	7.482	7.483
T(5)-H6	7.458	7.434	7.431	7.423	7.419	7.420
C(8)-H5	6.228	6.146	6.096	6.041	6.006	6.008
C(1)-H5	6.173	6.087	6.033	5.976	5.944	5.940
T(2)-CH3	1.850	1.842	1.837	1.832	1.830	1.829
T(5)-CH3	1.814	1.809	1.807	1.803	1.802	1.802
T(7)-CH3	1.761	1.742	1.737	1.722	1.718	1.718
T(4)-CH3	1.746	1.725	1.722	1.709	1.708	1.708

Proton	pH 8.5	9.2	10.0	11.2	12.2
G(6)-H8	7.955	7.954	7.925	7.889	7.883
G(3)-H8	7.949	7.954	7.925	7.889	7.883
C(8)-H6	7.812	7.813	7.810	7.812	7.815
C(1)-H6	7.737	7.731	7.697	7.654	7.646
T(7)-H6	7.481	7.485	7.459	7.426	7.416
T(4)-H6	7.481	7.485	7.459	7.397	7.388
T(2)-H6	7.481	7.485	7.438	7.397	7.388
T(5)-H6	7.419	7.415	7.368	7.300	7.287
C(8)-H5	6.008	6.009	6.005	6.009	6.011
C(1)-H5	5.942	5.938	5.930	5.920	5.919
T(2)-CH3	1.830	1.831	1.822	1.813	1.811
T(5)-CH3	1.803	1.803	1.786	1.762	1.758
T(7)-CH3	1.719	1.723	1.710	1.702	1.702
T(4)-CH3	1.710	1.723	1.723	1.722	1.724

Approximate pKa values for the various nitrogen atoms in the heterocyclic bases may be obtained from the chemical shift versus pH data for the non-platinated strands. With increasing acidity, the normal oligomers (i), (ii) and (iii), Figures 37, 38 and 39 respectively, the H-6 and H-5 signals of the terminal cytidine bases exhibit a downfield shift beginning at pH 6.5, with the inflection point of the sigmoidal curves centered about pH 5.0. This shift in the aromatic signals resulted from the protonation of N(3) of cytosine, pKa=4.4 (Martin, 1985). Inspection of the H-6 signal for the thymine bases in the different strands reveals deprotonation of the N(3)-H proton occurring at basic pH values above 9.0. The aromatic signals on the thymine bases appear somewhat insensitive to this deprotonation reaction. Of primary importance to this study is the movement of the guanine G-H8 signals to lower field upon protonation of the G-N(7) site, pKa= 2.4 (Martin,1985). This result has been observed for all of the non-platinated oligomers. The chemical shifts of the protons are influenced not only by the pH of the medium but also by the diamagnetic anisotropy of the adjacent bases. For example, in the basic pH region, the deprotonation of the guanine N(1) also occurs at pKa= 9.5. This alters the shielding effects of the base which in turn affects the chemical shifts of the protons on the neighbouring bases.

A comparison of the plots for the platinated and non-platinated species (see Figures 40, 41, and 42) reveals many similarities and several significant changes. Some of the chemical shift changes can be attributed to alterations in conformation and the effects these changes have on local environments. An example of this is seen when comparing

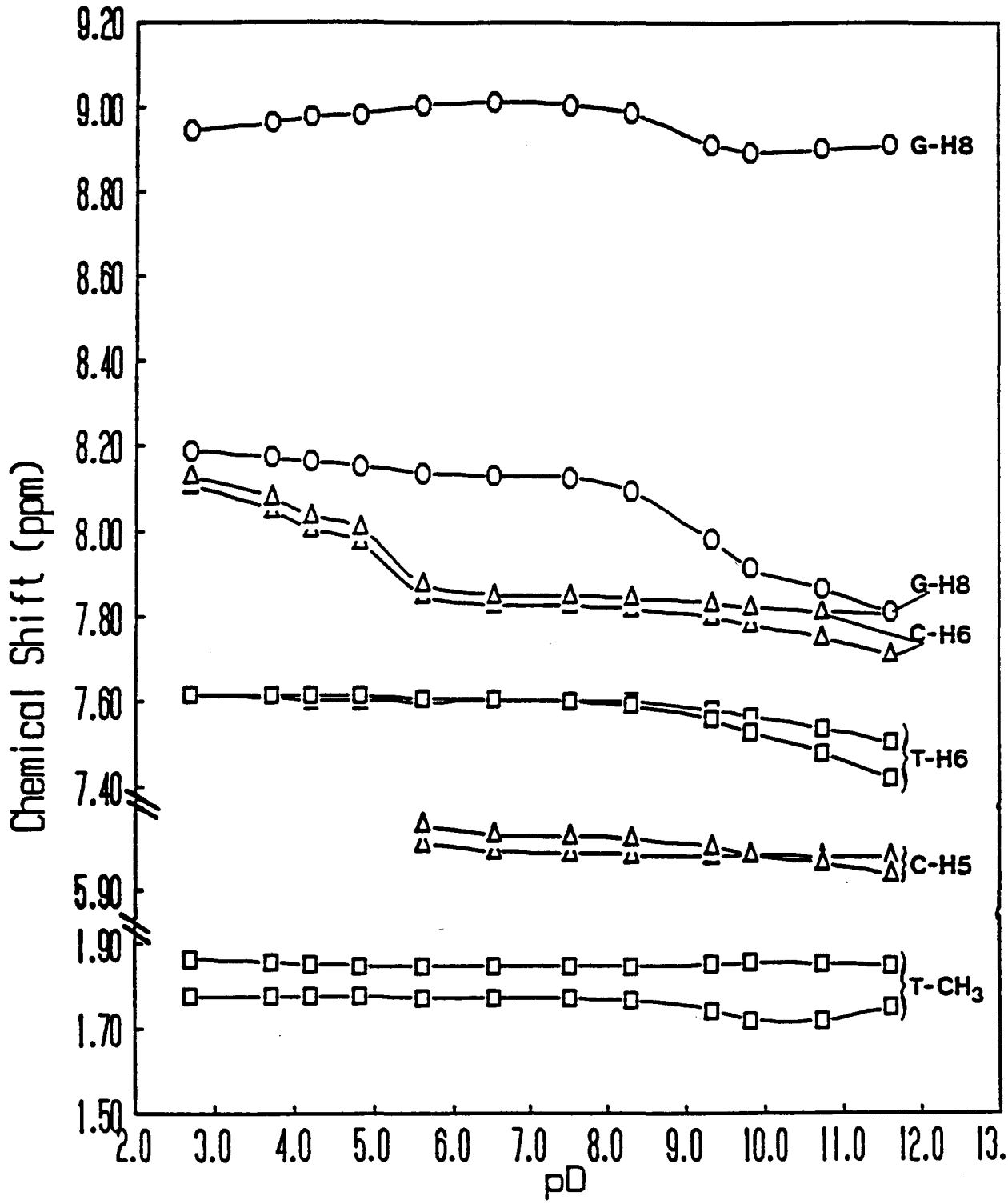


Figure 40

Chemical shift of aromatic and methyl protons as a function of pD for cis-$Pt(NH_3)_2$[d(CTGGTC)-N7(3),N7(4)].

TABLE 21

Chemical shift as a function of pH;
cis-$Pt(NH_3)_2$[d(CTGGTC)-N7(3),N7(4)], 1.7 mM,
25°C, no salt, no buffer.

Chemical Shift vs pH

Proton	pH 2.7	3.7	4.2	4.8
G-H8	8.945	8.964	8.978	8.985
G-H8	8.188	8.172	8.163	8.153
C-H6	8.129	8.080	8.038	8.010
C-H6	8.108	8.051	8.006	7.976
T-H6	7.614	7.615	7.615	7.614
T-H6	7.614	7.606	7.603	7.602
C-H5	-----	-----	-----	-----
C-H5	-----	-----	-----	-----
T-CH3	1.863	1.855	1.849	1.847
T-CH3	1.776	1.775	1.774	1.774

Proton	pH 5.6	6.5	7.5	8.3
G-H8	9.006	9.014	9.006	8.987
G-H8	8.135	8.130	8.126	8.096
C-H6	7.878	7.852	7.850	7.844
C-H6	7.852	7.829	7.828	7.820
T-H6	7.606	7.605	7.601	7.593
T-H6	7.600	7.602	7.601	7.602
C-H5	6.053	6.030	6.029	6.022
C-H5	6.010	5.992	5.989	5.985
T-CH3	1.845	1.845	1.846	1.846
T-CH3	1.769	1.768	1.768	1.764

Proton	pH 9.3	9.8	10.7	11.6
G-H8	8.914	8.895	8.903	8.912
G-H8	7.981	7.917	7.867	7.814
C-H6	7.833	7.824	7.815	7.807
C-H6	7.801	7.783	7.754	7.714
T-H6	7.561	7.531	7.485	7.426
T-H6	7.583	7.567	7.541	7.509
C-H5	6.005	5.986	5.969	5.943
C-H5	5.984	5.986	5.985	5.983
T-CH3	1.848	1.852	1.851	1.843
T-CH3	1.741	1.719	1.717	1.748

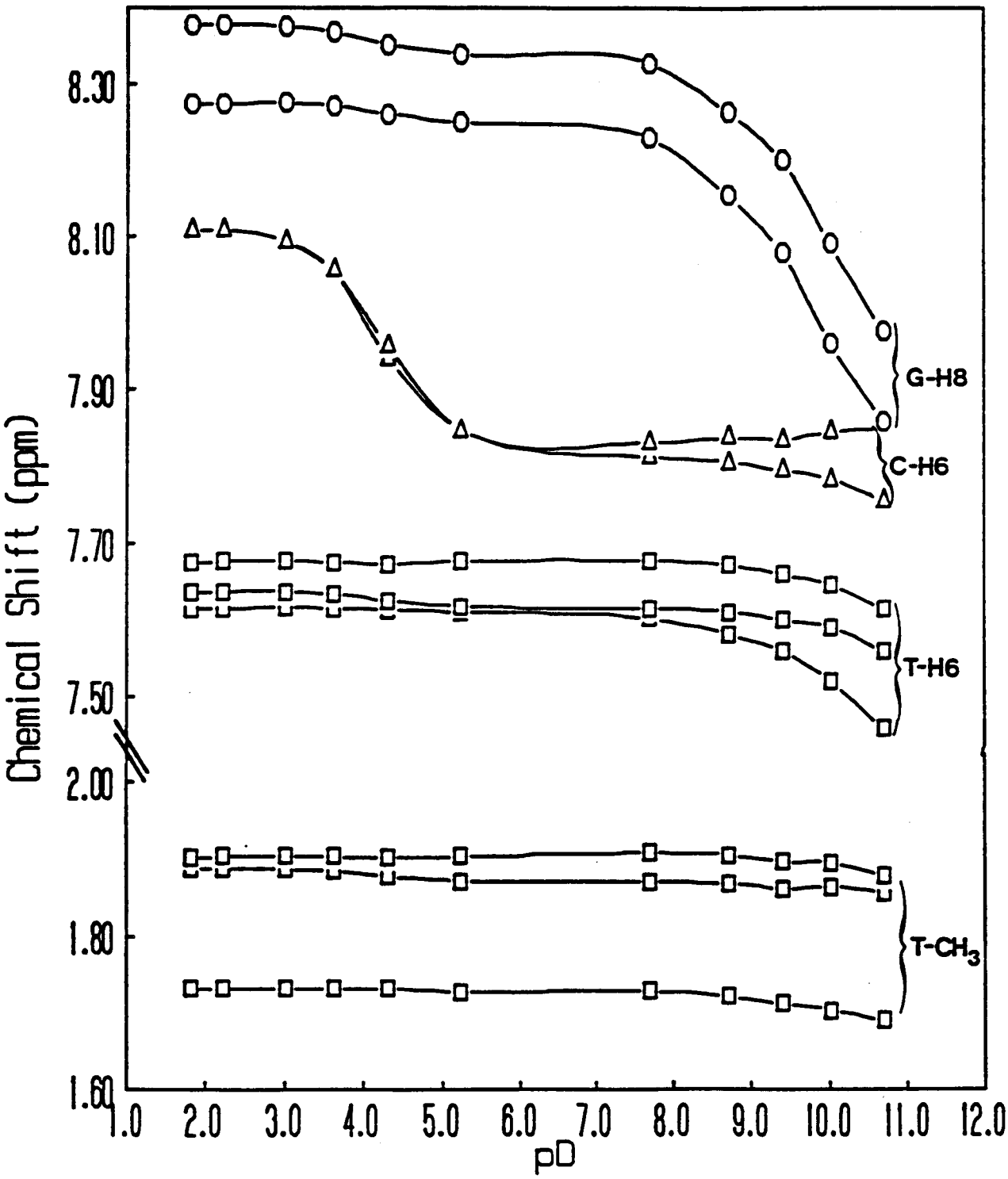


Figure 41

Chemical shift of aromatic and methyl protons as a function of pD for cis-$Pt(NH_3)_2$[d(CTGTGTC)-N7(3),N7(5)].

TABLE 22

Chemical shift as a function of pH;
cis-$Pt(NH_3)_2$[d(CTGTGTC)-N7(3),N7(5)], 1.6 mM,
25°C, no salt, no buffer.

Chemical Shift vs pH

Proton	pH 1.8	2.2	3.0	3.6	4.3	5.2
G-H8	8.378	8.378	8.376	8.369	8.351	8.339
G-H8	8.273	8.274	8.275	8.272	8.260	8.248
C-H6	8.109	8.108	8.093	8.056	7.957	7.846
C-H6	8.109	8.108	8.093	8.056	7.941	7.846
T-H6	7.675	7.676	7.677	7.675	7.673	7.677
T-H6	7.637	7.637	7.637	7.633	7.624	7.617
T-H6	7.614	7.614	7.616	7.614	7.612	7.609
C-H5	-----	-----	-----	-----	-----	-----
C-H5	-----	-----	-----	-----	-----	-----
T-CH3	1.902	1.903	1.903	1.903	1.901	1.903
T-CH3	1.887	1.887	1.886	1.884	1.877	1.871
T-CH3	1.731	1.731	1.732	1.732	1.731	1.727

Chemical Shift vs pH

Proton	pH 7.7	8.7	9.4	10.0	10.7
G-H8	8.328	8.263	8.200	8.091	7.976
G-H8	8.229	8.153	8.079	7.960	7.858
C-H6	7.830	7.837	7.834	7.844	7.850
C-H6	7.811	7.804	7.793	7.783	7.754
T-H6	7.677	7.672	7.659	7.646	7.613
T-H6	7.614	7.610	7.599	7.590	7.557
T-H6	7.601	7.580	7.557	7.520	7.458
C-H5	6.027	6.029	6.025	6.035	6.038
C-H5	5.985	5.982	5.974	5.976	5.967
T-CH3	1.908	1.905	1.896	1.895	1.878
T-CH3	1.870	1.867	1.861	1.863	1.855
T-CH3	1.728	1.721	1.711	1.703	1.690

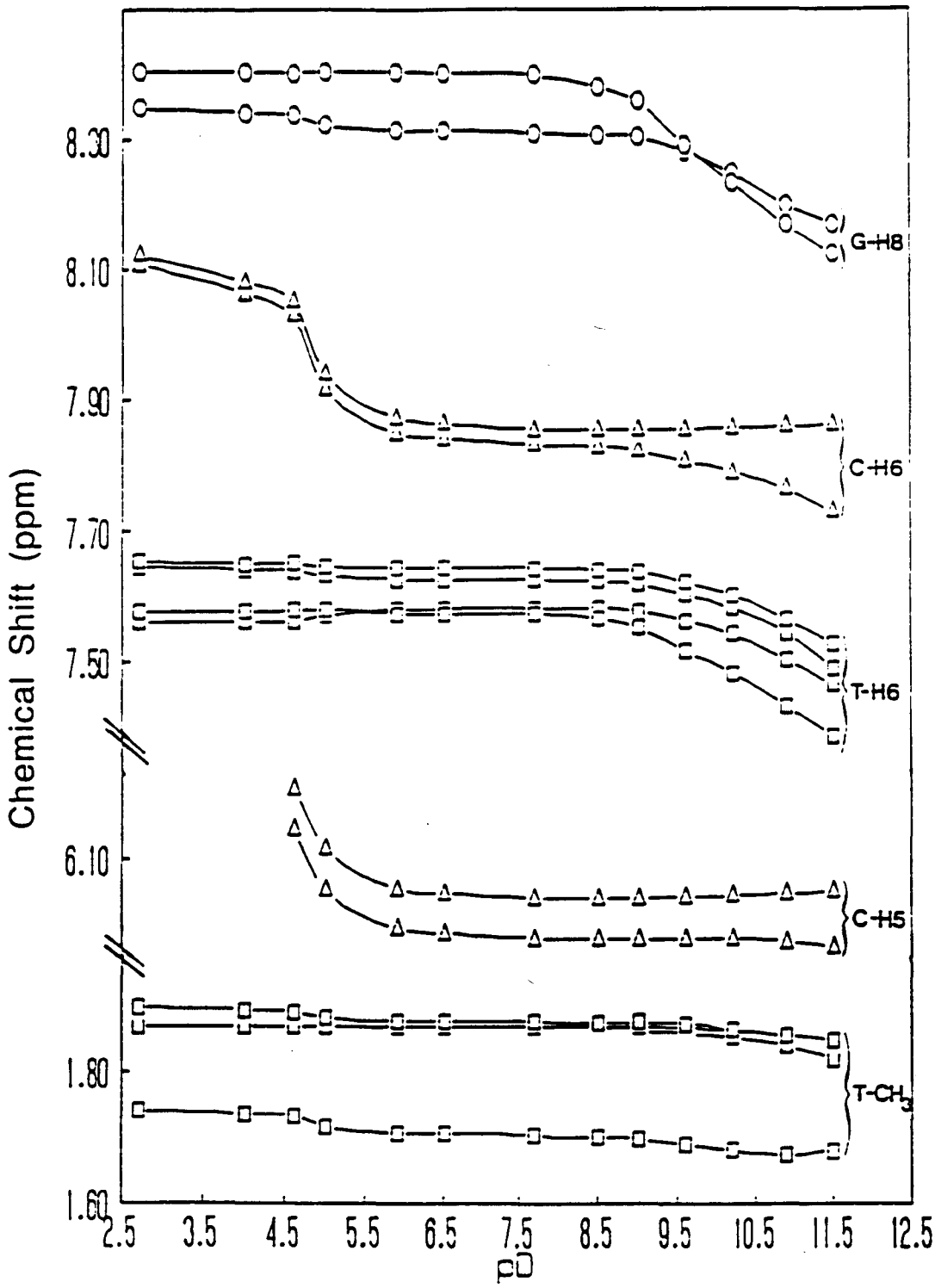


Figure 42

Chemical shift of aromatic and methyl protons as a function of pD for cis-$Pt(NH_3)_2$[d(CTGTTGTC)-N7(3),N7(6)].

TABLE 23

Chemical shift as a function of pH;
cis-$Pt(NH_3)_2$[d(CTGTTGTC)-N7(3),N7(6)], 1.3 mM, 25°C.

Chemical Shift vs pH

Proton	pH 2.7	4.0	4.6	5.0	5.9	6.5	7.7
G-H8	8.405	8.404	8.403	8.404	8.404	8.402	8.398
G-H8	8.348	8.341	8.337	8.322	8.313	8.314	8.309
C-H6	8.123	8.080	8.052	7.941	7.870	7.863	7.853
C-H6	8.108	8.063	8.029	7.917	7.847	7.840	7.830
T-H6	7.653	7.649	7.650	7.645	7.643	7.642	7.641
T-H6	7.645	7.640	7.638	7.631	7.625	7.625	7.623
T-H6	7.577	7.576	7.578	7.580	7.572	7.571	7.571
T-H6	7.559	7.560	7.561	7.570	7.579	7.580	7.580
C-H5	-----	-----	6.208	6.116	7.054	6.048	6.040
C-H5	-----	-----	6.146	6.052	5.994	5.987	5.980
T-CH3	1.897	1.893	1.890	1.881	1.876	1.875	1.875
T-CH3	1.867	1.867	1.867	1.867	1.867	1.869	1.868
T-CH3	1.867	1.867	1.867	1.867	1.867	1.869	1.868
T-CH3	1.741	1.736	1.734	1.717	1.706	1.707	1.703

Chemical Shift vs pH

Proton	pH 8.5	9.0	9.6	10.2	10.9	11.5
G-H8	8.380	8.358	8.290	8.232	8.167	8.124
G-H8	8.305	8.303	8.280	8.247	8.197	8.168
C-H6	7.853	7.853	7.853	7.855	7.857	7.860
C-H6	7.826	7.820	7.804	7.787	7.761	7.727
T-H6	7.638	7.635	7.618	7.598	7.561	7.524
T-H6	7.620	7.616	7.599	7.578	7.540	7.487
T-H6	7.562	7.549	7.513	7.479	7.428	7.383
T-H6	7.579	7.574	7.558	7.538	7.499	7.461
C-H5	6.040	6.041	6.042	6.044	6.047	6.051
C-H5	5.980	5.980	5.979	5.979	5.974	5.966
T-CH3	1.873	1.875	1.872	1.862	1.855	1.848
T-CH3	1.868	1.868	1.865	1.862	1.855	1.848
T-CH3	1.868	1.862	1.857	1.851	1.839	1.819
T-CH3	1.670	1.699	1.689	1.681	1.675	1.680

the curves for the T-H6 signals in the normal and the platinated material, i.e. Figures 37 and 40 respectively. The 0.2 ppm downfield shift of the whole T-H6 curve in the platinated species can be attributed to a conformational change in the oligomer resulting from platination of the guanine bases. Interestingly, all of the thymine bases, both internal to, as well as, 3′ and 5′ to the platinum chelate, exhibit deshielding when compared with their normal parent oligomers. The deshielding of the thymine bases which flank the platinated region as well as those located internal to the platinum chelate indicate that the intrastrand base stacking has been disrupted. Another chemical shift change which is important to the pH titration is the upfield shift in the basic pH region of the thymidine H-6 and, to a lesser extent, the 5-methyl group shift, indicating deprotonation of the N(3) position on the thymine base. This result confirms that the thymine N(3)-H position is intact after the reaction of cis-$Pt(NH_3)_2Cl_2$ with the oligomer. The observation was consistent over the three different 2′-deoxyoligomers studied.

The effects of guanine platination on the 3′ and 5′ terminal cytosine bases was minimal. The change in chemical shift (<0.1 ppm) of the C-H5 and C-H6 protons between the normal and platinated strands indicated little change in shielding from the adjacent thymidine residues; a result anticipated because of the poor shielding nature of the thymine base. Effects from the platinated region of the strand appear to fall off rapidly with distance as there is no apparent affect on the terminal cytosines, which are next nearest neighbours to the chelate. Protonation of the N(1) position on the cytosine bases causes

the downfield shift of both the H5 and H6 protons at pH values less than five. This result, observable for all strands studied, indicates that no reaction of cis-$Pt(NH_3)_2Cl_2$ has occurred on the cytosine base.

The signals assigned to the guanine H8 protons exhibit the most radical changes in chemical shift upon reaction of the oligomers with cis-$Pt(NH_3)_2Cl_2$. In the cis-$Pt(NH_3)_2$[d(CTGGTC)-N7(3),N7(4)] complex, Figure 40, the two H-8 signals show a large difference in chemical shift, 9.00 and 8.13 ppm at pH 7. This is an overall downfield shift upon platination when compared to the normal parent oligomer at a similar pH, which has shifts of 7.92 and 7.81 ppm. The platinum complexes of d(CTGTGTC) and d(CTGTTGTC) show similar results but the changes in G-H8 chemical shift are not as extreme. For cis-$Pt(NH_3)_2$[d(CTGTGTC)-N7(3),N7(5)], the G-H8 signals have shifts of 8.33 and 8.23 ppm, approximately 0.4 ppm downfield of the unplatinated material. With cis-$Pt(NH_3)_2$[d(CTGTTGTC)-N7(3),N7(6)], the G-H8 signals were again deshielded with chemical shifts of 8.40 and 8.31 ppm, approximately 0.4 ppm downfield when compared with the parent strand. This pattern in G-H8 chemical shift changes, resulting from the platination of the guanine N(7) site, is similar to that observed in the shorter strands discussed in Chapter 3. The G-H8 chemical shifts for the platinated hexamer, which contains a chelate of the form cis-$Pt(NH_3)_2$[d(GpG)], are similar to those observed for the dimer-platinum complex alone (den Hartog et al. 1982). In the cis-$Pt(NH_3)_2$[d(GpG)] dimer, both of the guanosine residues have been shown to maintain the normal anti base conformation. A similar conformation is suggested here for the platinated region of the hexamer.

The platinum-heptamer and platinum-octamer complexes have G-H8 chemical shifts which more closely resemble those observed in the shorter trimer and tetramer oligomers (Chapter 3). For cis-$Pt(NH_3)_2$[d(GCG)-N7(1),N7(3)], the conformation of the two guanine bases has been shown to be different (den Hartog et al. 1983). NOE results have shown that one guanine adopts the normal anti conformer while the second guanine exists in a syn-anti equilibrium. The chemical shifts for the G-H8 protons in the platinated oligomers d(CTGTGTC) and d(CTGTTGTC) are similar to those observed for the cis-$Pt(NH_3)_2$[d(GCG)] compound suggesting that the guanines adopt a comparable conformation with respect to their furanose moieties.

The shape of the pH titration curves, for all G-H8 protons in the three strands examined, was distinct and characteristic of platinum binding at guanine-N(7). Over the entire acid region of the curves, there was no significant change in chemical shift. The absence of a downfield shift for pH values less than four, indicative of N(7) protonation, reveals that the site is already occupied and no longer open to protonation. Deprotonation of the guanine N(1) site is indicated by the upfield movement of the G-H8 signal in the basic pH range. As observed with the shorter oligomers in Chapter 3, deprotonation appears to occur under less basic conditions when compared with the non-platinated parent molecules.

4.2.3 Conclusions to pH Titration Studies of Long Oligomers

The results of the pH titration studies of the deoxy oligomers d(CTGGTC), d(CTGTGTC), and d(CTGTTGTC), both before and after reaction

with cis-$Pt(NH_3)_2(Cl)_2$, are consistent with a reaction product which contains an intrastrand crosslink between the platinum atom and the N(7) positions of the guanine bases (Girault et al. 1984b; Caradonna et al. 1982). No reaction with cisplatin was observed at any other sites, on either the guanines, cytosines or thymines. A comparison of the G-H8 chemical shift data reported here and in the literature (Neumann et al. 1984; den Hartog et al. 1982), suggests that cis-$Pt(NH_3)_2$[d(CTGGTC)-N7(3),N7(4)], (GH-8 at 9.006 and 8.126 ppm) adopts an anti-anti type configuration about the glycosidic bonds of the chelated guanines. The cis-$Pt(NH_3)_2$[d(CTGTGTC)-N7(3),N7(5)], (GH-8 at 8.328 and 8.229 ppm) and cis-$Pt(NH_3)_2$[d(CTGTTGTC)-N7(3),N7(6)], (GH-8 at 8.402 and 8.314 ppm) complexes exhibit G-H8 chemical shifts ascribed to one guanine in the anti conformer while the second complexed guanine prefers a syn-anti equilibrium (den Hartog et al. 1983).

At neutral pH, the thymine bases in the platinated species are deshielded compared to the parent molecules, suggesting that the intrastrand stacking arrangement for these bases, both internal and external to the chelated region, has been disrupted. The terminal cytosine bases on all the platinum complexes show little change as a result of chelate formation. The structural effects of platination appear localized and decrease rapidly with distance from the reaction site.

4.3.1 Variable Temperature Studies of Single Stranded, Normal and Platinated Deoxy Oligomers.

Variable temperature studies on nucleic acids are useful in that they provide information pertaining to inter and intra molecular interactions over the temperatures examined. At high temperature, i.e. 70°C, most short oligomers maintain a single stranded, random orientation. As the temperature is lowered and molecular motion decreases, interaction within and between molecules increases. This interaction may take the form of intrastrand base stacking or, when duplexing occurs, hydrogen bonding upon base pair formation as well as interstrand base stacking. In this study, the oligomers examined were non-complementary and therefore no duplexing was expected. In the variable temperature study completed on the short cisplatin-oligomer complexes (Chapter 3), the effects of platination on the conformation of the bases in the internal loop were examined. With the sequences used in this work, not only can this effect be examined, but also, the effect on bases terminal to the platinated region of the molecule. It has been previously reported for cis-$Pt(NH_3)_2$[d(CGG)-N7(2),N7(3)], that with a decrease in the temperature, the 5'-terminal cytosine base stacked well on the adjacent guanine which was involved in the platinum chelate (den Hartog et al. 1985b). This section examines the results for the stacking abilty of thymine, both 3' and 5' to the platinated region. In addition the effect of platination on the conformational orientation about the guanosine glycosyl bond on intrastrand strand stacking in the heptamer and the octamer is discussed.

The deoxy oligomers d(CTGGTC), d(CTGTGTC), and d(CTGTTGTC), as well as their cis-$Pt(NH_3)_2(Cl)_2$ adducts, have been examined by proton NMR over the temperature range 10-70°C. All experiments have been carried out in 0.1 M NaCl, 0.01 M sodium phosphate buffer pH 7.0, and 0.5 mM EDTA. The data for the variable temperature experiments are given in Tables 24-29, and the results plotted on Figures 43-48.

4.3.2 Variable Temperature Results for Parent Oligomers

Examination of the diagrams illustrating the effects of temperature change on proton chemical shift for the oligomers d(CTGGTC), d(CTGTGTC), and d(CTGTTGTC), Figures 43, 44, and 45 respectively, reveals little change in chemical shift over the entire temperature range for any of the protons monitored. As previously mentioned, this result would be expected as the sequences are all non-complementary, as well as containing a high percentage of the poor stacking, thymine base. There appears to be some movement occurring below 10°C - possibly the beginnings of some intrastrand stacking, but this is minimal.

4.3.3 Variable Temperature Results for Platinated Oligomers

The variable temperature experiments, involving the sequences cis-$Pt(NH_3)_2$[d(CTGGTC)-N7(3),N7(4)], cis-$Pt(NH_3)_2$[d(CTGTGTC)-N7(3),N7(5)], and cis-$Pt(NH_3)_2$[d(CTGTTGTC)-N7(3),N7(6)], were carried out over the temperature range 70°C-0°C. Several notable changes in the results were observed for the non-platinated species when compared to the parent oligomers.

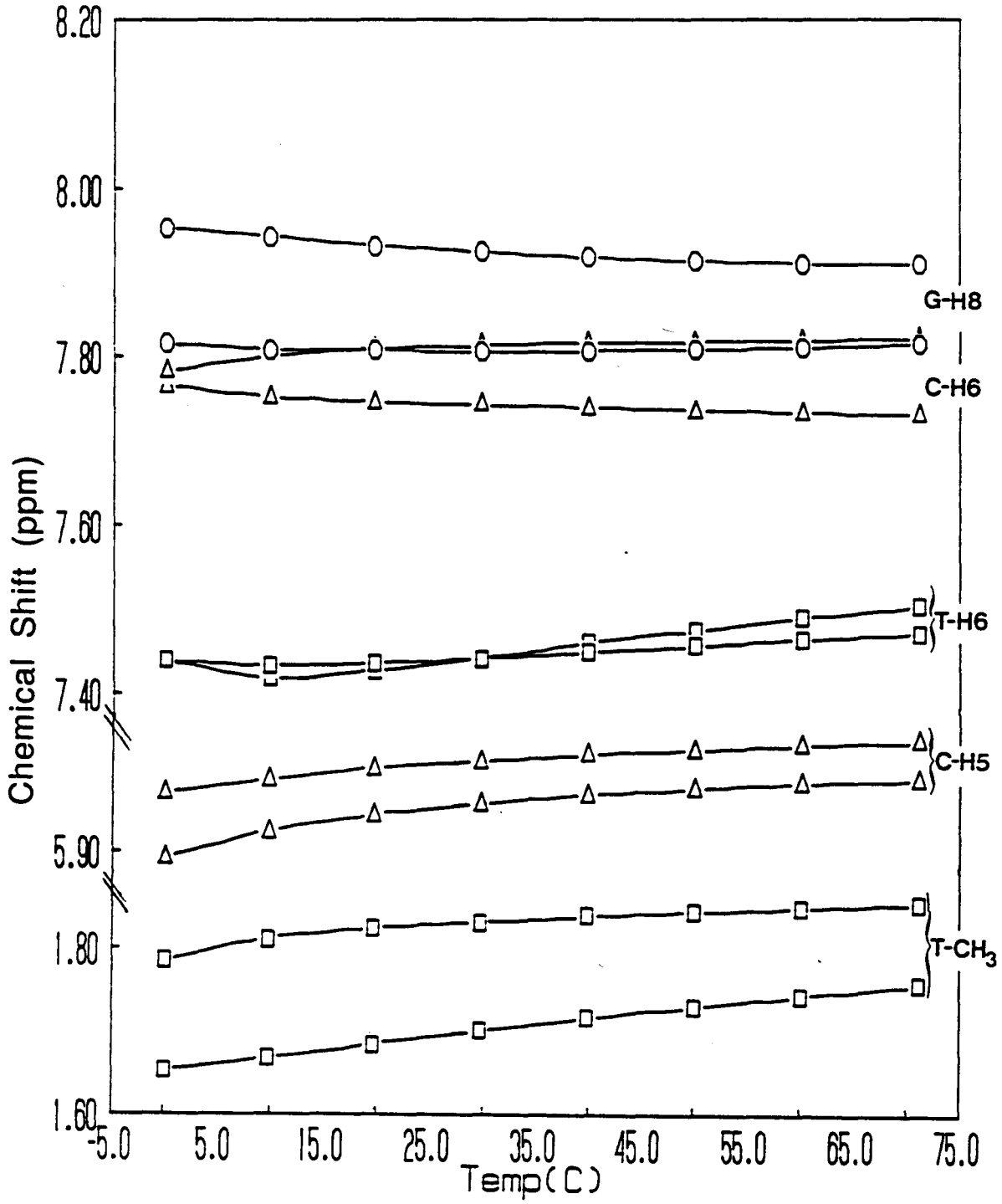


Figure 43

Chemical shift of aromatic and methyl protons as a function of temperature for d(CTGGTC).

TABLE 24

Chemical shift as a function of temperature (C);
d(CpTpGpGpTpC),
1-2-3-4-5-6
1.5 mM, 0.1 M NaCl, 0.01 M phosphate, pH 7.

Chemical Shift vs Temperature (C)

Proton	T(C) 0.0	9.6	19.6	29.5
G(4)-H8	7.953	7.942	7.931	7.923
G(3)-H8	7.814	7.807	7.806	7.804
C(6)-H6	7.782	7.800	7.808	7.812
C(1)-H6	7.765	7.751	7.745	7.741
T(2)-H6	7.438	7.432	7.435	7.440
T(5)-H6	7.438	7.417	7.426	7.440
C(6)-H5	5.969	5.985	5.999	6.008
C(1)-H5	5.892	5.924	5.944	5.957
T(2)-CH3	1.785	1.811	1.823	1.831
T(5)-CH3	1.653	1.668	1.685	1.701

Chemical Shift vs Temperature (C)

Proton	T(C) 39.8	50.0	60.0	71.0
G(4)-H8	7.917	7.912	7.909	7.907
G(3)-H8	7.805	7.806	7.809	7.813
C(6)-H6	7.815	7.816	7.818	7.819
C(1)-H6	7.738	7.734	7.731	7.728
T(2)-H6	7.447	7.454	7.461	7.468
T(5)-H6	7.458	7.473	7.487	7.500
C(6)-H5	6.016	6.021	6.027	6.031
C(1)-H5	5.968	5.975	5.981	5.985
T(2)-CH3	1.838	1.843	1.848	1.853
T(5)-CH3	1.717	1.731	1.744	1.757

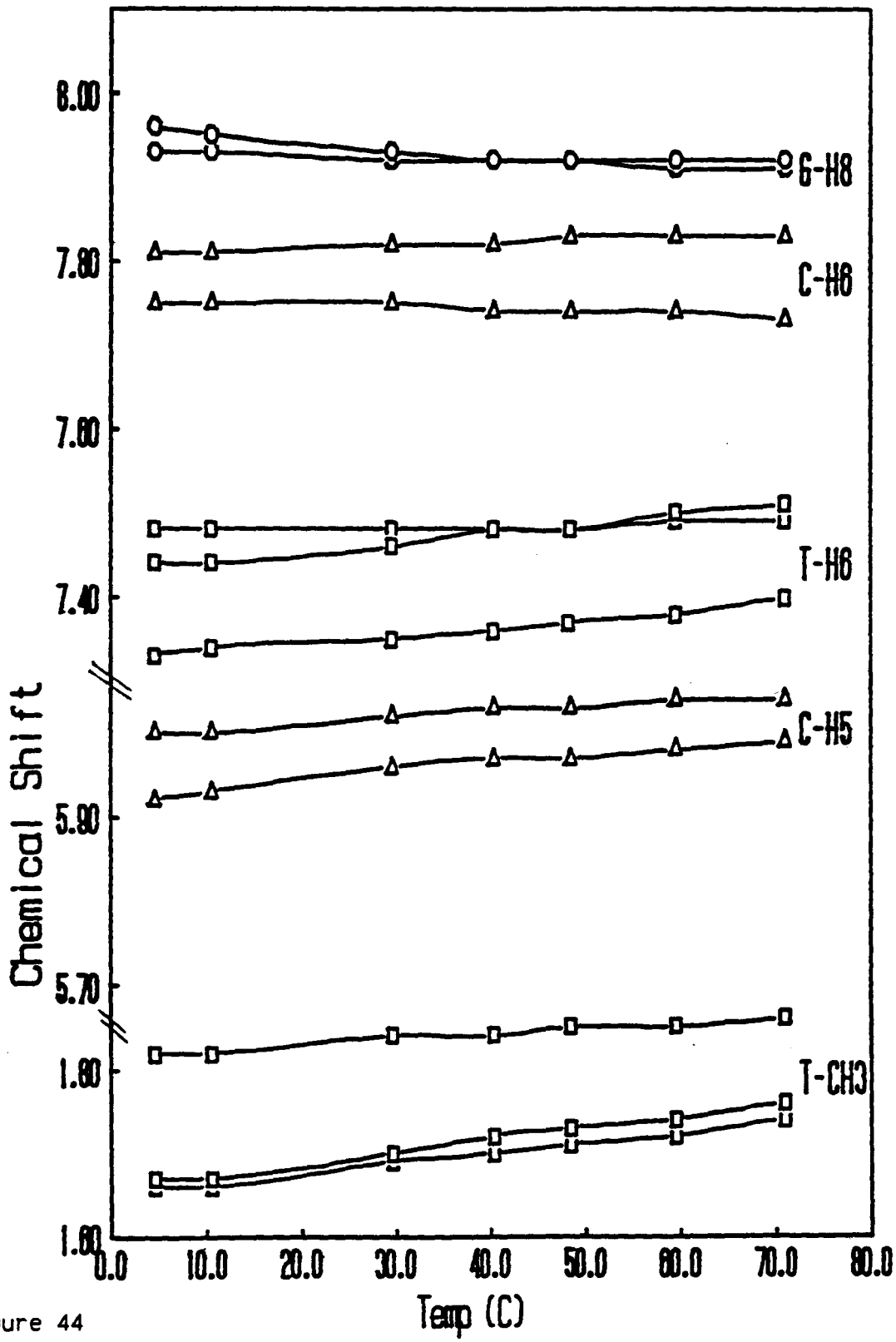


Figure 44

Chemical shift of aromatic and methyl protons as a function of temperature for d(CTGTGTC).

TABLE 25
Chemical shift as a function of temperature (C)
d(CpTpGpTpGpTpC),
1-2-3-4-5-6-7
1.5 mM, 0.1 M NaCl, 0.01 M phosphate, pH 7.

Chemical Shift vs Temperature (C)

Proton	T(C)	70.9	59.4	48.3	40.3
G(5)-H8		7.919	7.922	7.925	7.928
G(3)-H8		7.913	7.914	7.916	7.916
C(7)-H6		7.828	7.827	7.826	7.825
C(1)-H6		7.733	7.736	7.740	7.742
T(6)-H6		7.512	7.500	7.485	7.479
T(4)-H6		7.490	7.487	7.485	7.479
T(2)-H6		7.398	7.384	7.372	7.363
C(7)-H5		6.040	6.036	6.032	6.028
C(1)-H5		5.987	5.981	5.975	5.968
T(2)-CH3		1.856	1.851	1.847	1.843
T(6)-CH3		1.759	1.744	1.731	1.719
T(4)-CH3		1.736	1.723	1.711	1.701

Chemical Shift vs Temperature (C)

Proton	T(C)	29.5	10.3	4.5
G(5)-H8		7.934	7.954	7.964
G(3)-H8		7.919	7.927	7.930
C(7)-H6		7.822	7.815	7.813
C(1)-H6		7.746	7.752	7.755
T(6)-H6		7.463	7.444	7.444
T(4)-H6		7.479	7.481	7.484
T(2)-H6		7.352	7.337	7.333
C(7)-H5		6.022	6.004	5.998
C(1)-H5		5.957	5.930	5.920
T(2)-CH3		1.836	1.822	1.816
T(6)-CH3		1.702	1.673	1.666
T(4)-CH3		1.687	1.665	1.659

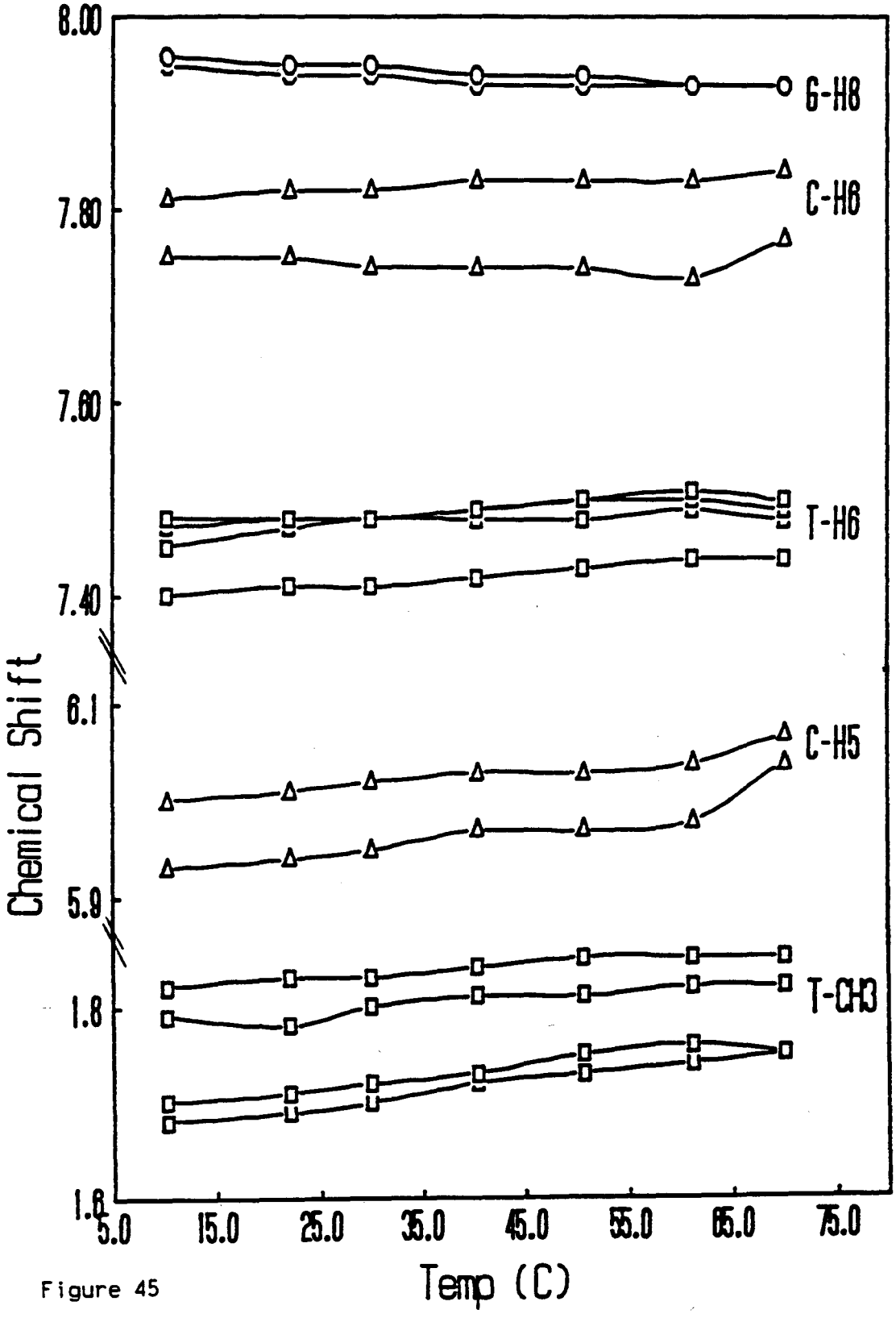


Figure 45

Chemical shift of aromatic and methyl protons as a function of temperature for d(CTGTTGTC).

TABLE 26

Chemical shift as a function of temperature (C);
d(CpTpGpTpTpGpTpC),
1-2-3-4-5-6-7-8
1.5 mM, 0.1 M NaCl, 0.01 M phosphate, pH 7.

Chemical Shift vs Temperature (C)

Proton	T(C)	70.0	61.0	50.5	40.2
G(6)-H8		7.932	7.933	7.938	7.941
G(3)-H8		7.927	7.926	7.929	7.933
C(8)-H6		7.837	7.828	7.827	7.825
C(1)-H6		7.767	7.734	7.737	7.740
T(7)-H6		7.502	7.509	7.497	7.487
T(4)-H6		7.494	7.503	7.497	7.487
T(2)-H6		7.479	7.487	7.483	7.480
T(5)-H6		7.437	7.441	7.432	7.423
C(8)-H5		6.069	6.038	6.033	6.028
C(1)-H5		6.037	5.982	5.975	5.966
T(2)-CH3		1.847	1.851	1.846	1.840
T(5)-CH3		1.817	1.819	1.814	1.807
T(7)-CH3		1.754	1.757	1.746	1.733
T(4)-CH3		1.747	1.743	1.731	1.719

Chemical Shift vs Temperature (C)

Proton	T(C)	29.8	22.0	10.2
G(6)-H8		7.948	7.952	7.963
G(3)-H8		7.939	7.943	7.952
C(8)-H6		7.822	7.819	7.813
C(1)-H6		7.744	7.746	7.752
T(7)-H6		7.477	7.477	7.479
T(4)-H6		7.477	7.477	7.474
T(2)-H6		7.477	7.466	7.456
T(5)-H6		7.414	7.410	7.405
C(8)-H5		6.020	6.013	6.001
C(1)-H5		5.954	5.944	5.927
T(2)-CH3		1.833	1.828	1.816
T(5)-CH3		1.800	1.797	1.790
T(7)-CH3		1.720	1.714	1.704
T(4)-CH3		1.705	1.695	1.677

4.3.4 cis-$Pt(NH_3)_2$[d(CTGGTC)-N7(3),N7(4)]

The platinated hexamer exhibited extensive line broadening below 30°C with no recovery in line shape by 0°C. This suggests the possibilities of either, aggregation of the sample at low temperatures or, a slow (with respect to the NMR time scale) unimolecular or bimolecular equilibrium. The only signals which did show any significant chemical shift change as a function of temperature were the G-H8 protons, which are directly involved in the platinum chelate. In particular, the lowfield G-H8 signal exhibited a steady downfield movement before becoming indiscernible below 30°C. Movement of proton resonances in a downfield direction with decrease in temperature is opposite to what would be expected for an increased intrastrand stacking interaction. This result may be attributed to a conformational change to relieve structural strain within the chelated region of the molecule - one guanine base rotating away from the second guanine despite motion restrictions enforced by the platinum atom.

The proton resonances expected to exhibit some temperature effect as a result of interaction with the cis-$Pt(NH_3)_2$[-d(GpG)-] region were the thymine H-6 and 5-CH_3 protons. However, there was no change in chemical shift over the entire temperature range examined, Figure 46. When these results are compared to the parent hexamer, the T-H6 signals are shifted downfield by 0.15 ppm. Intrastand base stacking by the 3' and 5' nearest neighbours to the central platinum chelate does not occur. If oligomer platination had any effect on intrastrand base stacking interactions it appears to have been disruptive. This result is contrary to the findings of den Hartog et al. (1985b) for the sequence

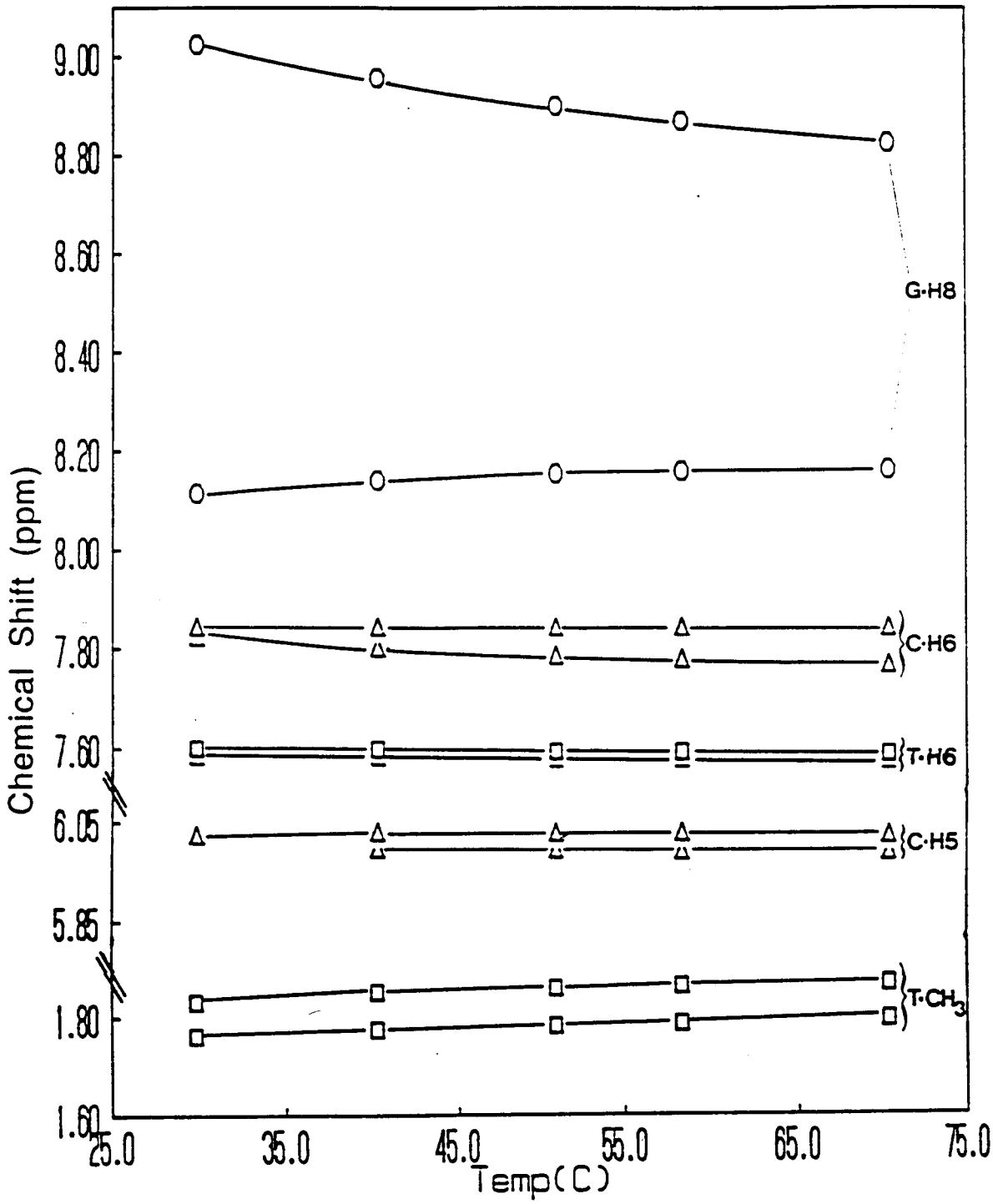


Figure 46

Chemical shift of aromatic and methyl protons as a function of temperature for cis-$Pt(NH_3)_2$[d(CTGGTC)-N7(3),N7(4)].

TABLE 27

Chemical shift as a function of temperature (C);
cis-$Pt(NH_3)_2$[d(CTGGTC)-N7(3),N7(4)],
1.7 mM, 0.1 M NaCl, 0.01 M phosphate, pH 7.

Chemical Shift vs Temperature (C)

Proton	T(C)	70.3	58.2	50.8	40.2
G-H8		8.825	8.869	8.900	8.957
G-H8		8.163	8.161	8.156	8.142
C-H6		7.839	7.841	7.841	7.841
C-H6		7.765	7.775	7.783	7.800
T-H6		7.592	7.594	7.597	7.598
T-H6		7.576	7.579	7.582	7.584
C-H5		6.043	6.040	6.037	6.032
C-H5		6.007	6.005	6.002	6.001
T-CH3		1.863	1.859	1.856	1.847
T-CH3		1.792	1.785	1.782	1.773

Chemical Shift vs Temperature (C)

Proton	T(C)	29.8	
G-H8		9.025	
G-H8		8.115	
C-H6		7.839	Lines broaden
C-H6		7.821	out below 30°C.
T-H6		7.598	
T-H6		7.584	
C-H5		6.023	
C-H5		-----	
T-CH3		1.830	
T-CH3		1.761	

cis-$Pt(NH_3)_2$[d(CGG)-N7(2),N7(3)]. In their work, after platination of the trimer, the 5'-terminal cytosine base was shown to have an increased stacking interaction with its 3'-neighbouring guanine.

It is unclear why the resonance signals broadened so drastically below 30°C. If the strand was going through a slow exchange between two different conformations, the resonance lines would sharpen once the low temperature conformation had been achieved. However, this was not observed, suggesting an aggregation of the sample in solution.

4.3.5 cis-$Pt(NH_3)_2$[d(CTGTGTC)-N7(3),N7(5)]

The results obtained for the sequence cis-$Pt(NH_3)_2$[d(CTGTGTC)] were in many ways similar to those observed for the platinated hexamer. Again, extensive line broadening was seen below 30°C the result of sample aggregation. Unlike the results for the hexamer complex, the G-H8 signals exhibit an insignificant change in chemical shift as a function of temperature, Figure 47. This suggests that once one guanine base has rotated to a more syn-like structure, as described by den Hartog et al. (1983) for cis-$Pt(NH_3)_2$[dGCG], the conformational strain on the system, resulting from platination, has been decreased to the extent that further conformational transitions are unnecessary. Alternatively, the structure of the platinated region of the molecule may be so rigid that, a change in temperature has no effect on molecular structure.

A comparison of these results with those obtained for the platinated hexamer with the trimer, cis-$Pt(NH_3)_2$[d(GTG)] (Chapter 3), allows the tentative assignment of the different T-H6 signals. The two higher field T-H6 signals have been assigned to the thymine bases which are 3' and 5'

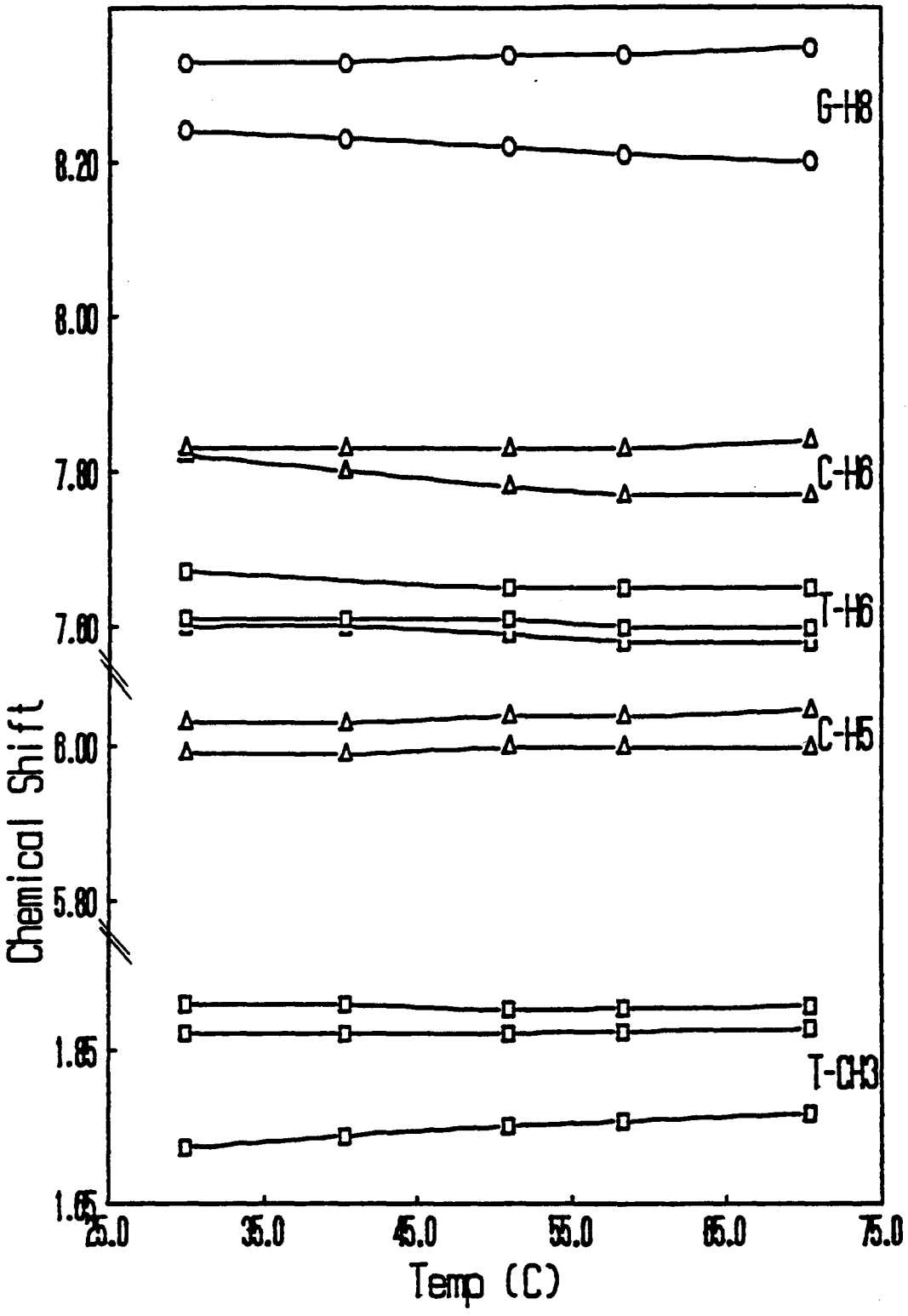


Figure 47

Chemical shift of aromatic and methyl protons as a function of temperature for cis-$Pt(NH_3)_2$[d(CTGTGTC)-N7(3),N7(5)].

TABLE 28

Chemical shift as a function of temperature (C);
cis-$Pt(NH_3)_2$[d(CTGTGTC)-N7(3),N7(5)],
1.6 mM, 0.1 M NaCl, 0.01 M phosphate, pH 7.

Chemical Shift vs Temperature (C)

Proton	T(C)	70.3	58.2	50.8	40.2
G-H8		8.348	8.344	8.341	8.335
G-H8		8.202	8.213	8.222	8.231
C-H6		7.835	7.834	7.831	7.832
C-H6		7.768	7.777	7.784	7.798
T-H6		7.646	7.649	7.650	-----
T-H6		7.604	7.604	7.606	7.608
T-H6		7.582	7.585	7.590	7.596
C-H5		6.047	6.040	6.037	6.032
C-H5		6.003	6.000	5.997	5.991
T-CH3		1.906	1.904	1.902	1.908
T-CH3		1.877	1.874	1.872	1.871
T-CH3		1.768	1.758	1.752	1.739

Chemical Shift vs Temperature (C)

Proton	T(C)	29.8	
G-H8		8.328	
G-H8		8.240	
C-H6		7.830	
C-H6		7.816	
T-H6		7.669	
T-H6		7.612	
T-H6		7.602	Lines broaden
C-H5		6.026	out below 30°C.
C-H5		5.987	
T-CH3		1.909	
T-CH3		1.871	
T-CH3		1.723	

to the platinated region, while the lowest field T-H6 signal has been assigned to the central thymine which has been looped out of the strand as a result of platination. The two higher field T-H6 signals show a similar chemical shift to those in the platinated hexamer as well as exhibiting a similar temperature effect. Again, these results indicate that the bases adjacent to the platinated region are less shielded than their non-platinated counterparts and demonstrate little stacking ability. The central thymine, which has been "bulged out" as a result of platination, exhibits insignificant change in chemical shift as a function of temperature. Conformationally, this base is no longer accessible to any intrastrand stacking and would be expected to have the most deshielded base protons of the three different thymines.

4.3.6 cis-$Pt(NH_3)_2$[d(CTGTTGTC)-N7(3),N7(6)]

The platinated octamer, cis-$Pt(NH_3)_2$[d(CTGTTGTC)], was the only platinum-oligomer adduct which did not exhibit total line broadening as a function of temperature in its proton NMR. This molecule was examined over the temperature range 70°C-10°C, with the G-H8 signals exhibiting the highest degree of line broadening at the lower temperatures. The chemical shift vs temperature plot, Figure 48, indicates some movement within the molecule as a function of temperature, but little base stacking is evident. The G-H8 signals display a similar pattern to those observed for the platinated hexamer (Section 4.3.4) as a result of temperature change, although the change in chemical shift is not as large. One of the G-H8 signals moves downfield by 0.1 ppm while the second resonance signal moves upfield by approximately the same amount.

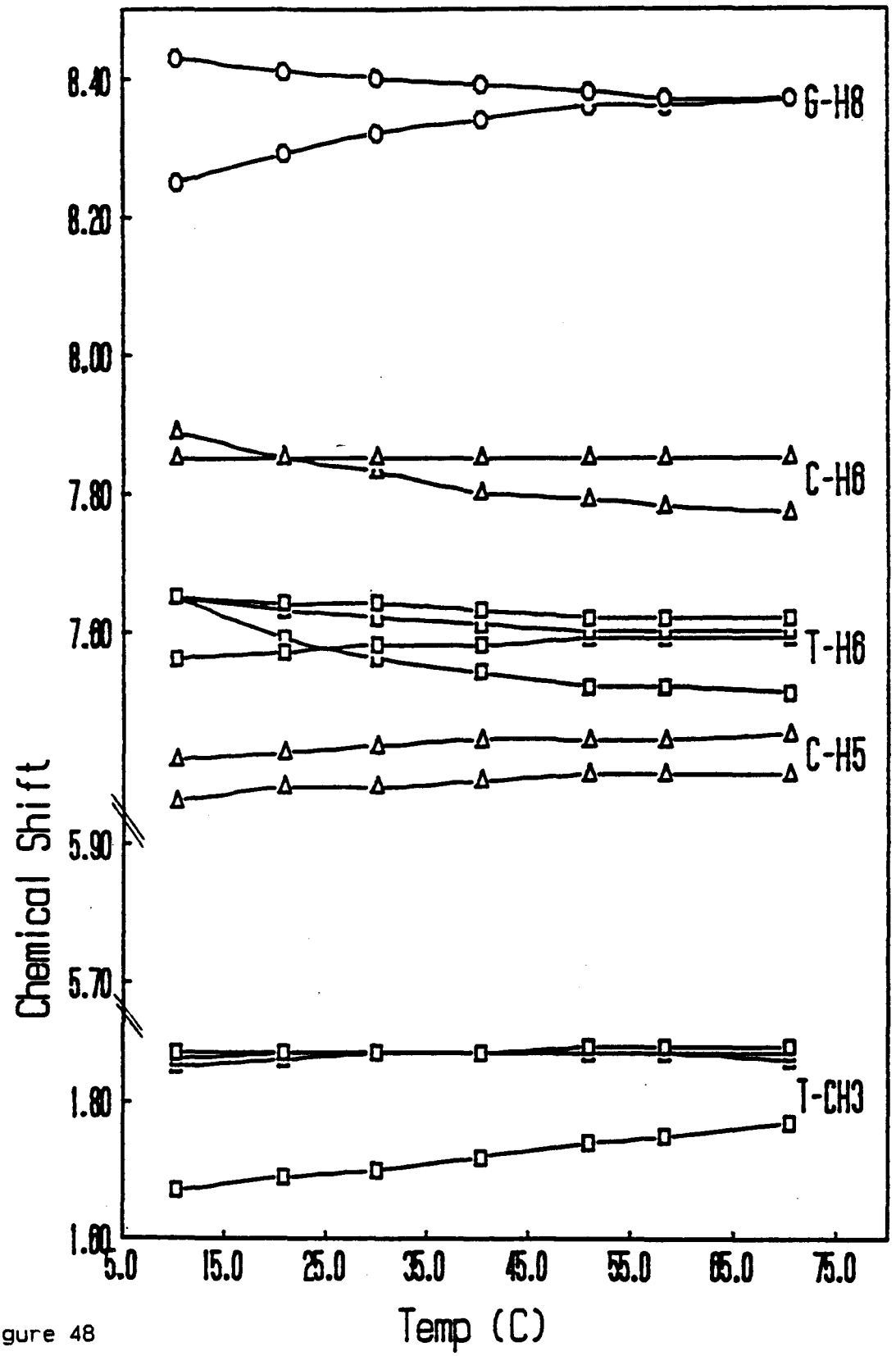


Figure 48

Chemical shift of aromatic and methyl protons as a function of temperature for cis-$Pt(NH_3)_2$[d(CTGTTGTC)-N7(3),N7(6)].

TABLE 29

Chemical shift as a function of temperature (C);
cis-$Pt(NH_3)_2$[d(CTGTTGTC)-N7(3),N7(6)],
1.3 mM, 0.1 M NaCl, 0.01 M phosphate, pH 7.

Chemical Shift vs Temperature (C)

Proton	T(C)	70.3	58.2	50.8	40.2
G-H8		8.373	8.375	8.379	8.388
G-H8		8.368	8.364	8.357	8.341
C-H6		7.847	7.848	7.849	7.849
C-H6		7.770	7.781	7.789	7.805
T-H6		7.617	7.620	7.624	7.629
T-H6		7.599	7.601	7.605	7.610
T-H6		7.589	7.589	7.587	7.583
T-H6		7.509	7.516	7.524	7.541
C-H5		6.057	6.055	6.052	6.047
C-H5		6.003	6.000	5.997	5.992
T-CH3		1.882	1.879	1.878	1.875
T-CH3		1.871	1.866	1.867	1.867
T-CH3		1.865	1.866	1.867	1.867
T-CH3		1.770	1.754	1.743	1.724

Chemical Shift vs Temperature (C)

Proton	T(C)	29.8	20.8	10.2
G-H8		8.399	8.411	8.431
G-H8		8.317	8.291	8.246
C-H6		7.849	7.850	7.849
C-H6		7.826	7.850	7.888
T-H6		7.636	7.644	7.648
T-H6		7.617	7.628	7.648
T-H6		7.578	7.570	7.558
T-H6		7.564	7.593	7.648
C-H5		6.040	6.032	6.021
C-H5		5.984	5.977	5.964
T-CH3		1.873	1.870	1.868
T-CH3		1.867	1.870	1.864
T-CH3		1.867	1.859	1.849
T-CH3		1.705	1.686	1.666

As was concluded for cis-$Pt(NH_3)_2$[d(CTGGTC)], the movement of the G-H8 signals is likely the attainment of the least structurally strained conformation within the chelated region of the molecule. This type of movement was not observed cis-$Pt(NH_3)_2$[d(CTGTGTC)], perhaps the result of decreased mobility within the smaller loop region in the heptamer.

Tentative assignments are made for the T-H6 signals by comparison to the hexamer and heptamer platinum complexes. At 70°C, the two thymines which are 3′ and 5′ to the chelated region, have been assigned to the signals at 7.599 and 7.589 ppm. The remaining two T-H6 signals at 7.617 and 7.509 ppm have been designated as those thymines which are found in the bulged section of the platinated region. Overall, the thymine T-H6 signals are deshielded with respect to their non-platinated parent. The two thymine bases flanking the chelated region of the oligomer do not exhibit any base stacking effects, as was observed for the previous two molecules. Of the two thymines which are in the bulged region, the highest field T-H6 signal has a deshielding temperature effect which parallels one of the G-H8 resonances. Again, this result is attributed to a reorganization of the conformer populations within the loop region of the strand to form the least sterically, and conformationally strained structure. In the case of the T-H6, it suggests further movement away from the shielding regions of adjacent bases as the proton resonance moved downfield when the temperature was decreased.

4.3.7 Conclusions to Variable Temperature Experiments

The examination of variable temperature effects on the sequences d(CTGGTC), d(CTGTGTC), and d(CTGTTGTC), as well as their

cis-$Pt(NH_3)_2(Cl)_2$ adducts has been carried out using proton NMR. The non-platinated sequences gave no indication of intrastrand base stacking as a function of temperature. This result may be a consequence of the high percentage of the poor stacking thymine bases in the sequences. The platinated sequences gave results similar to their parent molecules in that no base stacking effects were evident. The major changes in chemical shift could be attributed to a relief of structural and conformational strain within the platinated region of the different molecules. The bases immediately adjacent to the platinum chelate were generally less shielded than their non-platinated counterparts, indicating that any intrastrand base stacking interactions which may have been present in the normal strands were totally disrupted after reaction with cisplatin. The terminal cytosine bases on all three platinated strands exhibited an insignificant change in chemical shift with respect to their parent molecules. The effects of platination, although conformationally disruptive, appear localized and decrease rapidly with distance from the actual chelate. For the platinated sequences cis-$Pt(NH_3)_2$[d(CTGTGTC)] and cis-$Pt(NH_3)_2$[d(CTGTTGTC)], where the core thymines are looped out of the normal intrastrand stack, the bases in the loop regions were deshielded with respect to their non-platinated counterparts as well as being more deshielded than the thymine bases 3′ and 5′ to the complex.

It remains unclear as to why cis-$Pt(NH_3)_2$[d(CTGGTC)] and cis-$Pt(NH_3)_2$[d(CTGTGTC)] undergo line broadening in their proton NMR below 30°C. Perhaps the energy difference (and/or $\Delta\delta$ shift changes) between the allowed conformers of platinated oligomers is large and the

exchange process slows at lower temperature resulting in the line broadening. The oligomer cis-$Pt(NH_3)_2$[d(CTGTTGTC)] does not exhibit line broadening, perhaps the larger loop has more conformations available to it . The line broadening result does not appear to be an end effect as all three oligomers have the same base sequence both 3′ and 5′ to the platinated regions in the strands.

Chapter Five

5.1 Formation of Normal and non-complementary Duplexes; the effects of cisplatin on double helix formation.

The initial experiments on the interaction of cis-$Pt(NH_3)_2Cl_2$ with short DNA oligomers suggested that binding of the platinum moiety to the DNA caused complete disruption of hydrogen bonding in the chelated region of the molecule (Caradonna et al., 1982). It is clear that the drawback in much of this work was the oligomer sequences which were used and their self-complementary nature. After the reaction between the self-complementary oligomer and cisplatin, the resulting duplex, were it formed, would contain the cis-$Pt(NH_3)_2$ chelate on both strands. Results from these experiments indicated that duplex formation had not occurred, a consequence not only of platinum binding but also because the two, conformationally distorted, platinated regions were in such close proximity to one another (Caradonna et al. 1982; Girault et al. 1984b). The use of two complementary oligomer strands gave results which were contrary to these earlier conclusions using palindrome sequences. These experiments, utilizing complementary oligomers, indicated that it was possible to have duplex formation after the reaction of only one of the two strands with cis-$Pt(NH_3)_2Cl_2$. In addition, the results have shown that even those bases directly involved in the platinum-oligomer chelate are able to participate in normal base pair hydrogen bonding (Kozelka et

al. 1986; den Hartog et al. 1985a, 1984a, 1984b; van Hemelryck et al. 1984).

Based on these data we examined the effects of internal bulged loops, formed by reaction of cis-$Pt(NH_3)_2Cl_2$ with short DNA oligomers, on duplex formation using the compounds studied in Chapter 4.3. Examination of the structure of the cis-$Pt(NH_3)_2$[d(GpX_npG)] intrastrand crosslink, Figure 36, suggests several structural restrictions. The geometry about the platinum(II) atom is square planar and four coordinate, with the ligand binding sites approximately 90° apart. Once cis-$Pt(NH_3)_2Cl_2$ has reacted with the N(7) site on the guanine base and formed the intrastrand G-G crosslink, the distance between the two guanine bases is fixed by the square planar geometry of the metal. If one or more bases are located between the guanines involved in the chelate, as is the case in d(CTGTGTC) and d(CTGTTGTC), the bases central to the chelate are looped out of the strand, as was discussed in Chapter 4.3. The number of bases involved in the loop would only slightly alter the distance between the complexed guanine bases. However, platination of the oligomer will cause conformational alterations i.e. bond rotations, and changes in sugar pucker, as has been shown to occur in cis-$Pt(NH_3)_2$[dGCG] (den Hartog et al., 1983).

If the distance between the metalated guanine bases in the platinum chelate is fixed, then the presence of intervening bases should not significantly alter the ability of these bases to participate in G-C base pair formation. If the bulged loop regions in the chelate are sterically removed from the hydrogen bonding section of the duplex, it would not be necessary to maintain the complementary sequence for those bulged bases

in the opposite strand. It was proposed, therefore, to mix the platinated strands with their complementary sequence as well as with those which lack the base complements to the regions which have been looped out as a result of platinum binding. The overall scheme for the mixing experiments has been outlined in Table 15. In order that the full effects of platination on duplex formation be understood for the sequences studied, the non-platinated parent molecules were also examined in similar mixing experiments.

5.2 Mixing Experiments with Complementary Sequences

This section examines duplex formation between the complementary sets of deoxy oligomers of sequence: d(CTGGTC) + d(GACCAG), d(CTGTGTC) + d(GACACAG), d(CTGTTGTC) + d(GACAACAG). In all cases, the duplexed structures have been observed via the exchangeable imino protons involved in Watson-Crick base pairing. The duplexes were examined at low temperature and followed through complete unwinding as the temperature was increased. Wherever possible, the non-exchangeable aromatic protons have been monitored through the helix to coil transition and the T_m of the duplex determined. The assignment of the aromatic base protons on the individual nucleoside units in the complementary, non-platinated oligomers used in the mixing experiments is by comparison to calculated chemical shift values obtained using the method of Bell et al. 1981, Table 30.

Table 30

Assignment of aromatic proton chemical shifts (ppm) of oligomer complements for mixing experiments based on calculated values at 70°C.

		dGpApCpCpApG 1-2-3-4-5-6		dGpApCpApCpApG 1-2-3-4-5-6-7	
		calc.	obs.	calc.	obs.
5′					
G	H8	7.881	7.747	7.870	7.740
A	H8	8.266	8.208	8.277	8.267
	H2	8.116	8.053	8.079	8.074
C	H6	7.683	7.685	7.626	7.562
	H5	5.816	5.881	5.761	5.836
A	H8	-----	-----	8.192	8.188
	H2	-----	-----	7.996	8.037
A	H8	-----	-----	-----	-----
	H2	-----	-----	-----	-----
C	H6	7.662	7.592	7.602	7.532
	H5	5.840	5.981	5.769	5.856
A	H8	8.282	8.277	8.237	8.257
	H2	8.139	8.117	8.080	8.088
G	H8	7.981	7.911	7.895	7.903
3′					

5.2.1 d(CTGGTC) + d(GACCAG).

The complementary hexamers, d(CTGGTC) and d(GACCAG), were combined in a 1:1 ratio for an overall DNA concentration of 2.6mM, in 0.1 M NaCl, 0.01 M sodium phosphate buffer pH 7.0, and 0.5 mM EDTA. The chemical shift vs temperature results are given in Table 31 and the data plotted on Figure 49 for the aromatic non-exchangeable protons. A study of the two strands individually before mixing, in conjunction with the calculated chemical shift values, allowed the assignment of the guanine and cytosine signals to specific strands. The adenine and thymine bases are found in only one sequence - never in both - simplifying their assignment to a specific strand. The guanine and cytosine bases found in dCTGGTC have been designated in the data with an asterix (*) preceding the proton assignment.

The position of a specific heterocyclic base proton and its immediate surroundings in the right-handed double helix are reflected in the NMR chemical shift variations of that proton as a function of temperature. When the nucleic acid bases are in the normal anti conformation with respect to glycosidic torsion angle, the purine H-8, the pyrimidine H-6 and, to a lesser degree, H-5 and 5-CH_3 protons are directed out from the core of the helix into the major and minor grooves. As a result of this conformation the chemical shifts of these protons are less affected by the magnetic shielding regions of the adjacent bases. This effect is evident with the adenine H-8 resonances which do not exhibit a significant chemical shift change as a result of temperature variation. The H-8 resonances for the four guanine bases in the duplex do exhibit some variation in chemical shift as a function of

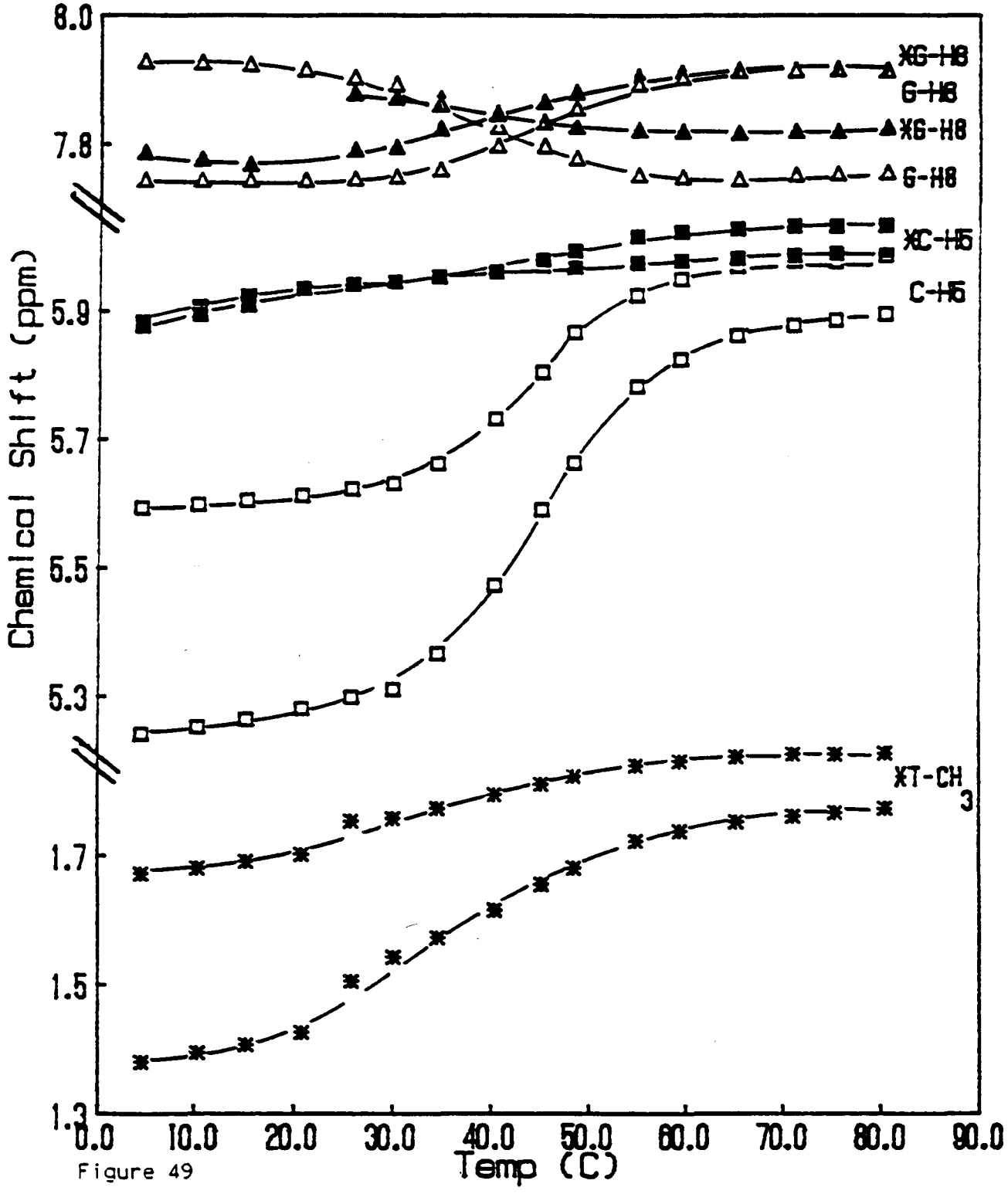


Figure 49

Chemical shift of the non-exchangeable aromatic and methyl protons as a function of temperature for the complementary hexamer mixture d(CTGGTC)* + d(GACCAG).

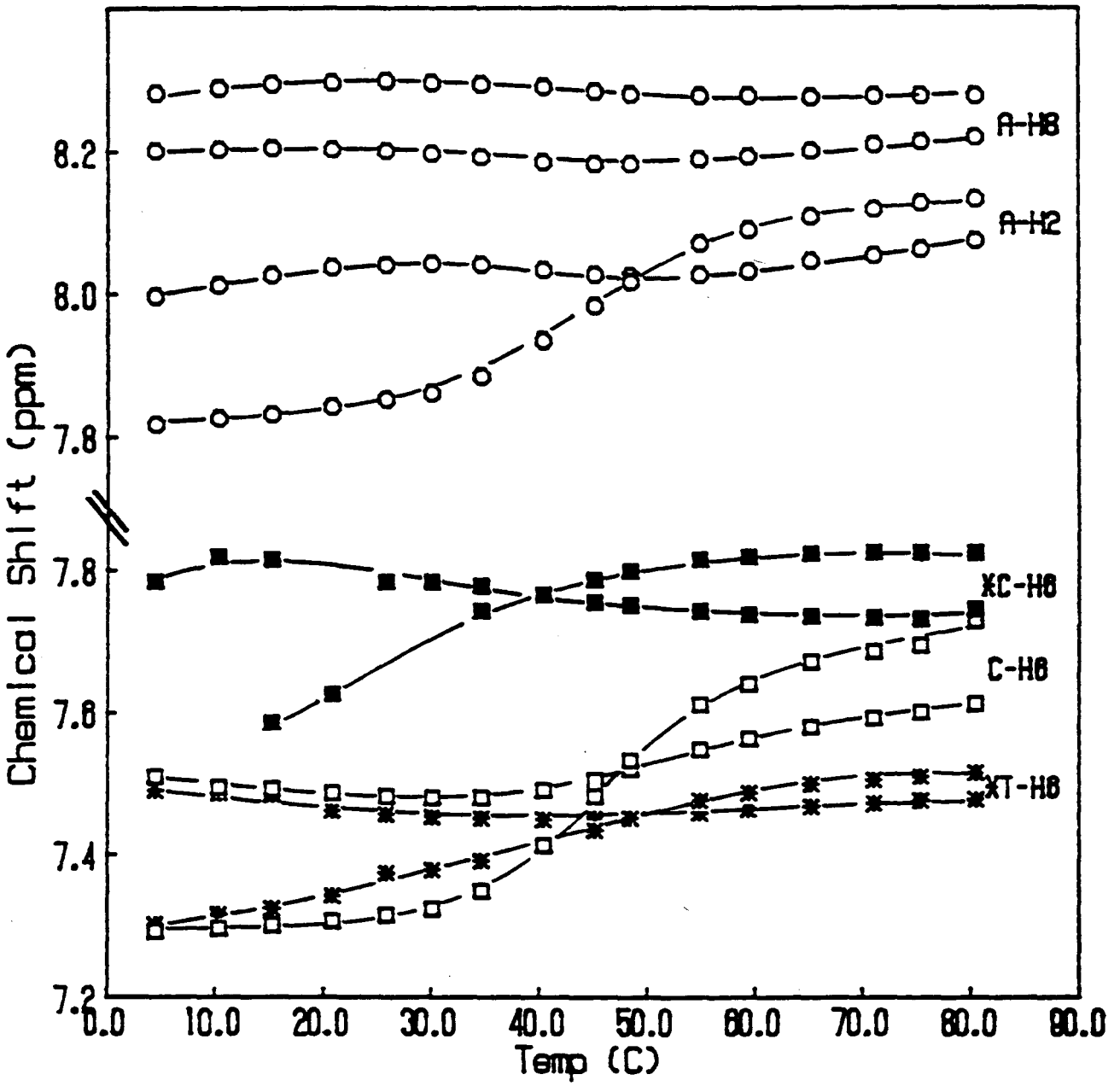


Figure 49 (continued)

Chemical shift of the non-exchangeable aromatic and methyl protons as a function of temperature for the complementary hexamer mixture d(CTGGTC)* + d(GACCAG).

TABLE 31
Chemical shift as a function of temperature;
d(CpTpGpGpTpC){*} + d(GpApCpCpApG),
(1-2-3-4-5-6) (7-8-9-10-11-12)
1.5 mM each strand, 0.1 M NaCl, 0.01 M phosphate, pH 7.

Chemical Shift vs Temperature

Proton	T(C) 80.4	75.3	70.9	65.2	59.4
A(11)-H8	8.278	8.277	8.277	8.276	8.277
A(8)-H8	8.219	8.212	8.208	8.201	8.192
A(11)-H2	8.131	8.124	8.117	8.107	8.087
A(8)-H2	8.072	8.061	8.053	8.043	8.030
*G(4)-H8	7.911	7.912	7.911	7.910	7.907
G(12)-H8	7.911	7.912	7.911	7.910	7.900
*C(6)-H6	7.824	7.824	7.824	7.823	7.819
*G(3)-H8	7.822	7.817	7.817	7.815	7.816
G(7)-H8	7.752	7.749	7.747	7.742	7.745
*C(1)-H6	7.730	7.732	7.734	7.735	7.738
C(9)-H6	7.745	7.694	7.685	7.670	7.639
C(10)-H6	7.612	7.600	7.592	7.580	7.562
*T(5)-H6	7.514	7.509	7.504	7.497	7.485
*T(2)-H6	7.477	7.474	7.471	7.467	7.462
*C(6)-H5	6.037	6.036	6.035	6.032	6.025
*C(1)-H5	5.992	5.994	5.989	5.986	5.982
C(10)-H5	5.992	5.982	5.981	5.972	5.953
C(9)-H5	5.899	5.890	5.881	5.865	5.827
*T(2)-CH_3	1.857	1.855	1.854	1.850	1.844
*T(5)-CH_3	1.770	1.764	1.759	1.751	1.735

Proton	T(C) 54.9	48.4	45.2	40.3	34.0
A(11)-H8	8.278	8.279	8.284	8.289	8.294
A(8)-H8	8.188	8.182	8.182	8.184	8.191
A(11)-H2	8.069	8.015	7.982	7.933	7.883
A(8)-H2	8.025	8.022	8.024	8.031	8.039
*G(4)-H8	7.900	7.877	7.862	7.843	7.820
G(12)-H8	7.889	7.852	7.832	7.793	7.757
*C(6)-H6	7.814	7.798	7.786	7.765	7.741
*G(3)-H8	7.818	7.826	7.832	7.843	7.858
G(7)-H8	7.748	7.774	7.793	7.826	7.867
*C(1)-H6	7.741	7.749	7.755	7.765	7.777
C(9)-H6	7.610	7.531	7.481	7.413	7.348
C(10)-H6	7.548	7.519	7.505	7.489	7.479
*T(5)-H6	7.475	7.450	7.434	7.413	7.390
*T(2)-H6	7.458	7.450	7.449	7.448	7.449
*C(6)-H5	6.019	5.996	5.981	5.963	5.955
*C(1)-H5	5.978	5.971	5.972	5.963	5.955
C(10)-H5	5.928	5.869	5.806	5.733	5.662
C(9)-H5	5.786	5.665	5.592	5.475	5.369
*T(2)-CH_3	1.838	1.820	1.809	1.792	1.770
*T(5)-CH_3	1.720	1.679	1.654	1.615	1.570

TABLE 31 (continued)
Chemical shift as a function of temperature;
d(CpTpGpGpTpC){*} + d(GpApCpCpApG),
(1-2-3-4-5-6) (7-8-9-10-11-12)
1.5 mM each strand, 0.1 M NaCl, 0.01 M phosphate, pH 7.

	Chemical Shift vs Temperature			
Proton	T(C) 30.0	25.8	20.8	15.1
A(11)-H8	8.296	8.297	8.296	8.293
A(8)-H8	8.196	8.199	8.202	8.204
A(11)-H2	7.861	7.852	7.842	7.832
A(8)-H2	8.041	8.040	8.035	8.024
*G(4)-H8	7.791	7.788	-----	7.764
G(12)-H8	7.745	7.741	7.739	7.739
*C(6)-H6	-----	-----	7.624	7.586
*G(3)-H8	7.869	7.876	-----	-----
G(7)-H8	7.890	7.901	7.913	7.921
*C(1)-H6	7.784	7.784	-----	7.815
C(9)-H6	7.323	7.315	7.307	7.301
C(10)-H6	7.479	7.482	7.486	7.492
*T(5)-H6	7.377	7.373	7.342	7.323
*T(2)-H6	7.453	7.456	7.461	7.484
*C(6)-H5	5.946	5.941	5.935	5.909
*C(1)-H5	5.946	5.941	5.935	5.922
C(10)-H5	5.632	5.622	5.612	5.604
C(9)-H5	5.313	5.301	5.282	5.265
*T(2)-CH_3	1.757	1.753	1.701	1.690
*T(5)-CH_3	1.541	1.504	1.424	1.406

Proton	T(C) 10.3	4.5	T_m(C)	
A(11)-H8	8.288	8.282		
A(8)-H8	8.203	8.201		
A(11)-H2	7.825	7.816	44.6	
A(8)-H2	8.011	7.995		
*G(4)-H8	7.772	7.783	38.8	
G(12)-H8	7.739	7.739	44.0	
*C(6)-H6	7.559	-----	23.9	
*G(3)-H8	-----	-----	36.5	
G(7)-H8	7.923	7.926	39.4	
*C(1)-H6	7.818	7.783		
C(9)-H6	7.296	7.291	45.8	
C(10)-H6	7.494	7.508	57.8	
*T(5)-H6	7.314	7.301	41.8	
*T(2)-H6	7.486	7.490		
*C(6)-H5	5.893	5.874		
*C(1)-H5	5.906	5.882		
C(10)-H5	5.598	5.591	43.4	average T_m
C(9)-H5	5.253	5.240	44.3	= 41.0°C
*T(2)-CH_3	1.680	1.670	35.4	
*T(5)-CH_3	1.394	1.380	36.9	

temperature, the average change in chemical shift being approximately 0.15 ppm. The position of the guanine base within the sequence does not seem to alter this result, as two of the guanines (G7, G12) are at terminal sites in the duplex, while the remaining two (G3, G4) are in the core. One significant difference between the four guanine H-8 resonances is that two move upfield with duplex formation while the remaining two become more deshielded with the decrease in temperature. The downfield shift has been explained by Borer et al. (1975) to be the result of several factors. The hydrogens in question lie approximately in the plane of an aromatic ring and may encounter cross-strand deshielding ring current effects, although the effects are small because the distances involved are large. Other factors, such as the change in the solvation about the proton and the effect of base-base hydrogen bonding on the electronic nature of the base, may also contribute to the deshielding environment.

The adenine H-2 protons are directed into the centre of the helix and are more ring current shifted as a result of duplex formation. The two adenine H-2 protons reflect this environment although A11 more so than A8, with chemical shift changes of 0.35 and 0.05 ppm respectively. The differences in the amount of shielding between the two adenine H-2 protons may be attributed to the variation in base sequence 3′ and 5′ to the specific proton and, consequently, the degree of base stacking surrounding the proton.

There are four cytosines in the duplex - two are located at terminal positions in the helix (C1, C6) and two are in the interior (C9, C10). The H-6 protons from the terminal sites exhibit chemical shifts in

opposite directions as a consequence of temperature variation. This illustrates the difference in the diamagnetic shielding at the two ends of the duplex and reflects the differences in the nature of the base stacking. The magnitude of the chemical shift change is 0.3 ppm for C(6)-H6 and 0.1 ppm for C(1)-H6. The H-6 protons on the cytosines located in the core of the duplex both move upfield upon helix formation, showing sigmoidal behaviour, with chemical shift changes of 0.1 and 0.4 ppm for C10 and C9 respectively. For the H-5 protons on the same bases there is a marked difference in the behaviour of those located in the core and those in the terminal positions. All H-5 protons show shielding effects as a result of helix formation. However, the core protons show large chemical shift changes of 0.4 ppm (C10) and 0.7 ppm (C9) while the shifts for those in the terminal sites are approximately 0.15 ppm. The curves for the terminal H-5 protons show a gradual linear shift with temperature change while the core resonances exhibit sigmoidal behaviour.

The aromatic protons on the two thymine bases, H-6 and 5-CH_3, also exhibit chemical shift changes with temperature variation. The 5-methyl group, like the cytosine H-5 proton, is directed towards the interior of the helix. This is evident here by downfield chemical shift changes of 0.2 and 0.4 ppm for the two methyl groups on T2 and T5 respectively. For the H-6 resonances, T2-H6 shows an insignificant change in chemical shift over the temperature range studied while T5-H6 moves upfield 0.2 ppm.

A general examination of the curves presented in Figure 49 reveals sigmoidal shaped lines for the majority of the protons studied. The shape of the melting curve indicates the degree of cooperation between

the two strands in the transition from double helix to single stranded coil. Individually, the T_m's of the various resonances differ depending on the position of the proton in the helix. The resonances associated with the core of the duplex range from a low of 36.5°C for G(3)-H8 to a high of 57.8°C for the C(10)-H6 signal, with the average Tm for the GG-CC core being 44.4°C. Moving out from the core in either direction, the A-T base pairs 2 and 5 show T_m values only one or two degrees lower than the core itself - with T_m's ranging from 35.4°C ($T2\text{-}CH_3$) to 44.6°C (A11-H2). The terminal G-C base pairs 1 and 6 again exhibited a slight drop in midpoint compared to the internal base pairs, with the overall duplex T_m having been determined to be 41.0°C. This decrease in midpoint temperature for the terminal base pairs when compared to the core region has been well documented and has been attributed to fraying of the terminal base pairs (Patel, 1975).

Line broadening of specific non-exchangeable aromatic resonances at different temperatures throughout the helix-coil transition gives an indication of the exchange rates between the two species. Over the temperature range 50-55°C there were major changes in line shape of several signals, particularly the cytosine H-5 and H-6 resonances for the core region. As the temperature was further decreased to 30°C, these same resonances began to narrow again while signals from bases in the terminal positions broaden. Below room temperature most of the resonances had narrowed again, while a further drop in temperature to below 10°C resulted in an overall broadening of the spectrum.

Similar results have been observed by Feigon et al. (1983) who attributed these effects to three factors. The line broadening which

occurs over the transition temperature region will depend on the difference between the chemical shift in the single stranded coil and the helical states; the rate at which the strands exchange between the two structures; and the population of each state at a particular temperature. If the exchange rate between the coil and helix is fast with respect to the NMR time scale, then the resonances will remain sharp. The observed resonance possesses a chemical shift which is an average between the proton chemical shift in the duplexed structure and that in the single stranded coil. The experimentally observed chemical shift reflects the populations of the two states at that particular temperature. However, if the rate of exchange between the coil and helix structures is slow, then line broadening will occur. The largest amount of broadening will occur for resonances which have a large chemical shift difference between the single stranded and the duplexed states. The line broadening which was observed around 45°C has been attributed to slow exchange between the coil and helix structures for the core region of the duplex. These resonances narrow again at lower temperatures, indicating that the exchange has stopped, at least in the core of the duplex. Below the T_m values where the proton resonance no longer shows a temperature dependence, the chemical shift reflects solely the duplexed structure. Because the resonances do not all broaden and narrow over the same temperature range i.e. the protons on terminal bases broadening at lower temperatures than the core protons - fraying of the terminal base pairs is suggested. The overall broadening at temperatures below 10°C may be caused by aggregation of the duplexes and/or solvent viscosity changes, both which would affect the tumbling rate of the molecules in solution.

5.2.2 Variable temperature study of the exchangeable imino protons in the hexamer duplex.

The same sample was examined in a solution of 9:1 H_2O:D_2O to observe the exchangeable imino protons involved in the hydrogen bonds of base pairs. Assignments of the imino proton signals to a specific base pair were made on the basis of sequential signal line broadening with temperature increase. The double helix unwinds from either end towards the middle of the duplex. When a base pair opens and exposes the imino protons to exchange with solvent, the NMR signal for that specific base pair broadens and eventually disappears as the exchange with solvent becomes rapid (Kan et al. 1975; Patel, 1975). For perfect duplexes, this type of melting experiment allows the assignment of the signals which broaden out at low temperature to terminal positions in the duplex and resonances which broaden at higher temperatures to base pairs in the core of the helix. When A-T base pairs exist at the terminal positions in a helix, imino protons may not be observed as a result of base pair fraying as discussed above. In the sequences used in this work, these end effects were not observed as the terminal base pairs in all cases were the more thermodynamically stable G-C pair.

In the variable temperature melting experiments, one A-T base pair, (either [2 or 5]), and one G-C, (either [1 or 6]), have signals which broaden and disappear between 15°C and 20°C as the helix begins to unwind (Figure 50). This result suggests that the duplex is preferentially unwinding from one end. The second terminal G-C base pair did not broaden until the temperature was above 20°C. At 25°C, the only resonances which had not broadened to the base line were the A-T signal

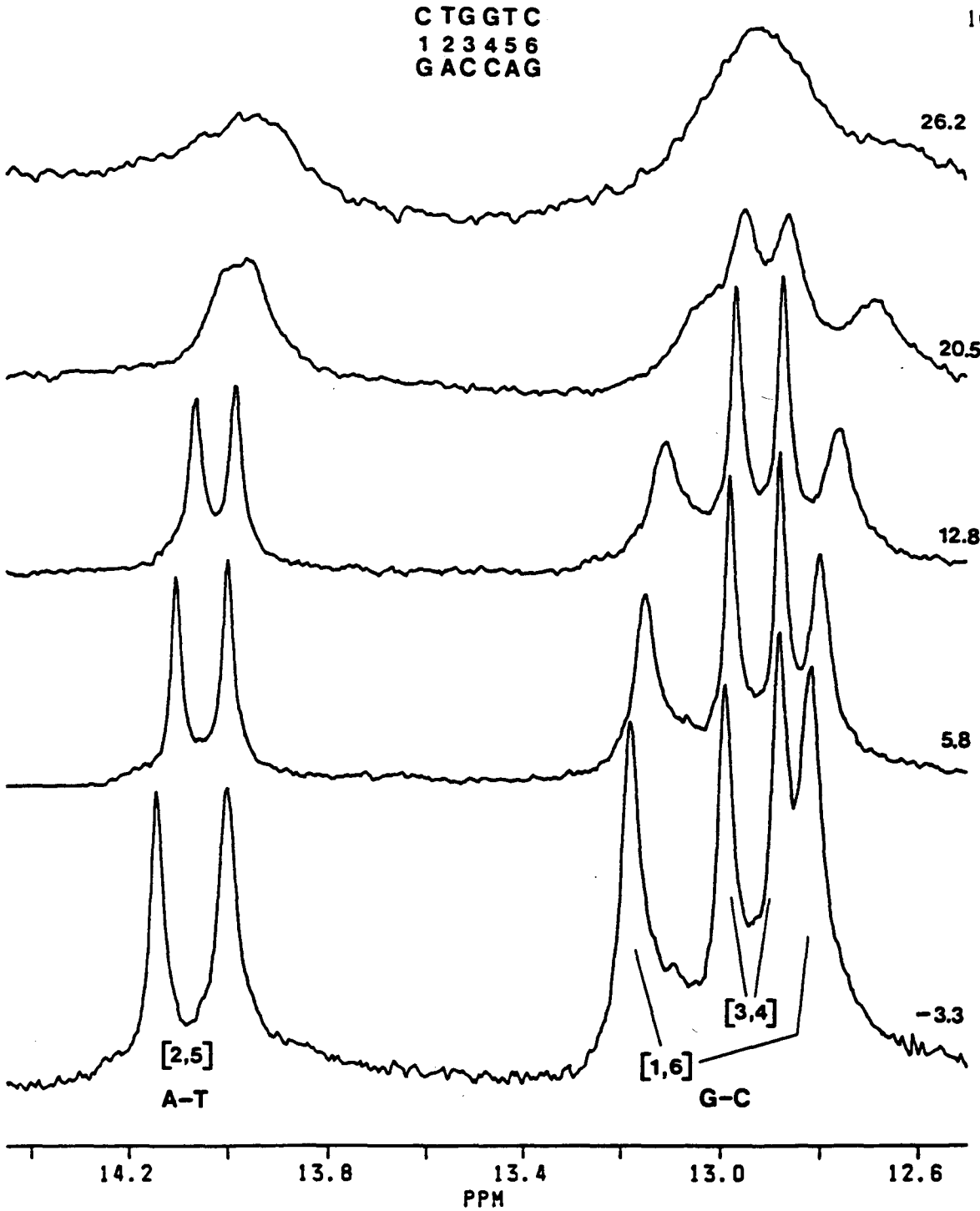


Figure 50

Exchangeable imino protons involved in base pairing as a function of temperature for the complementary hexamer mixture d(CTGGTC) + d(GACCAG).

at 13.95 ppm and the G-C signal, (either [3 or 4]), at 12.89 ppm. This observation also suggests that one end of the duplex was melting faster towards the core than the other.

Several factors contribute to the stability of the duplex i.e. hydrogen bonding, base stacking, and solvation effects (Patel et al. 1982a, 1982b, 1975, 1974; Kan et al. 1975). The observed non-symmetrical melting behaviour may reflect the difference in base stacking over the length of the duplex. Over the range 25°C to 30°C, all imino resonances broadened out, the result of a rapid exchange equilibrium between the base paired and open structures. The line broadening is assisted by a base catalyzed proton exchange in the open state between the imino base protons and the surrounding solvent (Patel and Hilbers, 1975).

Similar conclusions can be made from the line width versus temperature plot, Figure 51, which illustrates the effect of temperature on the imino resonance line width. This graph illustrates that the two resonances for the terminal G-C base pairs, [1,6], are broader than the remaining signals even at the lowest temperature. As the temperature was increased, the terminal base pairs broadened gradually until, around 20°C, they broadened out completely. Up until the terminal G-C base pairs began to broaden extensively, the neighbouring A-T base pairs [2,5] did not exhibit temperature line broadening effects. When the rate of opening of the terminal base pairs increased, as observed through increased line broadening, the line width of the base pairs [2,5] increased dramatically. This supports the extensive fraying effect observed for terminal A-T base pairs. Following the rapid opening of A-T base pairs [2,5], the GG-CC core of the duplex followed at approximately

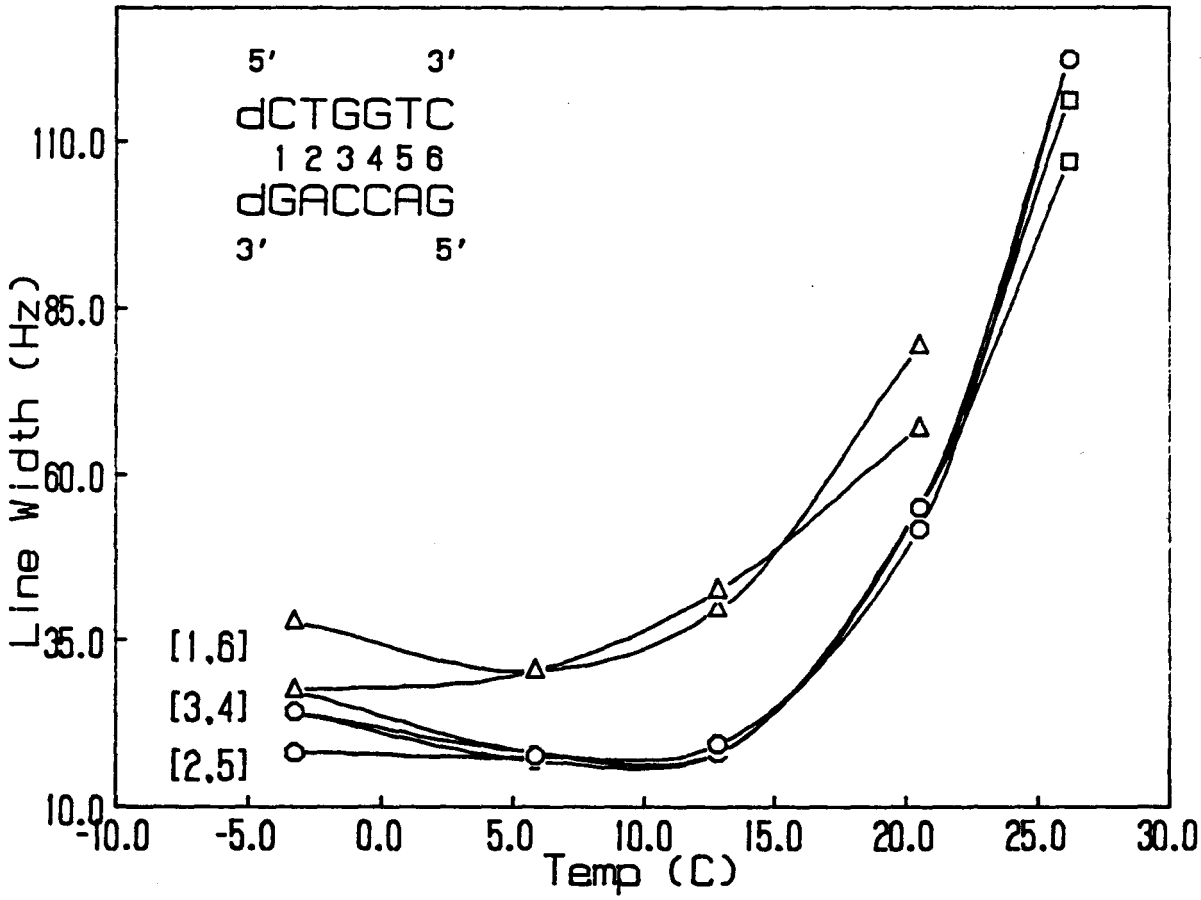


Figure 51

Line widths of exchangeable imino proton resonances as a function of temperature for the complementary hexamer mixture d(CTGGTC) + d(GACCAG).

the same rate of opening, as illustrated by the parallel nature of the curves for the A-T base pairs [2,5] and the core G-C base pairs [3,4]. The rate of exchange for all imino protons became too rapid above 30°C to observe any signals.

5.2.3 d(CTGTGTC) + d(GACACAG).

The complementary heptamers, d(CTGTGTC) and d(GACACAG), were combined in a 1:1 ratio at overall DNA concentration of 3.0 mM in 0.1 M sodium chloride, 0.01 M sodium phosphate pH 7, and 0.5 mM EDTA. The mixture of the two complementary strands was studied using both the exchangeable imino protons and the non-exchangeable aromatic base protons. Assignment of the non-exchangeable aromatic resonances to a specific strand was carried out by comparison with calculated chemical shift values and by comparison of the chemical shifts from the 70°C spectra for the two heptamers, before and after mixing. The aromatic protons belonging to the d(CTGTGTC) strand have been designated by an asterix (*), with the nucleotide units being numbered 5′ to 3′, 1 to 7. In the complementary oligomer, d(GACACAG), the nucleotides are numbered in the same fashion, 8 to 14. The NMR data for the non-exchangeable protons over the temperature range -5° to 80°C are given in Table 32. These results have been plotted in Figure 52.

Examination of the lines obtained from the variable temperature experiments reveals that many of the protons could not be followed throughout the entire temperature range. There were two major reasons for the incomplete curves. The first problem was that many of the resonances were found in the crowded region in the NMR spectrum between

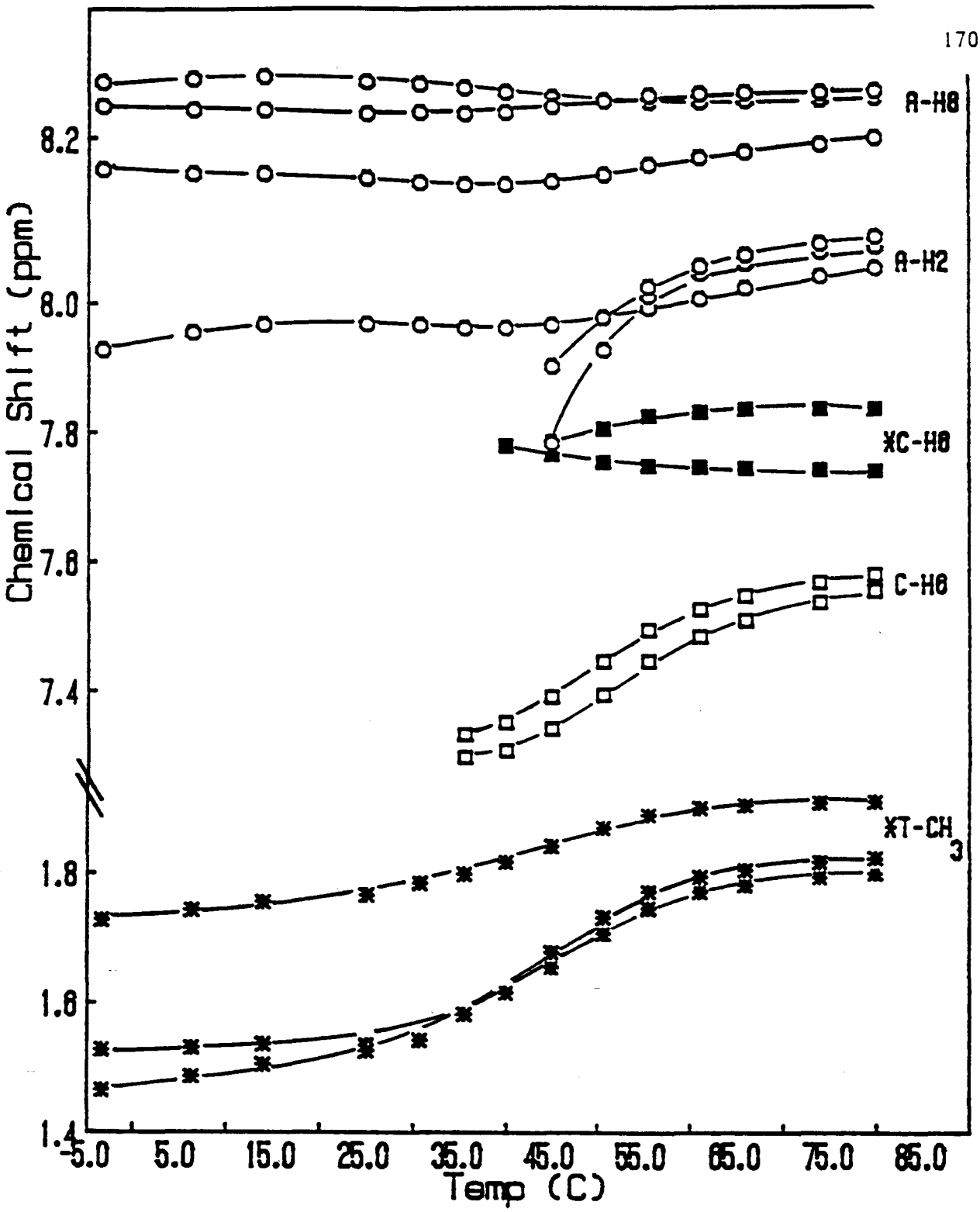


Figure 52

Chemical shift of the non-exchangeable aromatic and methyl protons as a function of temperature for the complementary heptamer mixture d(CTGTGTC)* + d(GACACAG).

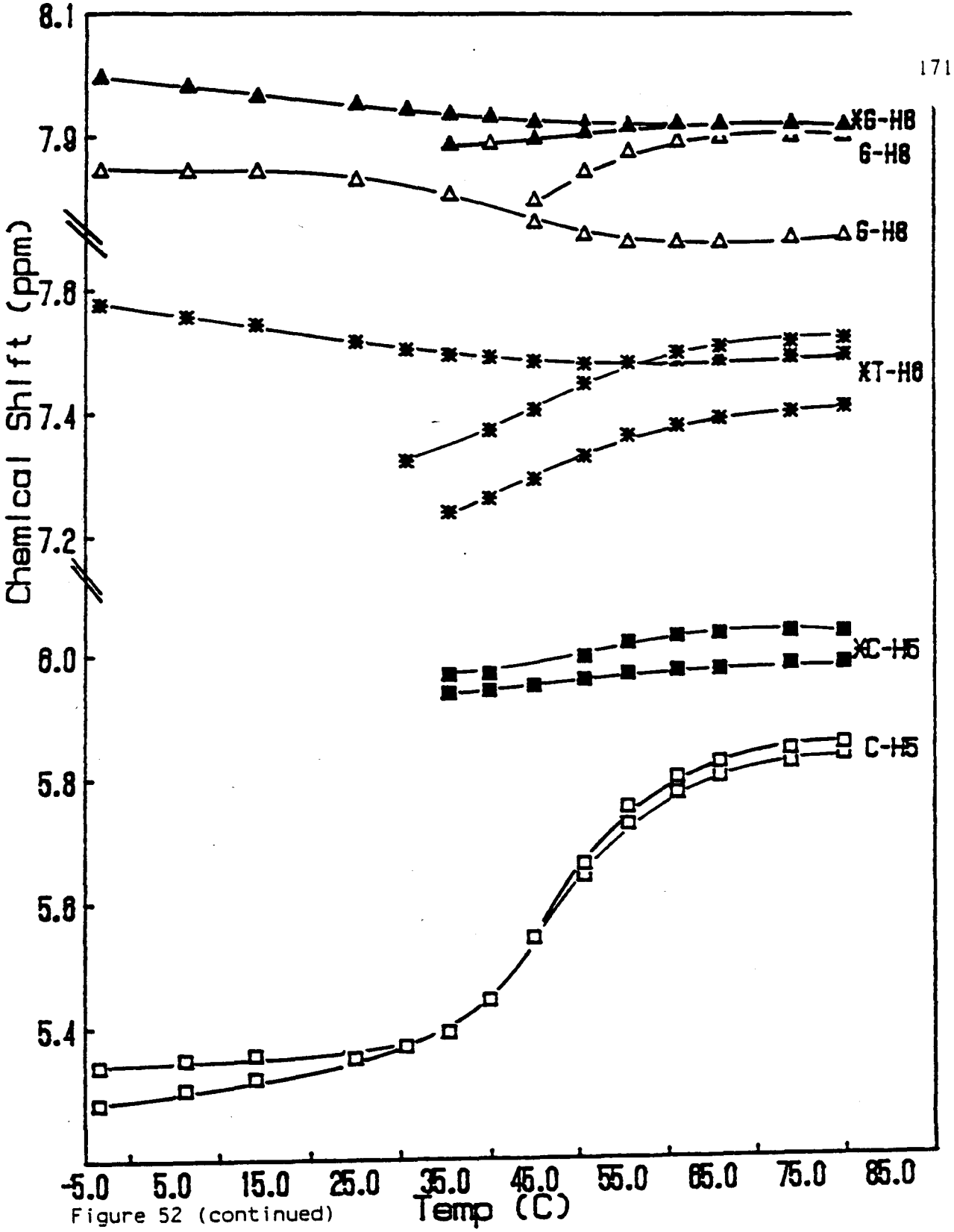


Figure 52 (continued)

Chemical shift of the non-exchangeable aromatic and methyl protons as a function of temperature for the complementary heptamer mixture d(CTGTGTC)* + d(GACACAG).

TABLE 32

Chemical shift as a function of temperature;
d(CpTpGpTpGpTpC){*} + d(GpApCpApCpApG),
(1-2-3-4-5-6-7) (8-9-10-11-12-13-14)
1.5 mM each strand, 0.1 M NaCl, 0.01 M phosphate, pH 7.

Chemical Shift vs Temperature

Proton	T(C) 79.8	73.7	65.8	61.0	55.5	50.6	44.9
A(9)-H8	8.268	8.267	8.266	8.265	8.262	8.255	8.246
A(13)-H8	8.259	8.257	8.254	8.252	8.252	8.255	8.261
A(11)-H8	8.196	8.188	8.176	8.167	8.154	8.140	8.129
A(13)-H2	8.098	8.088	8.071	8.053	8.020	7.972	7.897
A(9)-H2	8.084	8.074	8.058	8.041	8.006	7.922	7.781
A(11)-H2	8.050	8.037	8.018	8.003	7.987	7.972	7.962
*G(5)-H8	7.920	7.922	7.923	7.922	7.918	7.909	7.897
*G(3)-H8	7.916	7.916	7.917	7.918	7.918	7.922	7.926
G(14)-H8	7.905	7.903	7.900	7.894	7.878	7.845	7.799
*C(7)-H6	7.831	7.831	7.831	7.828	7.820	7.802	7.763
G(8)-H8	7.744	7.740	7.732	7.731	7.732	7.743	7.765
*C(1)-H6	7.736	7.737	7.739	7.741	7.744	7.751	7.763
C(10)-H6	7.576	7.562	7.541	7.520	7.489	7.442	7.388
C(12)-H6	7.549	7.532	7.504	7.479	7.442	7.390	7.338
*T(6)-H6	7.525	7.519	7.510	7.500	7.483	7.450	7.409
*T(4)-H6	7.497	7.494	7.490	7.488	7.486	7.481	7.485
*T(2)-H6	7.414	7.406	7.396	7.384	7.366	7.333	7.295
*C(7)-H5	6.046	6.045	6.042	6.038	6.028	6.005	-----
*C(1)-H5	5.995	5.993	5.986	5.984	5.977	5.967	5.956
C(12)-H5	5.866	5.856	5.836	5.811	5.762	5.671	5.553
C(10)-H5	5.850	5.836	5.813	5.786	5.736	5.653	5.553
*T(2)-CH_3	1.861	1.858	1.855	1.849	1.839	1.818	1.791
*T(6)-CH_3	1.773	1.766	1.754	1.743	1.721	1.682	1.627
*T(4)-CH_3	1.751	1.743	1.732	1.720	1.696	1.656	1.605

TABLE 32 (continued)

Chemical shift as a function of temperature;
d(CpTpGpTpGpTpC){*} + d(GpApCpApCpApG),
(1-2-3-4-5-6-7) (8-9-10-11-12-13-14)
1.5 mM each strand, 0.1 M NaCl, 0.01 M phosphate, pH 7.

Chemical Shift vs Temperature

Proton	T(C) 39.8	35.4	30.6	25.0	13.9	6.3	-3.3	T_m
A(9)-H8	8.238	8.235	8.237	8.236	8.242	8.242	8.248	47.5
A(13)-H8	8.269	8.275	8.281	8.286	8.293	8.289	8.286	38.0
A(11)-H8	8.125	8.124	8.129	8.135	8.143	8.143	8.149	58.0
A(13)-H2	-----	-----	-----	-----	-----	-----	-----	
A(9)-H2	-----	-----	-----	-----	-----	-----	-----	
A(11)-H2	7.959	7.958	7.963	7.965	7.965	7.952	7.925	64.5
*G(5)-H8	7.890	7.887	-----	-----	-----	-----	-----	
*G(3)-H8	7.933	7.938	7.944	7.953	7.965	7.982	7.996	
G(14)-H8	-----	-----	-----	-----	-----	-----	-----	
*C(7)-H6	-----	-----	-----	-----	-----	-----	-----	
G(8)-H8	-----	7.807	-----	7.831	7.844	7.844	7.843	38.2
*C(1)-H6	7.777	-----	-----	-----	-----	-----	-----	
C(10)-H6	7.348	7.330	-----	-----	-----	-----	-----	48.1
C(12)-H6	7.304	7.293	-----	-----	-----	-----	-----	50.6
*T(6)-H6	7.375	-----	7.325	-----	-----	-----	-----	45.3
*T(4)-H6	7.491	7.495	7.504	7.517	7.544	7.556	7.576	
*T(2)-H6	7.264	7.242	-----	-----	-----	-----	-----	46.7
*C(7)-H5	5.976	5.972	-----	-----	-----	-----	-----	
*C(1)-H5	5.948	5.943	-----	-----	-----	-----	-----	
C(12)-H5	5.453	5.399	5.378	5.357	5.321	5.303	5.277	50.5
C(10)-H5	5.453	5.399	5.378	5.357	5.358	5.350	5.338	50.2
*T(2)-CH_3	1.767	1.747	1.734	1.715	1.704	1.692	1.678	43.6
*T(6)-CH_3	1.564	1.531	1.491	1.475	1.454	1.436	1.414	44.2
*T(4)-CH_3	1.564	1.531	1.491	1.483	1.485	1.481	1.478	45.9

average T_m = 47.9°C

8.1 and 7.7 ppm, where many signal cross-overs occurred. Added to this was the problem of line broadening. As discussed in section 5.2.1, line broadening of many proton resonances occurred during the transition from single stranded coil to the duplex because of the slow exchange between the two structural conformations. Although several of the lines became sharp again below the transition temperature, it was not possible to disentangle the different resonances because of the many signal cross-overs which had occurred. Of the twenty-four non-exchangeable protons monitored, ten exhibited sigmoidal behaviour and their transition temperatures were obtained.

The majority of the aromatic protons present in the trimer core of the duplex [d-GTG-]-[d-CAC-], could be followed over the entire temperature range examined. The H-8 resonances for the core guanine bases (G3, G5) exhibited only minor changes in chemical shift as a function of temperature. This is expected, as the H-8 proton on a purine base in a double helix structure is directed into the major and minor grooves of the duplex. The end result of the H-8 proton orientation is that it is removed from the diamagnetic shielding region of the adjacent bases. Therefore, the major influence on purine H-8 chemical shift change is the alteration of the backbone and the accompanying solvation changes as well as nucleo-base geometry upon duplex formation. The 5-CH_3 protons of the central thymine base (T4) showed sigmoidal behaviour and gave a T_m value of 45.9^oC. The central adenine unit (A11) gave T_m values for it's H-2 and H-8 protons, with melting temperatures of 64.5^oC and 58.0 ^oC respectively. The H-6 and H-5 protons from the core cytosine bases (C10, C12) also showed sigmoidal behaviour, with T_m values of 50.2^oC and 50.5^oC

for the H-5 signals and 48.1°C and 50.6°C for the H-6 signals. The average T_m for the trimer core of the duplex was 52.5°C.

Moving from the core of the duplex to the next set of base pairs, A-T, H2 resonances for both adenine bases (A9, A13) were lost to line broadening and signal overlap. The H2 protons, which normally provide useful information as they are directed into the core of the duplex, disappeared as they move rapidly upfield. Although the adenine H-8 melting curves are shallow, they are sigmoidal and yeild T_m values of 47.5°C (A9) and 38.0 (A13). The H-6 and 5-CH_3 signals from the thymine bases (T2, T6) were followed over much of the temperature range studied. For the H6 resonances, both signals moved upfield showing sigmoidal behaviour and a T_m values of 46.7°C (T2) and 45.3°C (T6). The two 5-CH_3 groups shifted upfield with the decrease in temperature, giving T_m values of 43.6°C (T2) and 44.2°C (T6). Combining these transition temperature values with those obtained for the trimer core of the duplex, the T_m for the five internal base pairs was 48.7°C. This is a decrease of approximately four degrees over the trimer core alone.

Examination of the signals for the two terminal G-C base pairs [1 and 7] shows that only one proton resonance in the two base pairs exhibits sigmoidal behaviour. As the temperature was decreased, both C-H6 (C1, C7) signals shift - one upfield and the other downfield. Unfortunately, these proton signals could not be followed over a sufficiently wide temperature range to provide a transition temperature. The C-H5 proton resonances for the terminal base pairs moved upfield with temperature decrease but again, did not give a transition temperature. Similar to both C-H6 resonances, the G-H8 signals move in opposite

directions as a function of temperature, however only one H8 proton, from G8, gave a T_m value, which was 38.2°C. An overall T_m of 47.9°C for the helix to coil transition of the heptamer duplex was determined. The decrease of approximately five degrees in the T_m values from the core to the external base pairs of the duplex was the result of fraying of the terminal base pairs.

5.2.4 A comparison of variable temperature results for non-exchangeable aromatic protons in the hexamer and heptamer duplexes.

When compared with the hexamer mixture discussed in section 5.2.1, the addition of the core A-T base pair in the heptamer complex increased the T_m of the duplex by seven degrees. In general, many of the individual resonances in the two duplexes behave similarily, as they follow the same chemical shift trends as a function of temperature. The major differences in chemical shift for specific protons appear primarily in the core regions of the two duplexes - the extra base pair having been added in the centre of the heptamer mixture. For example, the chemical shifts of the two *G-H8 protons (G3, G5) which are separated by the central thymine base (T4) in the heptamer, are almost identical at 80°C whereas in the hexamer, Figure 49, where the two guanines are adjacent to one another (G2, G3), one H8 resonance is shielded by approximately 0.1 ppm. This diamagnetic shielding effect is the result of the purine-purine sequence and the larger diamagnetic shielding anisotropy of a purine base relative to a pyrimidine. This type of chemical shift result has been predicted by Bell *et al*. (1985) in which the calculation

of proton chemical shift were made based on strand sequence and the shielding effects of a base on its nearest and next nearest neighbours.

5.2.5 Variable temperature study of the exchangeable imino protons in the heptamer duplex.

The exchangeable imino protons involved in hydrogen bonding when base pair formation occurs were studied for the mixture of the complementary heptamers. Because there is no symmetry within the duplex, all seven imino protons are seen in the NMR. Figure 53 exhibits the spectra for the imino proton melting experiments. The assignment of specific resonance signals to terminal base pairs has been based on line broadening as a function of temperature. Those base pairs which open first and, as a result, their imino proton signals broaden and disappear at the lowest temperatures are assigned as the terminal base pairs. The imino proton signals which are visible at the highest temperatures have been assigned to core positions. The resonance signal for the central A-T base pair [4] has been assigned by the comparison of the heptamer results with those obtained for the hexamer duplex (section 5.2.2). The line widths for the different imino proton signals as a function of temperature have been calculated and the results given in Figure 54.

Below $0^{o}C$, all seven imino proton signals are visible. Two overlapping resonances at approximately 14 ppm have been assigned to the two A-T base pairs [2 and 6]. The imino resonance for the third A-T [4] base pair, which is the central base pair in the duplex, is located upfield at 13.6 ppm. The two lower field G-C imino proton signals have been assigned to the terminal base pairs [1 and 7], while the resonance

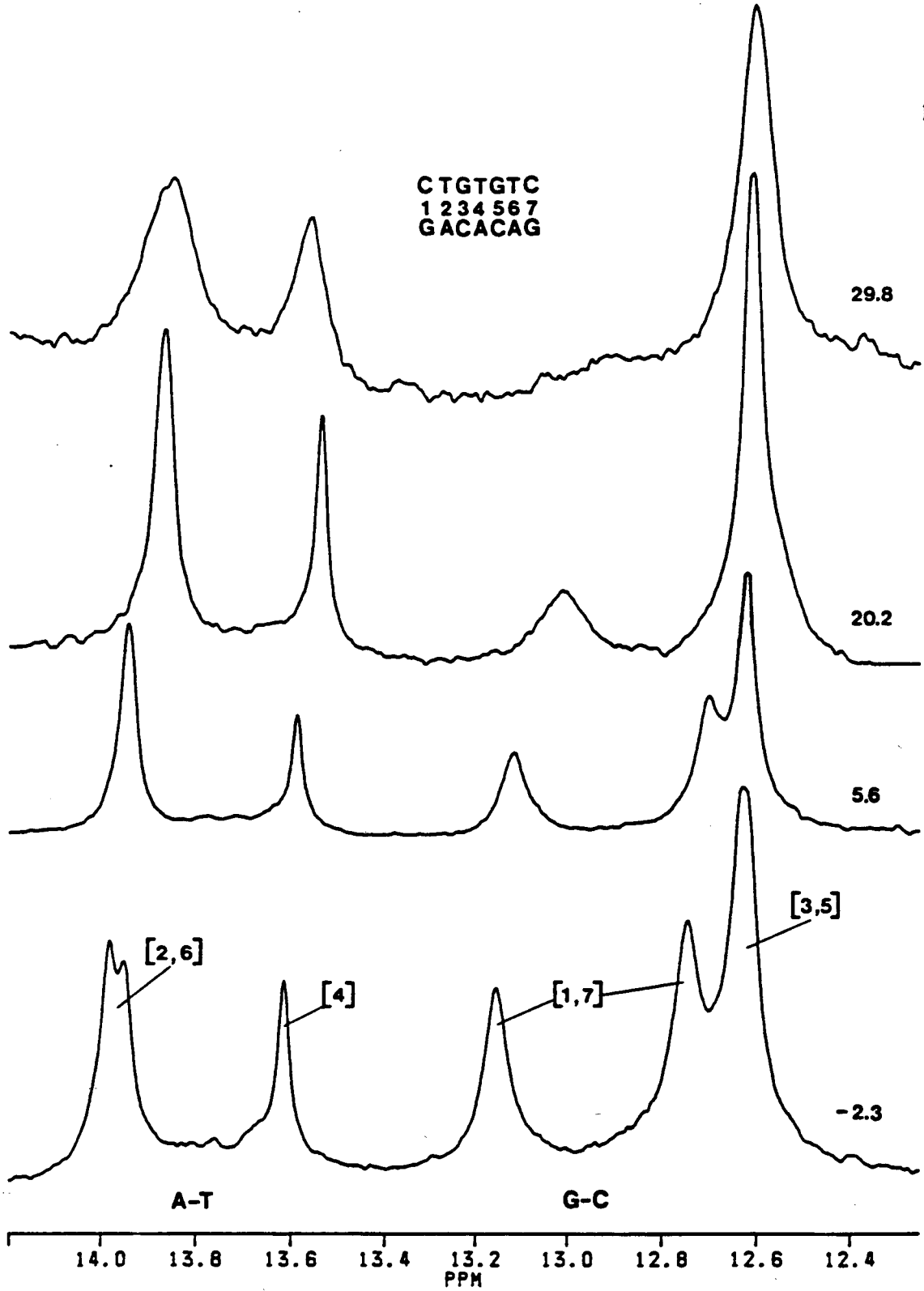


Figure 53

Exchangeable imino protons involved in base pairing as a function of temperature for the complementary heptamer mixture d(CTGTGTC) + d(GACACAG).

at 12.6 ppm contains both of the core G-C base pairs [3 and 5]. An increase in temperature resulted in the terminal G-C imino proton signals [1 and 7], and the resonances for the adjacent A-T base pairs [2 and 6] beginning to move upfield. All of the peaks associated with these protons shift approximately 0.2 ppm before broadening out. The imino protons which belong to the internal trimer core do not exhibit the variation in chemical shift as a function of temperature shown by the external base pairs. Similar to the results for the hexamer duplex, one of the terminal G-C imino proton signals begins to broaden before the other, although signal overlap prevents any direct comparison. By 30°C, both terminal G-C base pairs have disappeared along with one of the neighbouring A-T pairs. This suggests that although the duplex is unwinding from the two ends towards the middle, the stability of the base pairs i.e. A-T [2 and 6] is not the same. Again, this result is similar to that observed for the hexamer duplex and is attributed to differences in intrastrand base stacking interactions thoughout the helix, as well as solvation effects. All signals are gone by 35°C as the equilibrium between the helix and the single stranded coil structures begins to favour the open state and fast exchange of the base imino protons with the surrounding solvent occurs.

Similar conclusions are obtained from the line width versus temperature plot, Figure 54. It is evident that the line widths of the terminal G-C base pairs [1,7] increase more rapidly with increasing temperature than the line widths of the internal base pairs. One of the terminal G-C base pairs disappears above 5°C and the other not until approximately 20°C. The A-T base pairs [2,6] show no noticeable change

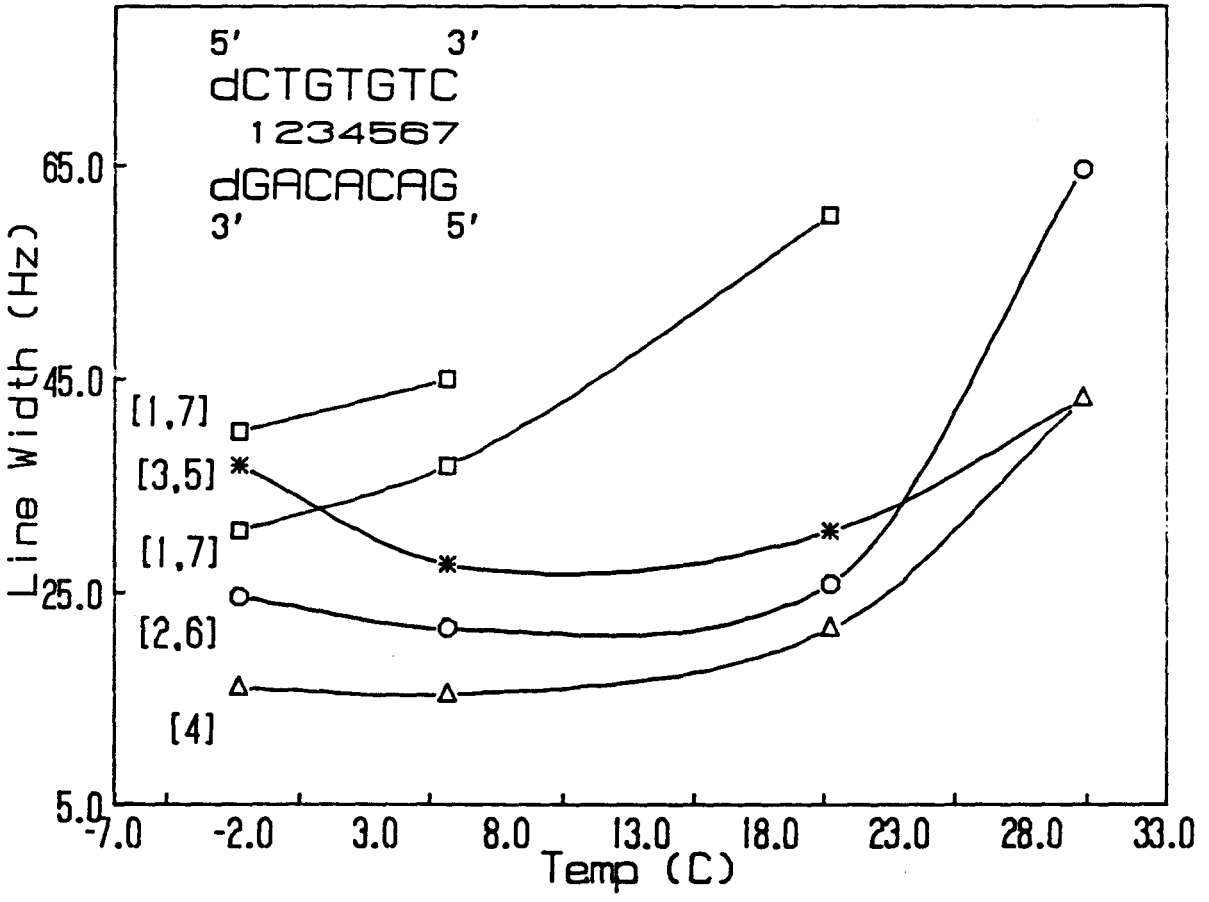


Figure 54

Line widths of exchangeable imino proton resonances as a function of temperature for the complementary heptamer mixture d(CTGTGTC) + d(GACACAG).

in line width until the terminal base pairs have opened. At this temperature, the line width curve for the these base pairs rapidly increases to a maximum at about 30°C, after which the signals disappear. In a similar fashion, the G-C [3,5] base pairs show little change in line width until the base pairs external to them have begun to broaden. The central A-T [4] base pair follows along in an almost parallel trend in line broadening as that exhibited by the neighbouring G-C base pairs.

5.2.6 d(CTGTTGTC) + d(GACAACAG).

The complementary octamers d(CTGTTGTC) and d(GACAACAG) were combined in 1:1 ratio for an overall DNA concentration of 3.0 mM. The oligomers were dissolved in 400 ul of 0.1 M sodium chloride, 0.01 M sodium phosphate pH 7.0, and 0.5 mM EDTA. The non-exchangeable aromatic protons could not be followed over the temperature range usually studied for melting experiments. The combination of multiple signal cross-overs and resonance overlaps, along with line broadening effects, prevented any reasonable interpretation of the variable temperature spectra.

5.2.7 Variable temperature study of the exchangeable imino protons for d(CTGTTGTC) + d(GACAACAG).

The exchangeable imino protons involved in base pair formation were studied. Again, as with the complementary hexamer (section 5.2.1) and heptamer systems (section 5.2.3), the lack of symmetry in the octamer duplex allowed all eight imino proton resonances to be observed. The results for the melting experiments have been plotted on Figure 55. The imino protons have been assigned to specific base pairs based on line

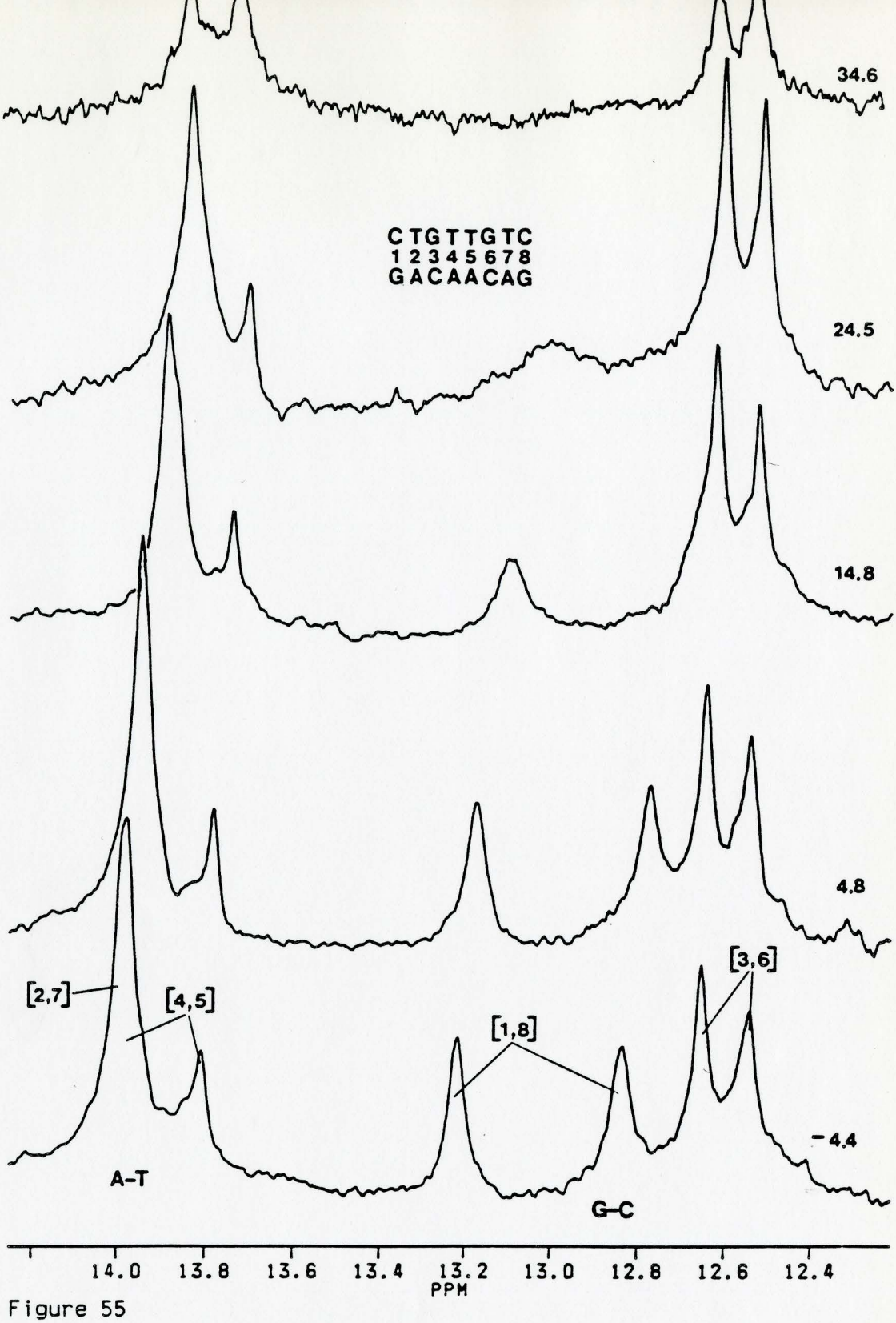


Figure 55

Exchangeable imino protons involved in base pairing as a function of temperature for the complementary octamer mixture d(CTGTTGTC) + d(GACAACAG).

broadening results, with the terminal and external base pairs broadening and disappearing at lower temperatures and the internal core signals broadening out at higher temperatures. The results of the line width measurements as a function of temperature for the different imino proton resonances are given in Figure 56.

At -5°C, the two external A-T base pairs [2 and 7], as well as one of the internal A-T base pairs, either [4 or 5], are overlapped at 13.99 ppm. The remaining internal A-T base pair is at 13.8 ppm. The two terminal G-C base pairs [1 and 8], located at 13.22 and 12.84 ppm, are at lower fields than the internal core G-C base pairs [3 and 6], found at 12.65 and 12.55 ppm. As the temperature was increased the terminal G-C imino resonances moved upfield with chemical shift changes on the order of 0.2 ppm before the resonances broadened out completely. The lowest field G-C terminal base pair broadens and disappears between 25°C and 30°C. The higher field terminal G-C resonance signal moved upfield and overlapped with the G-C core resonances as it melted. The exact region over which this signal disappeared completely was no longer discernible, but it appears to be between 20°C and 30°C. Of the two external A-T base pairs, [2 or 7], one melts between 20°C and 25°C, close behind the external G-C base pairs. The other external A-T signal did not broaden until the temperature was above 25°C. Examining the core region of the octamer duplex, base pairs [3 to 6], the G-C imino signals show little change in chemical shift (< 0.1 ppm) as a function of temperature, while the internal A-T base pairs exhibit a shift difference of between 0.15 and 0.2 ppm. By 35°C, only the imino proton resonances for the four core

base pairs remained. All signals had broadened out completely above 40°C.

As was observed for the heptamer mixing experiment, the line widths of the terminal G-C [1,8] base pairs showed a rapid increase in line width as a function of temperature, see Figure 56. Although both terminal G-C imino proton resonances appear to broaden at a similar rate, one signal disappears above 5°C while the second does not broaden out until the temperature is above 25°C. Above 5°C, the neighbouring A-T base pairs [2,7] began to gradually broaden, with the resonances broadening out above 25°C. Continuing inwards towards the core of the duplex, the G-C base pairs [3,6] showed very little line broadening until approximately 25°C. This temperature coincides with the melting of the external flanking 3' and 5' A-T base pairs. Above 25°C, the line width of the G-C base pairs [3,6] began to increase, with the resonances broadening out above 35°C. The two central A-T base pairs [4,5] exhibited a line broadening trend which paralleled that of the neighbouring G-C base pairs. This finding is similar to the results which were observed for the central A-T base pair [4] in the case of the heptamer mixture. Again, this indicates that the [-AA-][-TT-] core is not sufficiently stable on its own to hold the duplex together and, as a result, opens at approximately the same rate as the neighbouring G-C base pairs.

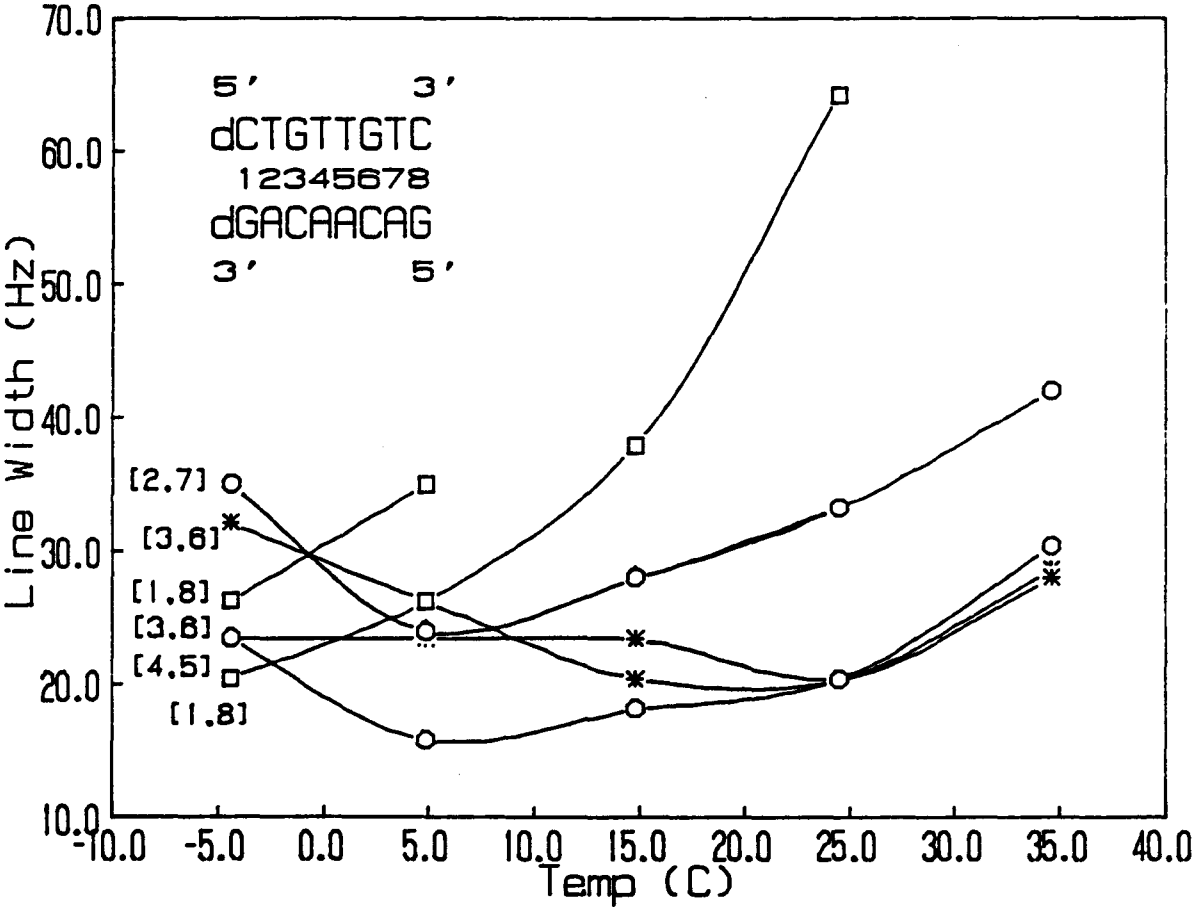


Figure 56

Line widths of exchangeable imino proton resonances as a function of temperature for the complementary octamer mixture d(CTGTTGTC) + d(GACAACAG).

5.2.8 Conclusions to the mixing experiments for fully complementary oligomers.

The mixing experiments involving the three sets of complementary deoxy oligomers resulted in several trends which appear to be common throughout the three different duplexes. These oligomers had common sequences with respect to their termini, the base additions occurring in the core of the duplexes. In general, the addition of bases to a nucleic acid sequence primarily affects the environment of the nearest neighbours 3′ and 5′ to the new base. This effect falls off rapidly with distance, with little affect noticable on the next nearest neighbours. Therefore, beginning with the dGG-CC core of the hexamer mixture (section 5.2.1), the addition of an intervening T-A base pair should show a major affect on the chemical shifts of the core G-H8 and C-H5 and H6, and to a lesser degree, the external A-T and the terminal G-C signals. The addition of a second T-A base pair to the core of the octamer mixture primarily affected its immediate nearest neighbours as was the situation with the heptamer. This type of sequence effect has been studied by several groups and empirical equations have been devised which can predict proton chemical shifts and aid in assignment of the various proton resonances (Bell et al. 1985).

A comparison of resonance signals assigned to similar protons from the melting experiments involving the non-exchangeable aromatic protons for the hexamer and heptamer mixtures, shows that many lines follow parallel patterns. The specific base protons which will be most affected by the transition from single strand to duplex, are those which are conformationally directed into the core of the helix and therefore, are

most influenced by the diamagnetic shielding anisotropy of the neighbouring bases. Models show that these protons are the pyrimidine H5 or 5-CH_3 protons and the purine H2 proton (Patel and Tonelli, 1975b; Kan et al., 1982).

In both of the two duplexes which could be monitored through their duplex melting, the central core cytosine H5 protons showed chemical shift changes of 0.4 to 0.7 ppm over the helix-coil transition. The same cytosine H5 protons, located in terminal positions in the helix, showed a smaller temperature dependence with chemical shift changes on the order of 0.1 ppm. This difference in temperature dependence reflects the fact that the terminal bases experience shielding effects from only one nearest neighbour and, is also the result of base pair fraying at the ends of the duplex. Bases occupying interior sites in the duplex have two nearest neighbours and, with the increased stability of core base pairs, experience localized changes to the magnetic environment to a greater extent. All of the thymine bases are located at internal sequence positions and the 5-CH_3 groups all exhibit similar chemical shift changes with temperature (0.2-0.4 ppm).

The base protons which are conformationally directed away from the central core of the helix, are less influenced by the diamagnetic shielding anisotropy of the neighbouring bases. As a result of this conformation, these particular protons exhibit smaller chemical shift changes between their single stranded and duplexed states. The purine H8 protons, and to a lesser extent the pyrimidine H6 protons, point into the grooves between the sugar-phosphate backbone and as a result, are more directly influenced by alterations in backbone conformation.

The comparison of similarily assigned signals in the hexamer and heptamer mixtures, for magnitude and direction of chemical shift change through the helix to coil transition, reveals little variation between the two duplexes. This observation suggests that the overall structure of the two duplexes in solution is quite similar and that the addition of the extra internal A-T unit did not substantially alter local environments of the various units throughout the duplex. The basic interpretations of structural changes, their effects on local environment, and in turn, proton chemical shift, will not completely cover the many complex motions and stuctural orientations which occur in the formation of the nucleic acid double helix.

Another similarity observed between the hexamer and heptamer mixtures for the non-exchangeable protons was the occurrence of line broadening of many signals as the mid point temperature for the helix to coil transition was approached. This effect is the result of a slow rate of exchange between the open, single stranded state and the closed duplex state with respect to the NMR time scale. The signals which have the largest chemical shift difference between the two states would be expected to exhibit the largest amount of line broadening. Once the temperature was below the transition temperature of the duplex, many of the broad signals sharpened again. This result indicates that little exchange between the two states was occurring and that the major structure in solution was that of the closed, duplexed species.

The experiments which examined the exchangeable imino protons involved in hydrogen bond formation gave some interesting results concerning the order in which the different signals broadened and

disappeared. Each of the three oligomer mixtures, the hexamers, heptamers and octamers, have common 3′ and 5′ terminal sequences. Because the mixtures of complementary sequences form normal duplexes, melting of the strands occurred from the terminal positions inward to the core base pairs. Inwards from both the 5′ and 3′ terminal base pairs the sequences were the same and, as a result, melting might be expected to occur at an equal rate from either end. However, this was not observed in any of the three systems examined, as the hydrogen bonding involved in base pairs is not the only stablizing factor involved in double helix formation. In all three cases, it appears that although melting began at the ends of the duplex at approximately the same temperature, one end of the duplex melted at a lower temperature than the other. The overall stability of the double helix is not only dependant on the hydrogen bonding involved in base pairing but also on inter and intrastrand base stacking and on solvation effects. Therefore, despite the apparent sequence similarities, the duplexes would not be expected to melt in a purely sequential fashion.

The overall T_m's for the hexamer and the heptamer were 41.0°C and 47.9°C respectively. Addition of the extra A-T base pair into the core of the heptamer increased the stability of the heptamer duplex by approximately seven degrees. This result is in line with stability predictions reported by Breslauer et al.(1986). Their results indicate that it is the base sequence, not the base composition, of an oligomer which determines the stability of the duplex. In our hexamer mixture, the core of the duplex contains the highly thermodynamically stable dimer unit, [-GG-][-CC-]. By adding the extra A-T base pair into the core of

the heptamer, [-GTG-][-CAC-], we have separated the stable dimer unit and, some of the extra stability gained in the extra base pair is in turn lost in the separation of the [-GG-][-CC-] core. The transition enthalpies were calculated according to the data listed by Breslauer, producing values of 36.7 and 38.0 kcal/mol for the hexamer and the heptamer respectively. This small difference, approximately 1 kcal/mol, between the hexamer and heptamer duplexes would suggest only a minor variation in the T_m values obtained by melting experiments. Again, the importance of pairwise interactions is evident in the results obtained from the enthalpy calculations for the octamer mixture. The transition enthalpy obtained for octamer duplex was 47.1 kcal/mol, an increase of almost 10 kcal/mol over that for the heptamer mixture. The primary reason for this result is the presence of the [-AA-][-TT-] core in the octamer which greatly aids in the stability of the duplex. Altough no T_m value was obtained for the octamer mixture from NMR experiments, the transition enthalpy value would suggest a significant increase over that obtained for the heptamer duplex.

5.3.1 Mixing Experiments with Non-Complementary Base Sequences.

Prior to carrying out the mixing experiments involving the platinated oligomers and their complementary and non-complementary oligomers, as outlined on Table 15, it was necessary to examine the non-platinated parent strands. The preceeding chapters gave results which characterized the formation and stability of the three different complementary duplexes involved in our work. This section mimics the oligomer mixtures which will be involved when the platinum derivatized strands are used, without the effects of platination. Upon completion, we will have examined the oligomer mixtures as fully complementary duplexes, as well as imperfect duplexes which contain extra internal thymine bases.

Several research groups have shown that it is possible to form a double helix between two strands which are only partially complementary and, as a result, form imperfect duplexes (Chu and Tinoco, 1983; Patel et al., 1982a; Roy et al., 1983; Woodson and Crothers, 1988). The formation of a double helix which includes a non-standard Watson-Crick opposition has also been demonstrated (Aboula-ela et al., 1985; Patel et al. 1984c; Tibanyenda et al. 1984). A few non-standard base pairs are still possible with only minor conformational changes, as is the case with the G-T wobble base pair (Early et al., 1978; Pardi et al., 1982; Patel et al., 1982b). Other base-base oppositions do not allow for base pair formation and the two bases either remain base stacked with adjacent normal base pairs or they are pushed out of the core of the duplex (Arnold et al., 1987). It is also possible to have duplex formation

occur when there is an extra base on one strand for which no opposing base exists on the other strand (Evans and Morgan, 1982;, Morden et al., 1983; Patel et al., 1982c). The extra base may be located in a terminal position on one strand, and is called a dangling base, or it may be found somewhere within the strand other than a terminal site. It is duplexes of the latter type which we are concerned with here.

5.3.2 d(CTGTGTC) + d(GACCAG) - A duplex containing one extra thymidine.

The heptamer d(CTGTGTC) , with the nucleotide units numbered sequentially from the 5' to 3' (1-7), was combined with the hexamer d(GACCAG), numbered (8-13), in a 1:1 concentration ratio to give an overall DNA concentration of 2.4 mM. The concentration of salt used in these experiments was 1.0 M NaCl, 0.01M sodium phosphate pH=7.0, and 0.5mM EDTA. Because the duplex formed in this experiment was not as stable as the fully complementary duplexes examined previously, the salt concentration was increased. The increased ionic strength would raise helix to coil transition temperature into a region which was experimentally observable. The data for the variable temperature NMR experiments for the non-exchangeable aromatic protons is given in Table 33 and the results plotted on Figure 57.

The aromatic protons on the d(CTGTGTC) strand have been designated with an asterix (*). Assignments of the various proton resonances to a particular strand have been based on 70°C chemical shift data for the oligomers prior mixing as well as calculated chemical shift values. A comparison between the heptamer d(CTGTGTC) and the hexamer d(CTGGTC), has permitted the specific assignment of the central thymine base protons in

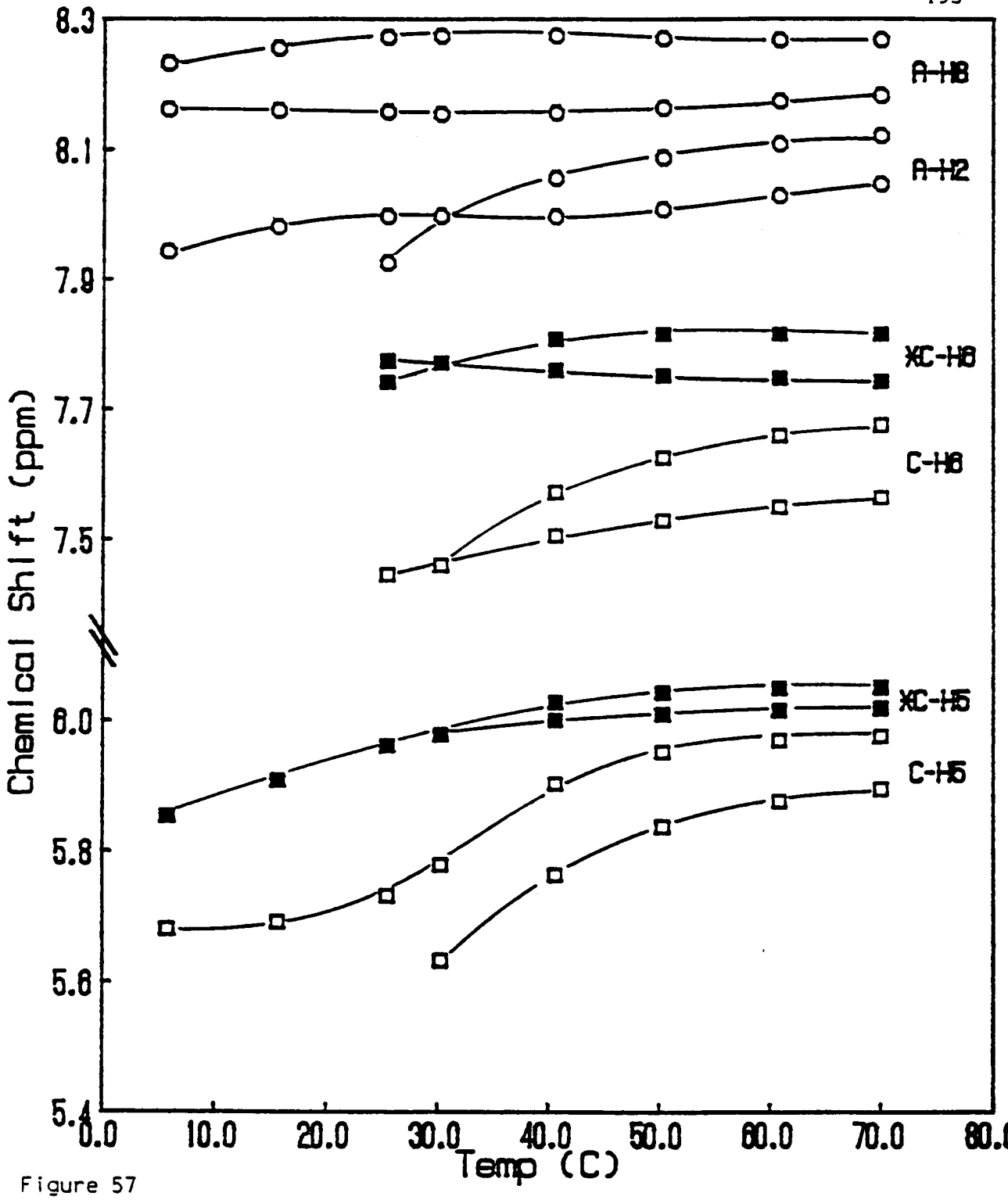


Figure 57

Chemical shift of the non-exchangeable aromatic and methyl protons as a function of temperature for the partially complementary heptamer-hexamer mixture d(CTGTGTC)* + d(GACCAG).

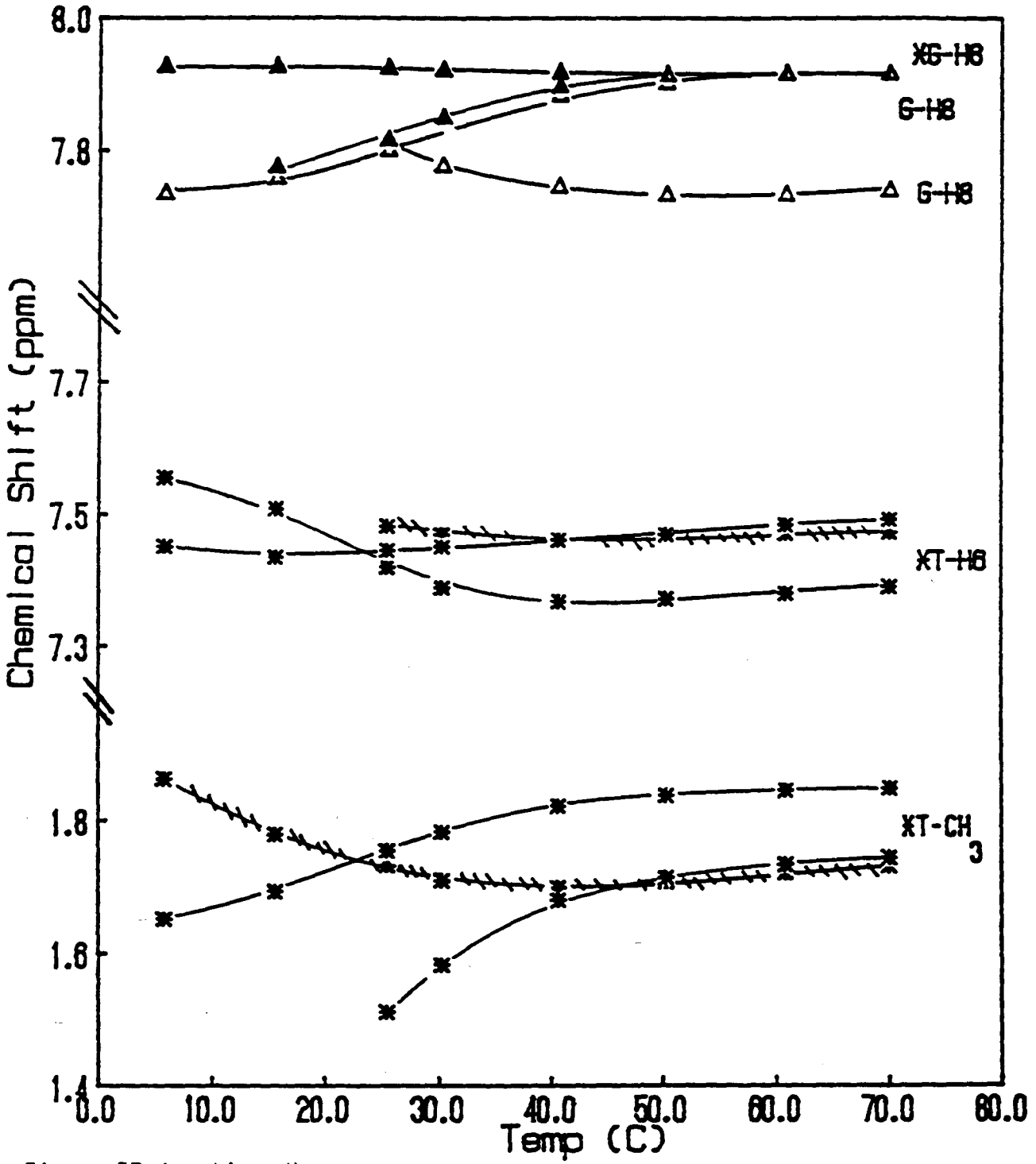


Figure 57 (continued)

Chemical shift of the non-exchangeable aromatic and methyl protons as a function of temperature for the partially complementary heptamer-hexamer mixture d(CTGTGTC)* + d(GACCAG).

TABLE 33
Chemical shift as a function of temperature;
d(CpTpGpTpGpTpC){*} + d(GpApCpCpApG),
(1-2-3-4-5-6-7) (8-9-10-11-12-13)
1.2 mM each strand, 1.0 M NaCl, 0.01 M phosphate, pH 7.

	Chemical Shift vs Temperature				
Proton	T(C) 70.0	60.8	50.3	40.6	30.2
A(12)-H8	8.266	8.266	8.268	8.272	8.273
A(9)-H8	8.182	8.172	8.162	8.155	8.153
A(9)-H2	8.119	8.106	8.085	8.054	7.996
A(12)-H2	8.045	8.027	8.007	7.996	7.996
*G(5)-H8	7.915	7.915	7.913	7.896	7.848
*G(3)-H8	7.913	7.915	7.913	7.916	7.920
G(13)-H8	7.908	7.906	7.899	7.881	-----
*C(7)-H6	7.814	7.814	7.814	7.807	7.769
*C(1)-H6	7.742	7.745	7.751	7.758	7.769
G(8)-H8	7.737	7.732	7.731	7.744	7.776
C(10)-H6	7.675	7.658	7.625	7.571	7.459
C(11)-H6	7.563	7.548	7.527	7.505	7.459
*T(6)-H6	7.492	7.483	7.468	7.461	7.449
*T(4)-H6	7.473	7.470	7.468	7.461	7.469
*T(2)-H6	7.390	7.380	7.370	7.366	7.388
*C(7)-H5	6.049	6.047	6.041	6.027	5.978
*C(1)-H5	6.018	6.014	6.008	5.999	5.978
C(11)-H5	5.975	5.968	5.949	5.903	5.777
C(10)-H5	5.893	5.876	5.836	5.762	5.631
*T(2)-CH_3	1.846	1.843	1.835	1.821	1.781
*T(6)-CH_3	1.742	1.731	1.712	1.679	1.582
*T(4)-CH_3	1.729	1.719	1.706	1.698	1.709

Proton	T(C) 25.5	15.6	5.8	T_m (C)
A(12)-H8	8.271	8.254	8.231	
A(9)-H8	8.156	8.158	8.161	
A(9)-H2	7.922	-----	-----	
A(12)-H2	7.996	7.979	7.942	
*G(5)-H8	7.814	7.772	-----	26.7
*G(3)-H8	7.922	7.924	7.925	
G(13)-H8	7.798	7.757	7.734	30.5
*C(7)-H6	7.740	-----	-----	
*C(1)-H6	7.773	-----	-----	
G(8)-H8	7.798	-----	-----	
C(10)-H6	-----	-----	-----	
C(11)-H6	7.444	-----	-----	
*T(6)-H6	7.444	7.434	7.450	
*T(4)-H6	7.481	-----	-----	
*T(2)-H6	7.418	7.508	7.555	18.0
*C(7)-H5	5.961	5.907	5.852	
*C(1)-H5	5.961	5.907	5.852	
C(11)-H5	5.730	5.690	5.679	34.4
C(10)-H5	-----	-----	-----	
*T(2)-CH_3	1.752	1.691	1.649	22.8
*T(6)-CH_3	1.510	-----	-----	
*T(4)-CH_3	1.732	1.778	1.862	av. T_m = 26.4°C

the heptamer. The assignment of the C5-methyl group and the H-6 proton on the extra thymine base in the duplex allows us to monitor the movements of this unpaired base through the helix to coil transition. At 70°C, the H-6 and 5-CH_3 protons of the central thymine base (T4) in the heptamer have chemical shifts of 7.473 ppm and 1.729 ppm respectively, and have been distinctively marked on the graph, Figure 57.

In many cases, individual protons could be monitored throughout the entire temperature range studied. As observed in the previous mixing experiments, many of the proton NMR signals exhibited line broadening effects below 30°C. Unlike the situations involving perfect duplexes, however, the broadened proton resonances did not sharpen below the T_m value for the helix to coil transition. This indicates that the duplex formed involving the extra base was not as stable as was formed with the complementary oligomers.

The adenine H-8 signals (A9, A12) exhibit normal behaviour for purine H-8 protons in that they show little chemical shift change as a result of temperature variation and duplex formation. The H-2 resonances for the two adenines exhibit a chemical shift change with a decrease in temperature. The lowest field H-2 signal (A9) began to show sigmoidal behaviour as a function of temperature, but broadened out before the base of the curve was achieved. The adenine (A12) H-2 signal did not exhibit sigmoidal behaviour, with an overall chemical shift change of only 0.1 ppm over the 70° temperature range. A comparison of these signals with similar resonances in the fully complementary hexamer mixing experiment, (Figure 49), shows only minor changes in the behaviour of the adenine aromatic signals in the both systems. The only difference between the

two duplexes is an extra thymidine unit in the non-complementary mixture and this extra nucleotide is separated by one base pair from the two adenines. This result illustrates how the structural and conformational effects of the extra base are localized and how this affect falls off rapidly with distance.

Continuing to focus on the hexamer strand in the non-complementary mixture at 70°C, the H-8 resonances of two terminal guanine bases (G8, G13) were well separated, with chemical shifts of 7.737 ppm and 7.908 ppm. As the temperature was decreased, the two signals moved in opposite directions, with the lowest field resonance (G13) moving upfield and the higher field signal (G8) moving downfield. The G(13)-H8 signal exhibited sigmoidal behaviour as a function of temperature, yielding a T_m of 30.5°C. The higher field G(8)-H8 signal appeared to have partial sigmoidal behaviour. However, this signal broadened out below 25°C leaving the sigmoidal curve incomplete. Again comparing these signals to similar resonances in the complementary hexamer system, (Figure 49), the terminal guanine bases appear to show little change as a result of the extra central thymine base on the opposite strand. As was the case with the adenine signals, this result suggests that the effect of the extra base is localised and dissipates quickly with distance from the altered region.

The cytosine bases on the hexamer strand (C10, C11) are nearest neighbours to the extra thymidine nucleoside on the opposite strand and would therefore be expected to show structural differences when compared with the complementary hexamer system. The two H-6 signals exhibited an upfield movement with a decrease in temperature, the C(10)-H6 resonance

moving more rapidly upfield than the C(11)-H6 signal. Again, line broadening effects did not allow the signals to be followed over the entire temperature with the resonances disappearing below 30°C. Comparing these signals to similarily assigned resonances in the hexamer system, it is evident that the trends in chemical shift for the cytosine H-6 protons in the two systems are similar except for the line broadening effects observed in the non-complementary system. This result suggests that even the nearest neighbours to the extra thymidine moiety are not significantly affected by the extra base on the heptamer strand.

Both of the two cytosine H-5 signals moved upfield with decreased temperature. The lowest field signal exhibited sigmoidal behaviour, while the higher field resonance line broadened below 30°C. The C(11)-H5 proton gave a T_m value of 34.4°C. A comparison of the cytosine H-5 resonances to their complements in the hexamer mixture shows almost identical behaviour with the exception of the line broadening effect. Again, this result suggests that the presence of the extra thymidine nucleotide has little affect with regards to structural changes when comparing the similar hexamer strands in the two different systems.

Concentrating on the heptamer strand in this bulged base system, we examined the behaviour of the non-exchangeable aromatic protons on the individual bases from the terminal sites inward to the extra thymine base. Comparisons are made to the similar strand in the complementary heptamer mixture, (section 5.2.3), as well as to the same strand minus the central thymidine in the hexamer mixture system, (section 5.2.1).

Both of the H-6 protons on the two terminal cytosine bases (C1, C7) had their resonance signals broaden below 25°C so that the curves for the

two protons could not be completed over the entire temperature range. The two H-6 resonances are less shielded, when compared with similar protons on the hexamer strand in this non-complementary mixture. The reason for this difference in shielding is because the cytosines located at terminal positions are shielded by only one neighbouring base. The cytosines, in the hexamer strand, are located in the interior of the sequence and have both 3' and 5' neighbours and as a result experience a greater amount of diamagnetic shielding. Comparing the cytosine H-6 signals to those in the same sequence used in the heptamer mixture, a small variation in behaviour is evident. In the heptamer mixture, (Figure 52), the cytosine H-6 protons exhibited a parallel temperature effect to each other, with their differences in chemical shift remaining fairly constant over the temperature range studied. In the hexamer mixture, (Figure 49), the terminal cytosine H-6 protons exhibited the same trend in chemical shift with temperature as was observed for the heptamer strand in the mixed system.

Examining the H-5 protons for the same cytosine bases in the three systems shows little change in chemical shift behaviour as a function of temperature between the various mixtures. In the bulged base system, the two terminal cytosine H-5 protons (C1, C7) show a slow upfield trend with a decrease in temperature. Neither of the signals exhibited sigmoidal behaviour. This may result from fraying of the terminal base pairs, i.e. the terminal cytosine bases and their complememts on the opposite strand do not complete the transition from single stranded coil to double helix. The same cytosine protons in the complimentary heptamer mixture system could not be followed over the entire temperature range. As observed in

the non-complementary mixture, these proton resonances slowly moved upfield as the temperature was decreased. The terminal cytosine residues in the hexamer mixture exhibited a similar trend. Overall, the terminal cytosine bases in all three systems exhibited similar chemical shift behaviour as a function of temperature. This observation suggests that the dominant factor operating with the terminal base pairs is fraying, and that variation of sequence in the interior of the oligomer has little effect at the terminal sites.

Moving inwards from the terminus of the duplex from both the 5′ and the 3′ directions, thymine bases (T2, T6) are found which have complements present on the opposite strand. A comparison of the complementary hexamer and heptamer systems, and the hexamer-heptamer mixture, along with calculated chemicla shift values, allows the differentiation of these two thymine bases from the central thymine (T4) on the heptamer strand. The central thymine base proton resonances have been marked on Figure 57.

The H-6 resonance of the (T6) thymine base exhibited a change in chemical shift as a function of temperature of less than 0.1 ppm, while the T(2)-H6 resonance moved downfield 0.16 ppm. A comparison of these signals with the same thymine protons in the complementary heptamer system as well as the two thymines in the complementary hexamer mixture, it is evident that the observed trend in chemical shift as a function of temperature is similar in all three systems. However, the best correlation was again with the hexamer system.

For the thymine (T2, T6) methyl groups in the bulged base system, their resonances moved upfield with a decrease in temperature. The lower

field methyl group of the two (T2) exhibited sigmoidal behaviour and gave a T_m of 22.8°C. A comparison of these curves with those obtained for similar thymine methyl groups in the complementary heptamer and hexamer systems again showed similar results, with differences between the three duplexes being negligible.

Guanine bases (G3, G5) are located inwards from the thymine bases in both the 5′ and 3′ directions. These guanines are also 3′ and 5′ nearest neighbours to the bulged central thymine base. At 70°C, the H-8 proton resonance signals, for both guanine bases, were overlapped. As the temperature was decreased, one of the H-8 signals moved upfield through a sigmoidal transition, with a T_m of 26.7°C. The second guanine H-8 signal showed little change in chemical shift (less than 0.05 ppm) with temperature variation. A comparison of these resonances to the complementary hexamer and heptamer systems shows a similar temperature effect in the hexamer mixture. However, the heptamer mixture shows that neither of the guanine H-8 proton resonances has a significant temperature dependence. One signal shows a minor downfield shift with temperature decrease while the second H-8 signal could not be followed below 30°C.

The remaining nucleotide unit on the heptamer strand, which does not have an opposing complementary base on the hexamer oligomer, is the central thymidine unit (T4). If the hexamer and heptamer strands in the mixing experiment form normal Watson-Crick base pairs, this leaves the central thymine base without an opposing base with which to hydrogen bond, either in a Watson-Crick type fashion or otherwise. If this is the situation, the extra thymine base has the option of existing either

inside or outside of the double helix, or in an equilibrium between the two possible conformations upon duplex formation.

The H-6 and 5-CH_3 proton resonances of the central thymine base have been specifically assigned and are the thymine H-6 at 7.473 ppm and 5-CH_3 resonance at 1.729 ppm at 70°C. Following the H-6 proton from 70°C, the chemical shift remained fairly constant to approximately 30°C, at which point it moved slightly downfield before broadening out beyond recognition. In a similar fashion, the chemical shift of the methyl group resonance on the same thymine base reacted to the decrease in temperature with a downfield shift beginning around 40°C and becoming more deshielded with temperature drop. This base is not present in the complementary hexamer mixture and therefore no comparison can been made. However, this central thymine base is present in the complementary heptamer mixture and comparison of these results show large differences between the two systems at this site. In the heptamer mixture, (Figure 52), the central thymine base was involved in normal base pairing. Both the H-6 and the 5-CH_3 protons experienced increased shielding as the central A-T base pair stacked between adjacent base pairs with duplex formation. This behaviour is contrary to the observed result in the bulged base mixture where the central thymine base became more deshielded with duplex formation. These observations indicate that the extra thymine base does not remain within the base-stacked core of the helix. Instead, the thymine (T4) has been pushed out of any base stacked conformation to form a structure in which the extra base is no longer influenced by the diamagnetic shielding anisotropy of its nearest neighbours, Figure 58. This conclusion further illustrates why the

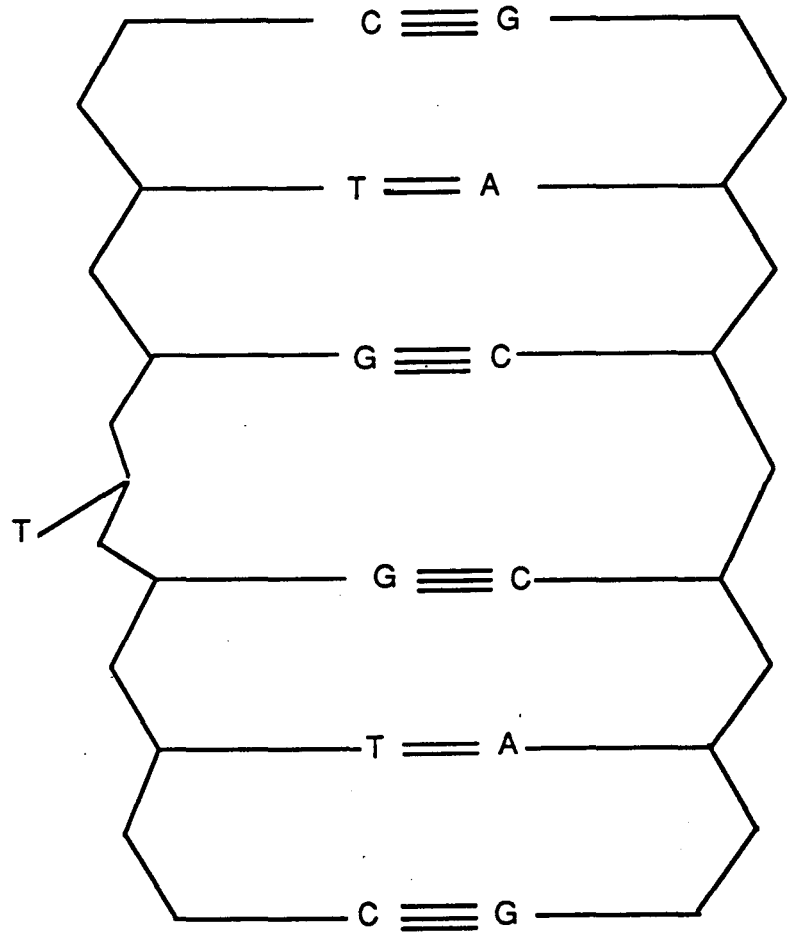


Figure 58

Schematic of proposed base pairing involved in heptamer-hexamer duplex containing central bulged out thymidine base.

behaviour of many of the protons closely resemble the trends observed in the complementary hexamer system throughout the duplex to coil transition, as discussed above. The heptamer-hexamer mixture essentially mimicks the hexamer mixing experiment by bulging the extra thymine base out of the duplex, and forming a hexamer-like double helix which is as close to a normal helix as possible.

5.3.3 Conclusions to variable temperature studies of the non-exchangeable aromatic protons of d(CTGTGTC) +d(GACCAG).

In the heptamer-hexamer mixing experiment, twenty-two protons have been monitored throughout the coil to helix transition, with only five of these signals exhibiting sigmoidal behaviour and yielding T_m values. The average T_m for the duplex was 26.4°C in 1.0 M salt. A comparison of this helix to coil transition temperature to the average T_m value for the complementary hexamer system, where a T_m of 41.0°C was obtained in 0.1 M salt, illustrates the destablising effect of the extra bulged thymine base. This large decrease in the T_m (-14.6°C) of the bulged base duplex is in agreement with published work in which the most unstable duplexes were shown to be those which contain an extra nucleotide in one strand (Aboul-ela et. al. 1985).

Similar experiments have been carried out which incorporate an extra nucleotide unit into a duplex structure. The extra-helical thymine agrees with results observed where an extra cytosine was present and was shown to exist outside of the stacked duplex (Morden et al., 1983). When an extra adenine was inserted into the core of the duplex without an opposing base, results indicated that the extra base remained stacked

within the core of the duplex and contibuted to the overall base stacking structure of the double helix (Patel et al. 1982c; Pardi et al. 1982). These results are in agreement with the variation in stacking ability of purine and pyrimidine bases.

Researchers have also examined non-complementary base-base oppositions. In these situations, the bases were either unable to base pair or were found to form non-Watson-Crick wobble base pairs (Patel et al. 1984c, 1982b). One system, which contained a T-T opposition in the double helix, revealed that the two opposing thymine bases did not form a hydrogen bonded base pair. However, the two thymine bases remained stacked within the core of the duplex and did not significantly disturb the helical structure (Mellema et al., 1984). With the two opposing thymine bases, both strands have the same number of sugars and phosphates along their respective backbones. Mellema's results illustrate how a duplex can form and maintain a constant winding angle while incorporating the non-hydrogen bonded thymine. These results contrast those which were observed in our work for the single extra thymine base, which was bulged out from the duplex core. With the single extra base, the two sugar-phosphate backbones are of different lengths, making a consistent winding angle difficult. Rather than incorporate the extra nucleotide unit into the duplex, the thymidine is ignored, with normal helical structures on either side of the bulge.

5.3.4 Variable temperature study of the exchangeable imino protons for d(CTGTGTC) + d(GACCAG).

The exchangeable imino protons involved in the hydrogen bonds in base pair formation have been examined using the same sample for the bulged base system in a 90:10 H_2O:D_2O solvent mixture. The results for the melting experiment are plotted on Figure 59. The imino proton resonance line widths as a function of temperature for the duplex have been determined and the results plotted on Figure 60.

Figure 59 shows three major chemical shift regions for the imino protons: the A-T base pairs centred around 14.2 ppm; the G-C base pairs around 13.0 ppm; and the N(3)-H proton in the bulged thymine base around 11.2 ppm. The thymine N(3)-H on the extra bulged base was seen even though it was not involved in hydrogen bonding. This observation is similar to the results obtained for the single stranded platinated oligomers which contained internal, bulged thymine bases as a consequence of chelation of the strand by cis-$Pt(NH_3)_2Cl_2$. The presence of the non-hydrogen bonded imino resonance indicates a steric hinderance to exchange of the N(3) proton with solvent. The rate of proton exchange has been slowed sufficiently for the proton to be observed by NMR. Haasnoot and coworkers (1980) have observed similar resonances for thymine bases which have been looped out of a duplex in the formation of small hairpin loops.

The spectra for the bulged base mixing experiments differ in several ways when compared to those spectra obtained for complementary oligomer mixing experiments. The major and most important difference is that as the temperature was increased, the bulged base system did not exhibit the sequential opening of base pairs from the terminal positions inwards to

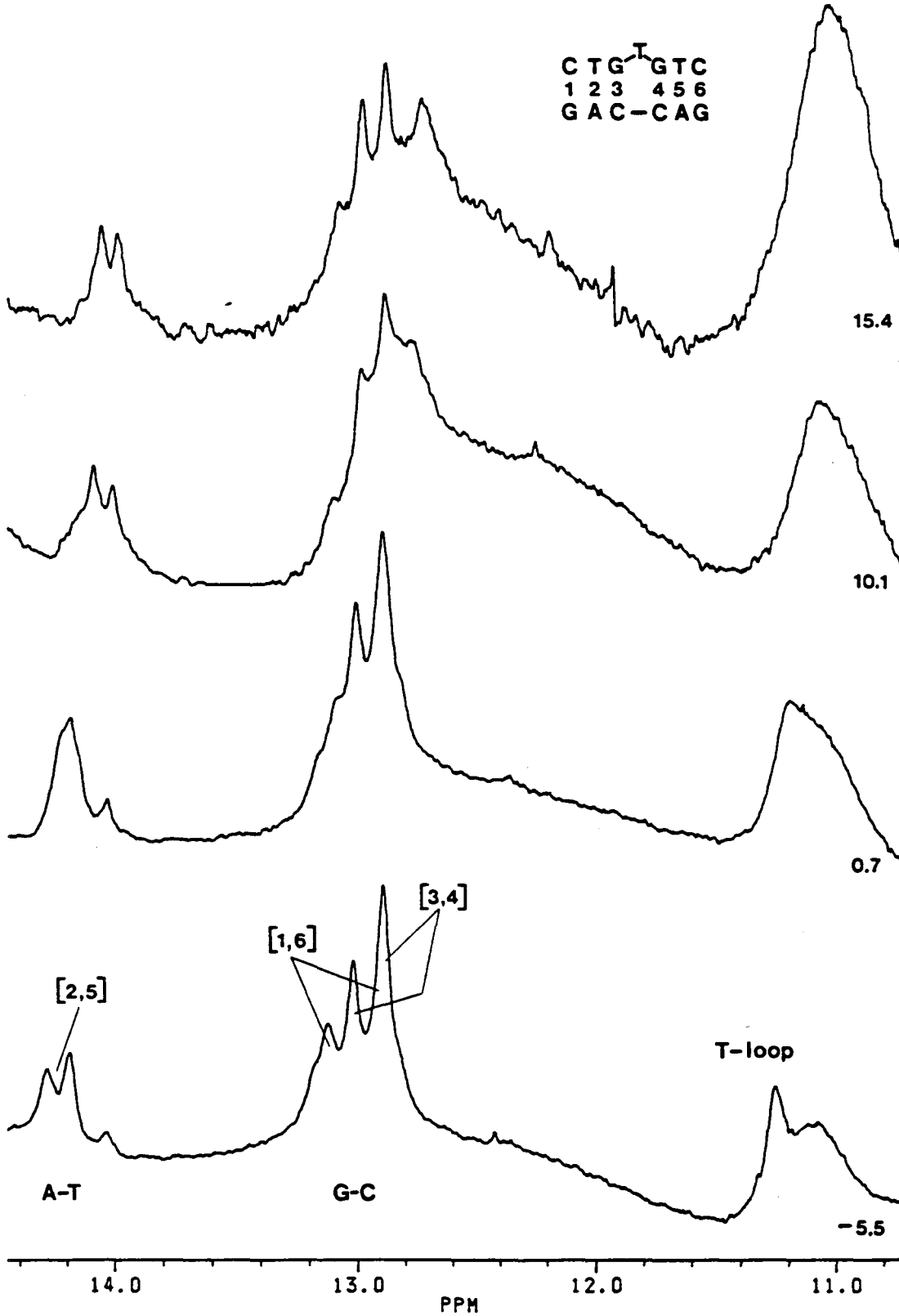


Figure 59

Exchangeable imino protons involved in base pairing and extra thymine bulge as a function of temperature for the partially complementary heptamer-hexamer mixture d(CTGTGTC) + d(GACCAG).

the core of the helix. All of the normal Watson-Crick base pairs were visible at -5.5°C and underwent line broadening as the temperature was increased. Unlike the complementary mixing experiments, all imino signals in the bulged base system disappeared simultaneously above 15°C, rather than in the normal sequential fashion. Because of the non-sequential order of base pair opening, assignment of the imino protons to a specific base pair could not be made. As a result, the assignment of the imino protons have been made by the comparison of the -5.5°C spectrum for the bulged base system with similar spectra in the complementary hexamer and heptamer mixtures. The explanation for the non-sequential duplex opening reflects the presence of the bulged thymine base, which has added internal ends to the helix. As a result, rather than having fraying of the base pairs occurring only at the terminal ends of the duplex, base pair fraying also takes place with the base pairs which are adjacent to the thymine bulge. The effect of this extra source of duplex instability is that helix begins to unwind not only from the ends of the duplex inwards towards the core, but also from the bulged region outwards. When the G-C base pairs [1,3,4,6] opened, the entire duplex disintegrated at once and did not go through the normal, cooperative melting transition.

The non-cooperative melting transition experienced by the bulged base duplex is also seen in the plot of imino resonance line widths as a function of temperature, Figure 60. The plot does not exhibit the normal sequential broadening of the signals as a function of temperature, as was observed for the fully complementary duplexes (Figures 51. 54, 56). Although not identical, the rates of line broadening do show a trend of

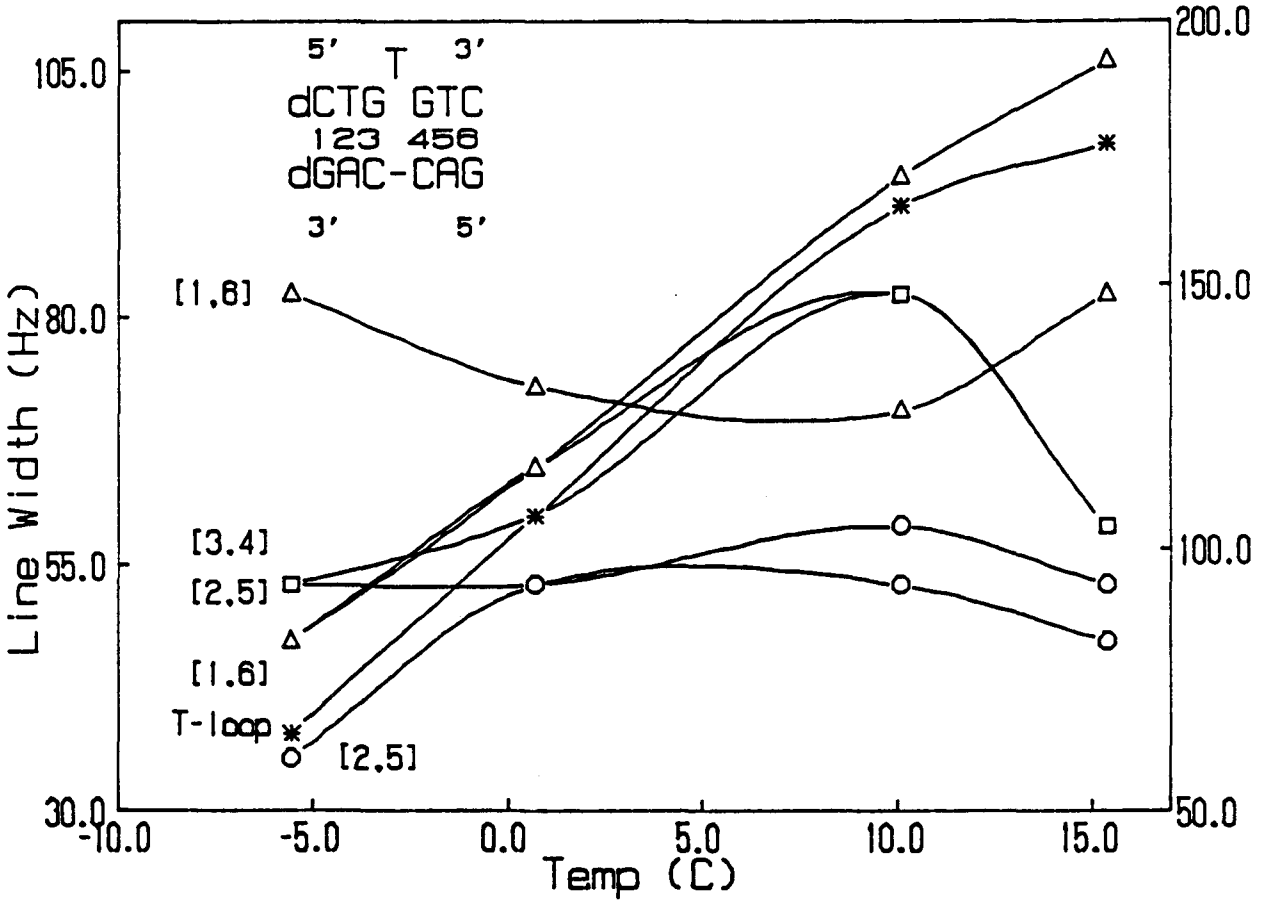


Figure 60

Line widths of exchangeable imino proton resonances as a function of temperature for the partially complementary heptamer-hexamer mixture d(CTGTGTC) + d(GACCAG).

broadening of both the terminal G-C base pairs [1,6] and the internal G-C base pairs [3,4] with increased temperature. The internal A-T base pairs [2,5] did not broaden significantly over the melting experiment. This suggests that both the internal and terminal G-C base pairs opened at approximately the same rate, leaving only the two well separated A-T base pairs to hold the duplex together. Once the four G-C pairs were open, the two remaining A-T base pairs opened quickly before they could go through a normal melting transition. Therefore, the helix to coil transition in the case of the bulged base mixing experiment was non-cooperative.

The imino proton line width of the central, looped out thymine N(3)-H resonance, showed a steady rise with increasing temperature. This result reflects an increase in the mobility of the looped out thymine base accompanied by a more efficient interaction between the exchangeable base proton and solvent molecules. The consistently broadening line width is an indication of the increasing rate of exchange of the thymine N(3)-H proton with the surrounding solvent.

5.3.5 Mixing experiments involving the partially complementary oligomers d(CTGTTGTC) + d(GACACAG).

In this set of mixing experiments the octamer d(CTGTTGTC), nucleotide units numbered 5'-3' (1-8), was combined with the heptamer d(GACACAG), numbered in the same fashion (9-15), in a 1:1 ratio at an overall DNA concentration of 3.0 mM in 1.0 M NaCl, 0.01 M sodium phosphate pH 7.0, and 0.5 mM EDTA. As was the case in the previous mixing experiments, the two strands are not completely complementary in that the octamer contains an extra thymidine nucleotide in the core of its sequence. Unlike the previous situation, however, there is a single adenine base (A12) located in the centre of the shorter strand which, in this mixing experiment, will have the choice of forming a base pair with either of the two thymine bases located in the centre of the octamer, (T4) or (T5).

5.3.6 Variable temperature studies of the non-exchangeable aromatic protons.

The non-exchangeable aromatic protons as well as the methyl groups on the thymine bases were monitored over the temperature range 5°C-70°C. Not all of the proton resonances could be followed over the entire temperature range because of line broadening problems. As the mixture of the two partially complementary oligomers approached the apparent transition temperature for the duplex, several of the proton signals broaden out. Unlike the fully complementary duplex mixtures, (section 5.2), which exhibited line broadening effects through the transition temperature but whose resonances sharpened up once the temperature was

below the mid-point temperature, most of the signals involved in this imperfect duplex did not return to being sharp signals. The data for the melting experiments has been given in Table 34 and the results have been plotted on Figure 61.

Examination of the various individual proton resonances reveals several new results not observed in the previous examples. In all of the duplexes which have been studied in this work, the adenine H-8 signals have shown little or no alteration in chemical shift as a result of temperature change. This was anticipated as the purine H-8 protons are conformationally directed out from the core of the duplex and, therefore, do not experience the diamagnetic shielding effect from neighbouring bases in the core of the helix. As a result of having only a small change in chemical shift between the single stranded and the double stranded states, the adenine H-8 signals have not shown any line broadening. In the octamer-heptamer mixture, two (assigned as A12, A14) of the three adenine H-8 resonances melt in what has been seen to be a typical pattern. The third adenine H-8 signal (A10), which, at 70°C, is the lowest field of the three - did not exhibit "normal" behaviour. The signal showed little chemical shift change down to 25°C, where the resonance split into two signals, each with only half the intensity of the original resonance. Below 20°C the signal was no longer visible and did not show any signs of recovering at 5°C. This region of the ^{1}H NMR spectrum is shown over several temperatures in Figure 62.

The corresponding adenine H-2 resonances would be expected to provide useful information for interpreting what was occurring in the central region of the duplex being formed. The three adenine H-2 signals

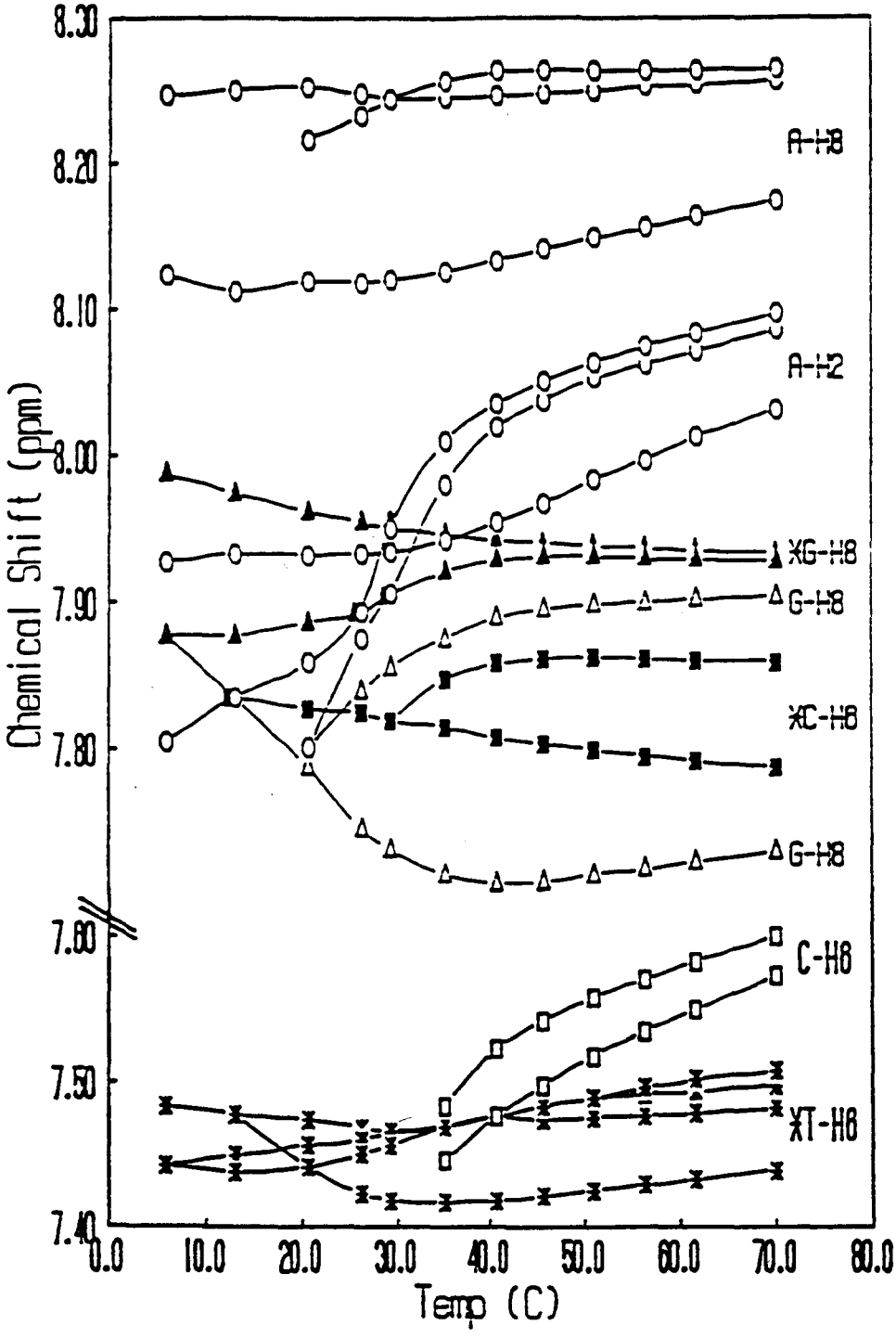


Figure 61

Chemical shift of the non-exchangeable aromatic and methyl protons as a function of temperature for the partially complementary octamer-heptamer mixture d(CTGTTGTC)* + d(GACACAG).

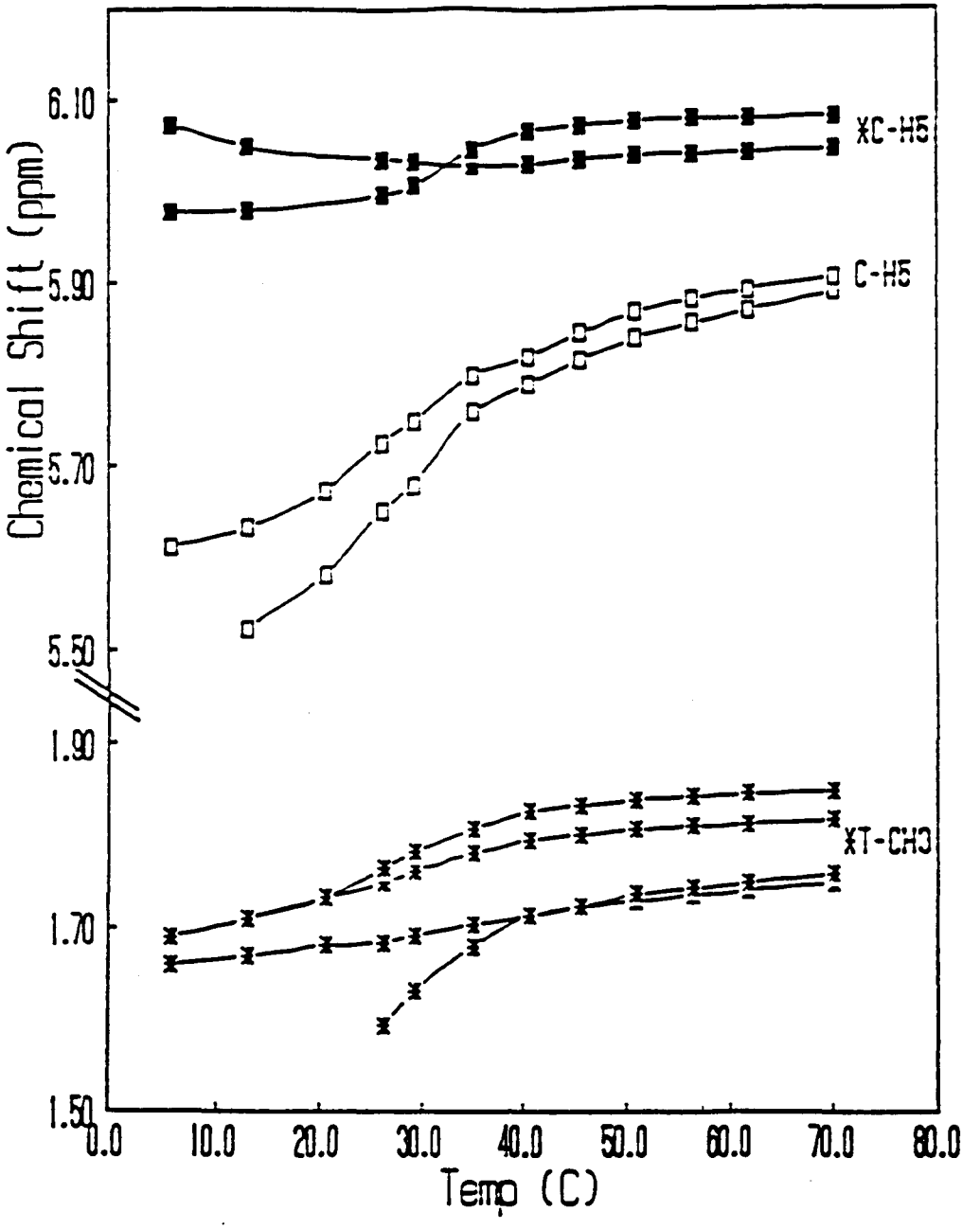


Figure 61 (continued)

Chemical shift of the non-exchangeable aromatic and methyl protons as a function of temperature for the partially complementary octamer-heptamer mixture d(CTGTTGTC)* + d(GACACAG).

TABLE 34

Chemical shift as a function of temperature;
d(CpTpGpTpTpGpTpC){*} + d(GpApCpApCpApG),
(1-2-3-4-5-6-7-8) (9-10-11-12-13-14-15)
1.5 mM each strand, 1.0 M NaCl, 0.01 M phosphate, pH 7.

Chemical Shift vs Temperature

Proton	T(C)	70.0	61.6	56.2	50.8	45.6
A(10)-H8		8.265	8.264	8.264	8.264	8.264
A(14)-H8		8.257	8.254	8.253	8.250	8.248
A(12)-H8		8.175	8.164	8.156	8.149	8.141
A(14)-H2		8.097	8.083	8.074	8.063	8.050
A(10)-H2		8.085	8.071	8.062	8.052	8.037
A(12)-H2		8.031	8.012	7.997	7.983	7.967
*G(6)-H8		7.933	7.935	7.937	7.938	7.940
*G(3)-H8		7.928	7.929	7.930	7.931	7.931
G(15)-H8		7.904	7.902	7.900	7.898	7.895
*C(8)-H6		7.859	7.860	7.861	7.862	7.861
*C(1)-H6		7.787	7.791	7.795	7.799	7.803
G(9)-H8		7.731	7.724	7.719	7.715	7.710
C(11)-H6		7.600	7.583	7.571	7.558	7.542
C(13)-H6		7.573	7.550	7.535	7.518	7.498
*T(7)-H6		7.508	7.502	7.496	7.489	7.484
*T(4)-H6		7.498	7.494	7.492	7.489	7.484
*T(2)-H6		7.482	7.479	7.477	7.476	7.474
*T(5)-H6		7.440	7.434	7.430	7.426	7.422
*C(8)-H5		6.082	6.081	6.080	6.077	6.073
*C(1)-H5		6.046	6.043	6.041	6.039	6.035
C(13)-H5		5.906	5.892	5.881	5.867	5.845
C(11)-H5		5.889	5.869	5.855	5.839	5.816
*T(2)-CH_3		1.849	1.846	1.842	1.839	1.833
*T(5)-CH_3		1.818	1.813	1.810	1.807	1.801
*T(7)-CH_3		1.758	1.749	1.742	1.736	1.723
*T(4)-CH_3		1.749	1.741	1.735	1.729	1.723

TABLE 34 (continued)

Chemical shift as a function of temperature;
d(CpTpGpTpTpGpTpC){*} + d(GpApCpApCpApG),
(1-2-3-4-5-6-7-8) (9-10-11-12-13-14-15)
1.5 mM each strand, 1.0 M NaCl, 0.01 M phosphate, pH 7.

Chemical Shift vs Temperature

Proton	T(C) 40.6	35.1	29.1	26.2	20.5
A(10)-H8	8.263	8.257	8.244	8.233	8.216
A(14)-H8	8.246	8.245	8.244	8.247	8.252
A(12)-H8	8.134	8.126	8.120	8.118	8.119
A(14)-H2	8.035	8.009	7.950	7.893	7.858
A(10)-H2	8.019	7.980	7.905	7.875	7.800
A(12)-H2	7.954	7.942	7.934	7.933	7.932
*G(6)-H8	7.942	7.946	7.950	7.954	7.961
*G(3)-H8	7.929	7.921	7.905	7.893	7.886
G(15)-H8	7.889	7.875	7.855	7.839	7.800
*C(8)-H6	7.858	7.847	7.819	-----	-----
*C(1)-H6	7.807	7.814	7.819	7.824	7.827
G(9)-H8	7.709	7.715	7.732	7.746	7.787
C(11)-H6	7.523	7.484	-----	-----	-----
C(13)-H6	7.477	7.446	-----	-----	-----
*T(7)-H6	7.477	7.470	7.457	7.451	7.442
*T(4)-H6	7.477	7.470	7.466	7.461	7.457
*T(2)-H6	7.477	7.470	7.466	7.469	7.474
*T(5)-H6	7.419	7.418	7.419	7.423	7.442
*C(8)-H5	6.067	6.045	6.006	5.997	-----
*C(1)-H5	6.030	6.026	6.032	6.035	-----
C(13)-H5	5.819	5.798	5.749	5.725	5.673
C(11)-H5	5.789	5.757	5.679	5.650	5.581
*T(2)-CH_3	1.826	1.807	1.783	1.764	1.733
*T(5)-CH_3	1.795	1.781	1.761	1.749	1.733
*T(7)-CH_3	1.713	1.680	1.631	1.594	-----
*T(4)-CH_3	1.713	1.704	1.692	1.684	1.681

TABLE 34 (continued)

Chemical shift as a function of temperature;
d(CpTpGpTpTpGpTpC){*} + d(GpApCpApCpApG),
(1-2-3-4-5-6-7-8) (9-10-11-12-13-14-15)
1.5 mM each strand, 1.0 M NaCl, 0.01 M phosphate, pH 7.

Chemical Shift vs Temperature

Proton	T(C) 12.8	5.8	T_m
A(10)-H8	-----	-----	
A(14)-H8	8.250	8.246	
A(12)-H8	8.112	8.123	
A(14)-H2	7.835	7.804	28.5
A(10)-H2	-----	-----	
A(12)-H2	7.933	7.927	
*G(6)-H8	7.974	7.987	
*G(3)-H8	7.877	7.877	28.5
G(15)-H8	-----	-----	
*C(8)-H6	-----	-----	
*C(1)-H6	7.835	-----	
G(9)-H8	7.835	7.877	
C(11)-H6	-----	-----	
C(13)-H6	-----	-----	
*T(7)-H6	7.438	7.443	
*T(4)-H6	7.450	7.443	
*T(2)-H6	7.478	7.484	
*T(5)-H6	7.478	-----	
*C(8)-H5	5.979	5.976	31.2
*C(1)-H5	6.048	6.073	
C(13)-H5	5.632	5.611	28.5
C(11)-H5	5.522	-----	28.3
*T(2)-CH_3	1.710	1.690	
*T(5)-CH_3	1.710	1.690	
*T(7)-CH_3	-----	-----	
*T(4)-CH_3	1.669	1.660	

average T_m = 29.0°C

exhibited a much larger temperature dependence than was observed for the H-8 resonances. The lowest field H-2 signal (A14) could be followed over the entire melting range and exhibited sigmoidal behaviour with a T_m value of 28.5°C. The remaining two H-2 resonances also showed changes in their chemical shift as a function of temperature, both moving upfield as the temperature was decreased. Unfortunately, both of these signals, one of which has been assigned to the central adenosine moiety on the heptamer strand, broaden out below twenty-five degrees. This is not unusual behaviour for adenine H-2 signals but this result does not yield further structural information on the orientation in the core of the duplex.

Also involved in the core region of the duplex are two of the four thymine bases located on the octamer strand. The four thymine H-6 signals were crowded together and exhibited little change in chemical shift as a function of temperature. One of the thymine H-6 resonances, which has been assigned as one of the core thymidine nucleosides (T5), 7.440 ppm at 70°C, did show a slight downfield shift as the temperature was decreased. The remaining three thymine H-6 resonances remained closely grouped together with little chemical shift change throughout the helix to coil transition. The four thymine methyl groups did exhibit some interesting temperature effects in that one or two of the methyl resonances abruptly changed appearance around the transition temperature region. The alkyl region of the ^1H NMR spectra has been provided in Figure 62 for the temperatures of interest. None of the four methyl resonances exhibited downfield shifts as the temperature was decreased.

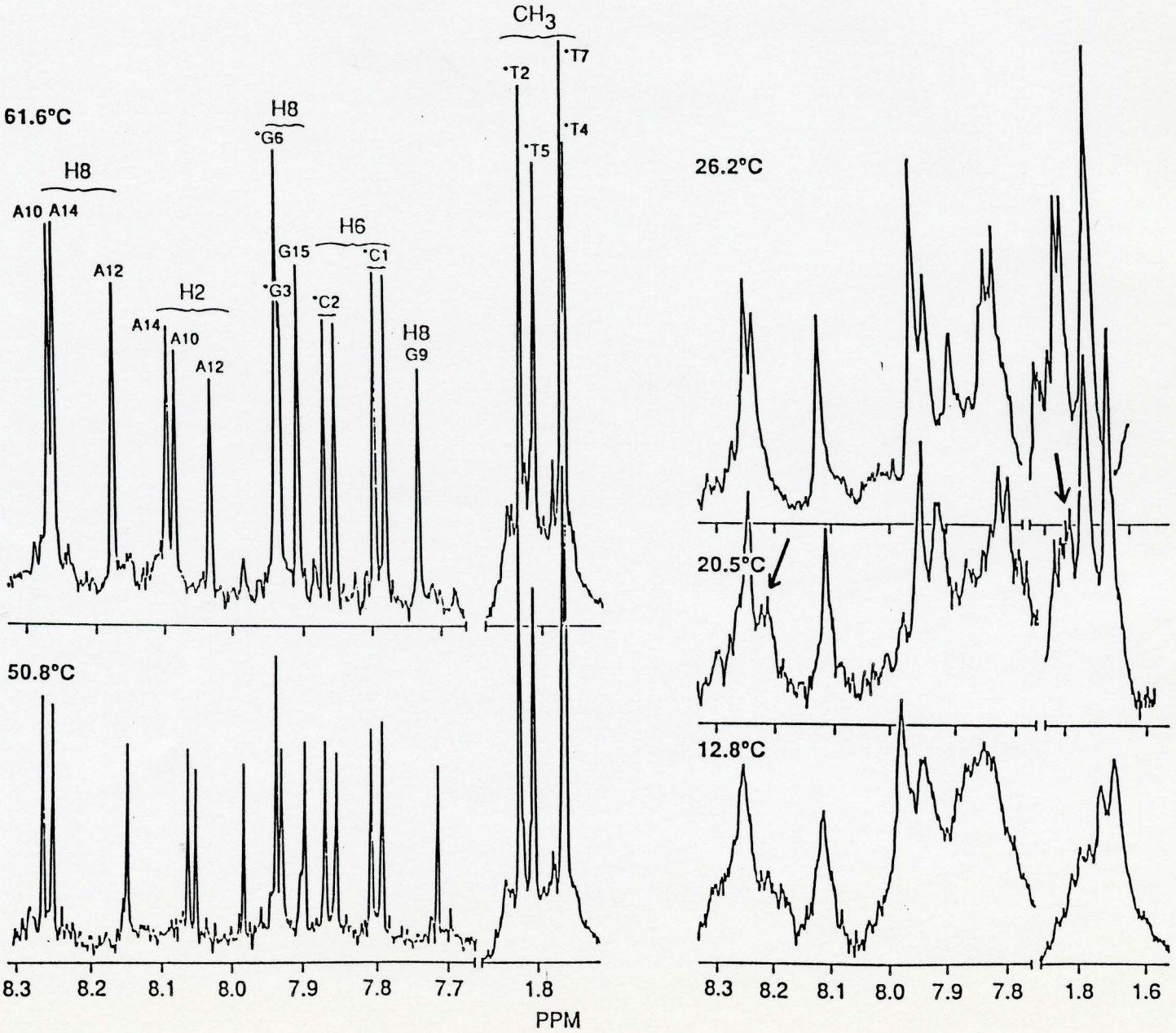


Figure 62 ^{1}H NMR spectra illustrating the aromatic and methyl regions as a function of temperature for the oligomer mixture d(CTGTTGTC) + d(GACACAG). Note the collapse of A-H2 and T-CH_3 below 25°C.

These results contrast those observed in the heptamer-hexamer mixture, where the extra, central thymine base was found to be "looped out" of the duplex structure. The result which suggested that the central thymine base was in a "looped out" conformation was the downfield shift of the resonance for the (T4) methyl group as a function of temperature. The results obtained from the octamer-heptamer variable temperature experiments suggest that the two central thymine bases (T4, T5) remain "stacked in" the duplex, even though the opposing strand contains only one of the two complementary adenine bases. The splitting and eventual line broadening of the core thymine methyl signals, along with the result observed with the adenine H-8 resonances, suggests an exchange equilibrium in the core of the duplex. It appears that the single adenine base in the core of the duplex is being shared between the two core thymine bases on the octamer strand, with the adenine moving like a pendulum between the two thymines, Figure 63.

An examination of the remaining non-exchangeable aromatic resonances indicates that many of the resonances exhibit changes in their chemical shifts as a function of temperature. Unfortunately, because of the non-complementary nature of the duplex being formed, most of the resonances broadened out over the transition temperature region and did not recover. This type of behaviour was anticipated as the formation of the duplex would not be completely cooperative between the two oligomers. Although many of the signals appeared to exhibit the beginnings of sigmoidal behaviour, complete curves were not possible and as a result very few lines provided T_m values.

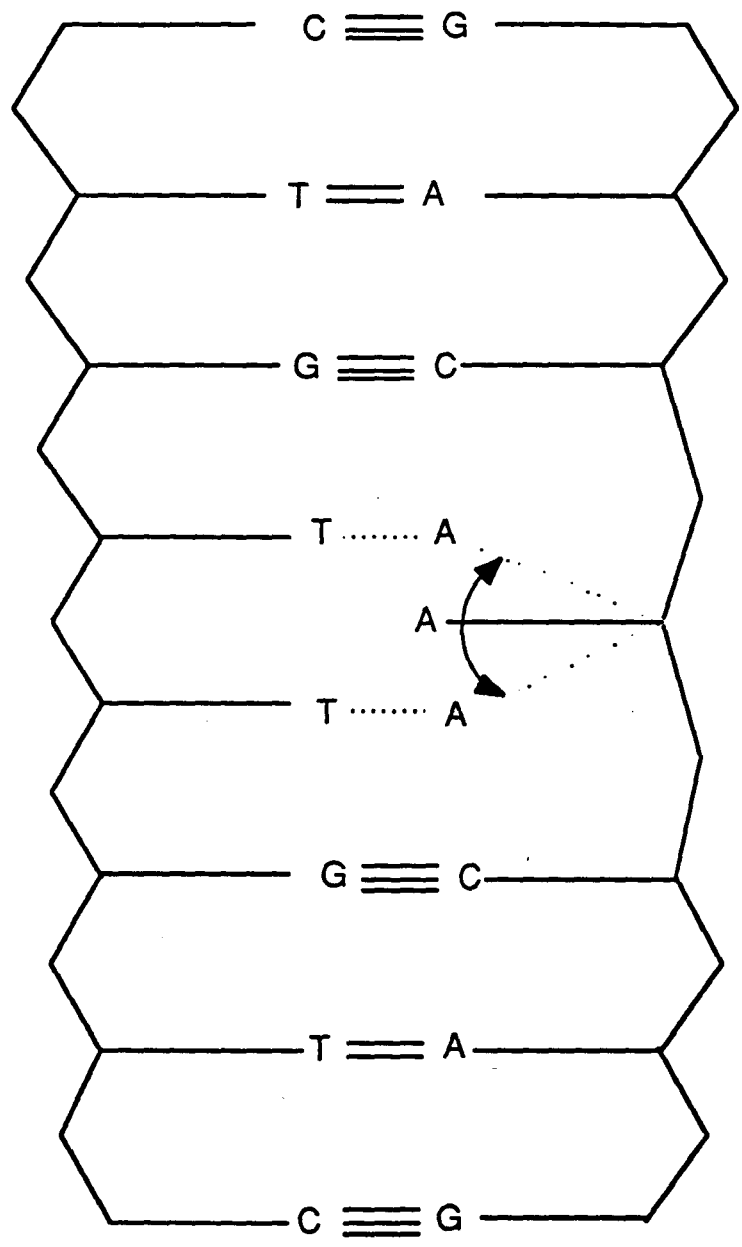


Figure 63

Schematic of proposed base pairing involved in octamer-heptamer duplex containing stacked-in central thymidine bases and oscillating adenosine base.

Several studies have been conducted which have examined the effects of mixing partially non-complementary oligomers on duplex stability. Some of these experiments have addressed the stability of non-Watson-Crick base pairs, i.e. the G-T wobble base pair (Patel et al., 1982b). Other studies, which are more closely related to the work done here, have examined the effects of extra bases within the duplex and their conformation and stability changes with respect to the normal duplex (Patel et al., 1982a; Morden et al., 1983; Woodson and Crothers, 1988). The octamer-heptamer mixture discussed here incorporates the presence of the extra base within the duplex structure, as well as an equilibrium in the core of the duplex between the one extra base and a neighbouring base pair. Although there is no direct evidence for the back and forth movement by the central adenine base in the heptamer between the two central thymine bases on the octamer, this is one plausible interpretation of the NMR results.

A second interpretation of the splitting of the resonance signals is that a mixture of duplex structures were formed. Half of the duplexes contained a central A-T base pair which utilized one of the core thymines, while the second component in the mixture used the second core thymine in the base pair. This explanation is less likely knowing that internal fraying of the base pairs will occur as a result of the non-complementary sequences. In addition, an A-T base pair adjacent to a bulge would fray easily and may not form at all, as is known with terminal A-T base pairs.

5.3.7 Variable temperature study of the exchangeable imino protons in the duplex d(CTGTTGTC) + d(GACACAG).

The exchangeable imino protons involved in the hydrogen bonds of the base pairs have been examined using the same sample studied in the previous section in a 90:10 H_2O:D_2O solution. The results for the imino proton variable temperature experiments are plotted in Figure 64. The line widths of the various imino proton resonances have been measured and plotted as a function of temperature in Figure 65.

At -3.1°C only six imino proton resonances were visible for the duplex. If a normal A-T base pair were formed in the core of the duplex, seven imino proton signals would be expected. In the region of the proton spectrum centred about 14.0 ppm - the location of the imino proton for A-T base pairs - only two signals were observed and not the expected three. A comparison of this region of the spectrum with the same region in the complementary hexamer mixture (Figure 50) and the complementary heptamer mixture (Figure 53) indicated which A-T base pair was missing from the octamer-heptamer mixture. For the two complementary systems, the terminal A-T [2,5] base pair imino proton resonances were both located at approximately 14.0 ppm. The central A-T [4] base pair in the heptamer mixture was found at approximately 13.6 ppm, slightly upfield of the terminal signals. In the octamer-heptamer mixture, it was the high field A-T signal which was absent, even at the lowest temperature studied.

Although there was the possibility of forming an A-T base pair between the central adenine base on the heptamer strand with one of the two central thymine bases on the octamer strand, no such base pair was

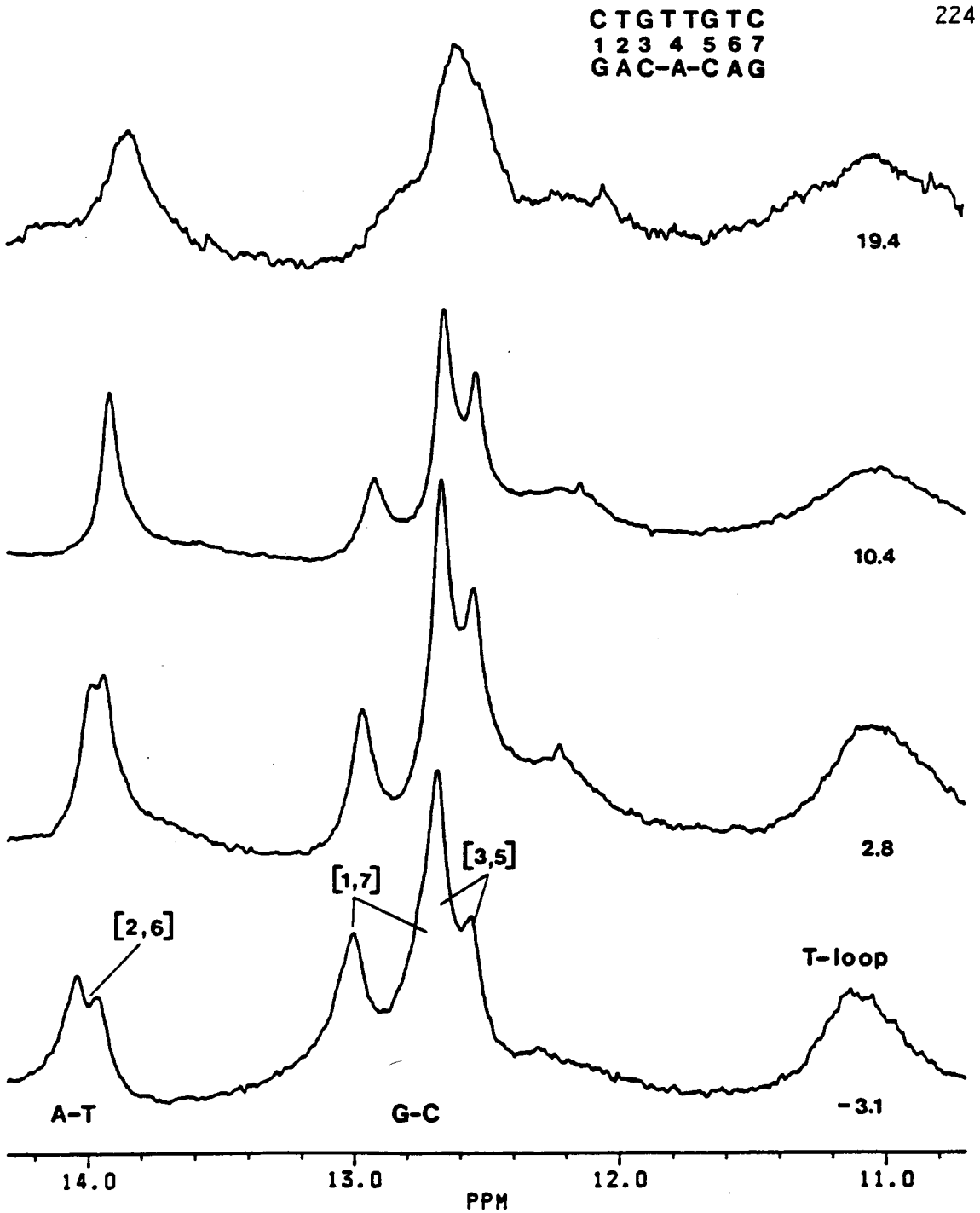


Figure 64

Exchangeable imino protons involved in base pairing and extra thymine units as a function of temperature for the partially complementary octamer-heptamer mixture d(CTGTTGTC) + d(GACACAG).

observed. This result is in agreement with the conclusions of the variable temperature studies of the non-exchangeable aromatic protons. An interpretation of the combined results again suggests that an equilibrium exists between the central adenine and the two thymines on the opposite strand, with the single adenine shifting back and forth between the two thymines. If the central adenine was involved in an base pairing equilibrium between the two core thymines (T4, T5), the observation of an imino proton signal for an A-T base pair would be dependent on the rate of exchange of the adenine between the two thymine bases. If the exchange rate between the two different A-T base pairs was slow, two signals or, more likely, one broad signal would be observed. If the rate of exchange were fast with respect to the NMR time scale, no signal would be observed. The splitting of the two thymine methyl signals and the one adenine H-8 resonance, as observed in the non-exchangeable aromatic protons experiments, combined with the absence of an imino proton signal for the central A-T base pair in this experiment, led to the conclusion that a rapid exchange of association between the one central adenine base on the heptamer oligomer with the two adjacent thymine bases on the octamer strand was occurring.

As observed with the heptamer-hexamer mixture, the line width versus temperature plot for this non-complementary oligomer mixture did not exhibit normal melting behaviour, Figure 65. In the normal complementary duplex mixing experiments, line broadening of the imino proton resonances displayed a sequential broadening of signals beginning with the 5′ and 3′ terminal base pairs, continuing inwards towards the core of the duplex. The terminal G-C [1,7] base pairs, in the octamer-heptamer mixture,

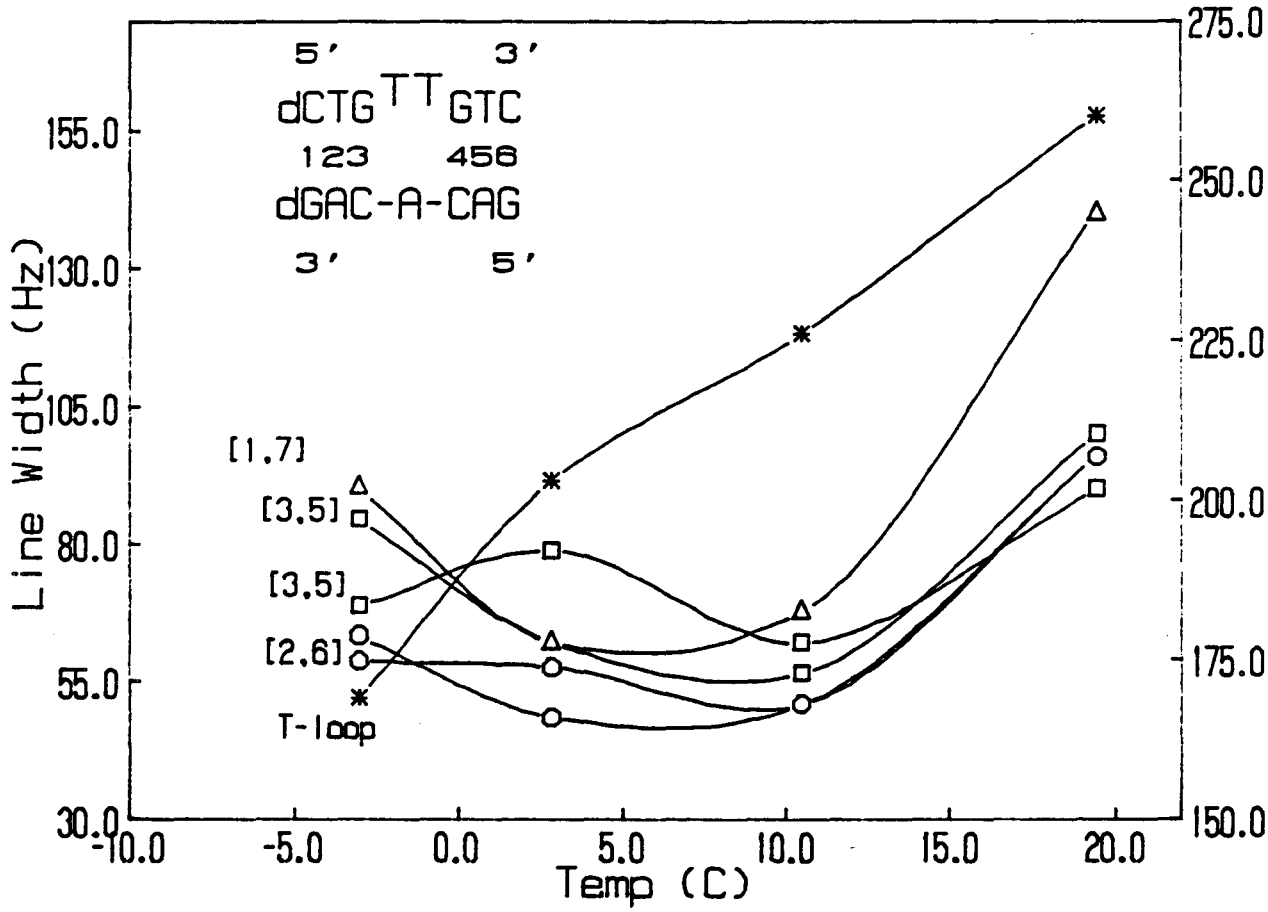


Figure 65

Line widths of exchangeable imino proton resonances as a function of temperature for the partially complementary octamer-heptamer mixture d(CTGTTGTC) + d(GACACAG).

exhibited the steepest rise in line width curve as a function of temperature. The internal G-C base pairs [3,5] did not broaden as quickly, although all signals had disappeared above 20°C.

The results for this set of mixing experiments parallels those discussed in the previous section. Again, the terminal G-C base pairs, as well as those next to the mismatched core region, broadened over the same temperature range. This is an indication of base pair fraying at the sequence terminal base pairs as well as the base pairs flanking the mismatched core region. As a result, the duplex was opening from the centre of the duplex outwards as well as the normal opening of the ends of the double helix. Once the terminal and internal G-C base pairs exchanged rapidly between the closed duplex and the open single stranded states, the remaining two A-T [2,6] base pairs in the duplex opened. This observation is reflected in the parallel trend in line broadening between the A-T [2,6] imino resonances when compared with the neighbouring, internal G-C [3,5] base pairs. The constantly increasing line width of the resonance assigned to the thymine N(3)-H protons on the extra bases in the duplex core, 11.2 ppm, reflects the increased interaction between the exchangeable imino protons on the thymine bases and the surrounding water molecules. As the temperature was increased there was more motion within the oscilating base pair core of the duplex. The added motion allows for a less structurally restricted path of interaction between the exchangeable thymine N(3)-H imino protons and the surrounding solvent.

5.3.8 Mixing experiments involving the partially complementary oligomers d(CTGTTGTC) + d(GACCAG) - Two extra thymine bases.

This was the final mixing experiment carried out in the series of model studies involving normal oligomers before examination of similar experiments with oligomers treated with the anti-tumor compound cis-$Pt(NH_3)_2Cl_2$. The interaction between the two partially complementary oligomers was examined as a function of temperature.

In this experiment, the octamer d(CTGTTGTC), numbered sequentially 5′-3′ (1-8), was combined with the hexamer d(GACCAG), numbered in the same fashion (9-14). The two oligomers are partially complementary in the Watson-Crick sense in that the terminal trimers from both the 5′ and 3′ directions are complementary. As previously discussed in sections 5.3.2 and 5.3.5, the core regions of the two oligomers are not complementary. In the other examples examined there was only one extra thymine base in the core region of the duplex, whereas in this experiment there are two extra thymine bases (T4, T5) which do not have complementary bases on the opposite strand.

The interaction between the two oligomers was examined using variable temperature ^1H NMR. Temperature effects on the duplex to coil transition were studied using both the non-exchangeable aromatic base protons, as well as the exchangeable imino protons involved in hydrogen bonding. The two oligomers were combined in a 1:1 ratio for an overall DNA concentration of 2.4 mM. The sample was dissolved in 1.0 M sodium chloride, 0.01 M sodium phosphate pH 7, and 0.5 mM EDTA. The high salt concentration was used in an effort to raise the T_m value for the duplex into a region within the range of experimental temperatures examined.

5.3.9 Variable temperature studies of the non-exchangeable aromatic protons.

The non-exchangeable aromatic base protons for the mixture of d(CTGTTGTC) and d(GACCAG) were examined over the temperature range of +5°C-70°C. The ^{1}H NMR chemical shift results for the experiments are given in Table 35 and the chemical shift versus temperature data have been plotted in Figure 66. The chemical shift assignments are based on calculated chemical shift values as well as on spectra of the individual oligomers at 70°C before the two strands were mixed. This allowed for the assignment of a resonance to a specific base on a strand.

For this set of results it is not necessary to examine the various aromatic protons individually. The variable temperature - chemical shift diagram, Figure 66, clearly shows that there was little or no duplex interaction between the two oligomers. For the majority of the proton resonances, there were only small changes in chemical shift as a function of temperature. Even those protons whose chemical shift are the most sensitive to duplex formation i.e. the purine H-2 and the pyrimidine H-5, show only small changes. The adenine H-2 signals exhibited chemical shift changes of less than 0.1 ppm. The H-5 resonances for the cytosine bases located in the core of the hexamer sequence, showed the largest chemical shift changes, approximately 0.2 ppm. This upfield shift of the cytosine H-5 resonances can be interpreted in two ways - firstly as an indication of the initial interactions leading to duplex formation between the two strands; or secondly, as the result of intrastrand stacking within the hexamer strand. Based on these results, it may be concluded that the presence of two extra thymidine nucleosides on the

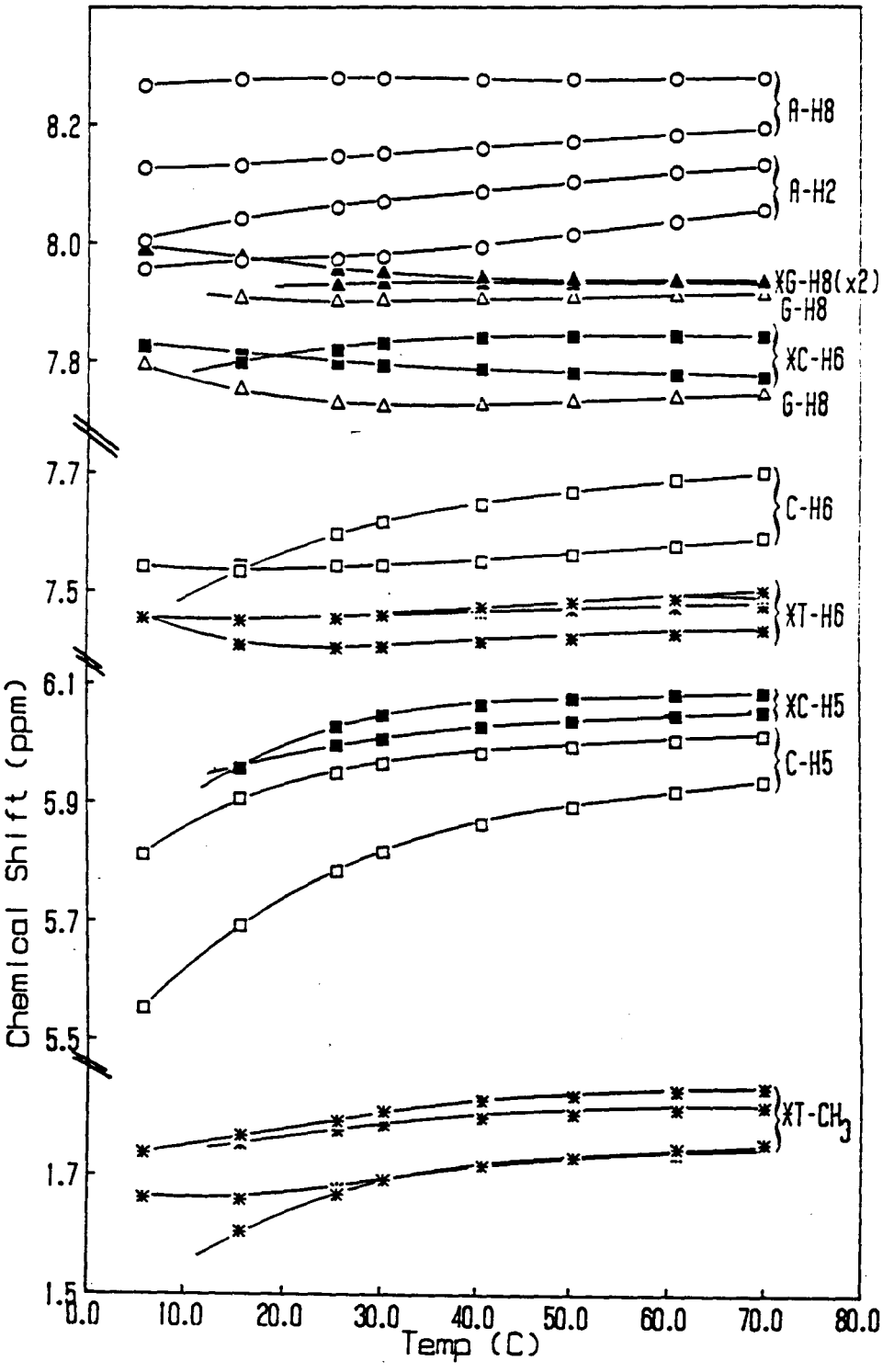


Figure 66

Chemical shift of the non-exchangeable aromatic and methyl protons as a function of temperature for the partially complementary octamer-hexamer mixture d(CTGTTGTC)* + d(GACCAG).

TABLE 35

Chemical shift as a function of temperature:
d(CpTpGpTpTpGpTpC){*} + d(GpApCpCpApG),
(1-2-3-4-5-6-7-8) (9-10-11-12-13-14)
1.3 mM each strand, 1.0 M NaCl, 0.01 M phosphate, pH 7.

Chemical Shift vs Temperature (C)

Proton	T(C)	70.0	60.8	50.3	40.6	30.2
A(13)-H8		8.274	8.274	8.275	8.276	8.277
A(10)-H8		8.189	8.180	8.169	8.159	8.149
A(10)-H2		8.129	8.117	8.102	8.086	8.069
A(13)-H2		8.052	8.034	8.012	7.992	7.975
*G(6)-H8		7.932	7.934	7.937	7.941	7.949
*G(3)-H8		7.927	7.929	7.930	7.932	7.931
G(14)-H8		7.911	7.909	7.907	7.904	7.902
*C(8)-H6		7.837	7.839	7.839	7.838	7.829
*C(1)-H6		7.767	7.772	7.778	7.784	7.791
G(9)-H8		7.740	7.734	7.728	7.722	7.721
C(11)-H6		7.705	7.691	7.671	7.649	7.619
C(12)-H6		7.593	7.580	7.564	7.552	7.545
*T(7)-H6		7.502	7.490	7.483	7.475	7.461
*T(4)-H6		7.494	7.490	7.483	7.475	7.461
*T(2)-H6		7.477	7.474	7.470	7.467	7.461
*T(5)-H6		7.437	7.431	7.422	7.416	7.409
*C(8)-H5		6.069	6.068	6.064	6.057	6.040
*C(1)-H5		6.037	6.034	6.028	6.019	6.001
C(12)-H5		5.997	5.992	5.984	5.975	5.959
C(11)-H5		5.920	5.906	5.884	5.857	5.810
*T(2)-CH_3		1.847	1.843	1.836	1.827	1.808
*T(5)-CH_3		1.817	1.812	1.805	1.797	1.785
*T(7)-CH_3		1.754	1.745	1.731	1.717	1.692
*T(4)-CH_3		1.747	1.738	1.726	1.713	1.692

TABLE 35 (continued)

Chemical shift as a function of temperature;
d(CpTpGpTpTpGpTpC){*} + d(GpApCpCpApG),
(1-2-3-4-5-6-7-8) (9-10-11-12-13-14)
1.3 mM each strand, 1.0 M NaCl, 0.01 M phosphate, pH 7.

Chemical Shift vs Temperature (C)

Proton	T(C) 25.5	15.6	5.8
A(13)-H8	8.277	8.275	8.265
A(10)-H8	8.143	8.130	8.126
A(10)-H2	8.059	8.039	8.109
A(13)-H2	7.971	7.968	7.954
*G(6)-H8	7.954	7.974	7.987
*G(3)-H8	7.927	-----	-----
G(14)-H8	7.900	7.906	-----
*C(8)-H6	7.817	7.795	-----
*C(1)-H6	7.796	7.808	7.824
G(9)-H8	7.725	7.749	7.793
C(11)-H6	7.598	7.534	-----
C(12)-H6	7.543	7.544	7.541
*T(7)-H6	7.455	7.450	7.454
*T(4)-H6	7.455	7.450	7.454
*T(2)-H6	7.455	7.450	7.454
*T(5)-H6	7.406	7.411	7.454
*C(8)-H5	6.021	5.952	-----
*C(1)-H5	5.990	5.952	-----
C(12)-H5	5.944	5.903	5.811
C(11)-H5	5.780	5.687	5.551
*T(2)-CH_3	1.791	1.766	1.737
*T(5)-CH_3	1.775	1.753	-----
*T(7)-CH_3	1.667	1.605	-----
*T(4)-CH_3	1.678	1.659	1.660

octamer strand cannot be accomodated in a duplex of the sequence length examined, Figure 67.

Studies which have examined this type of duplex structure have only incorporated one extra nucleotide unit in the duplex (Morden et al., 1983; Patel et al., 1982a), as was the situation in our previous partially non-complementary mixing experiments. Although not directly comparable, hairpin loop and cruciform structures have some similarities to the present mixing experiment. In hairpin type structures, single stranded oligomers fold back on themselves to form double helices which have one end joined by a single stranded loop region. Studies have shown that there is an optimal minimum and maximum number of nucleotide units which may be present in the loop region. The minimum number of nucleotides in the loop was found to be three (Haasnoot et al., 1983; Hilbers et al., 1985). Less than three nucleotides in the loop proved to be too much strain on the duplex whereas more than seven was also found to create too much strain for the system. In the octamer-hexamer mixture, there were two extra bases in the bulge region of the proposed duplex. More bases in this region of the complex might allow increased structural freedom and the ability to adjust to the geometric constraints of the system. However, the section of the hexamer oligomer which was in opposition to the extra thymine bases in the duplex, may not have been able to stretch its backbone sufficiently to accomodate both of the extra bases and to continue to participate in hydrogen bonding in the complementary regions. Further work will be necessary to determine the answers to these questions.

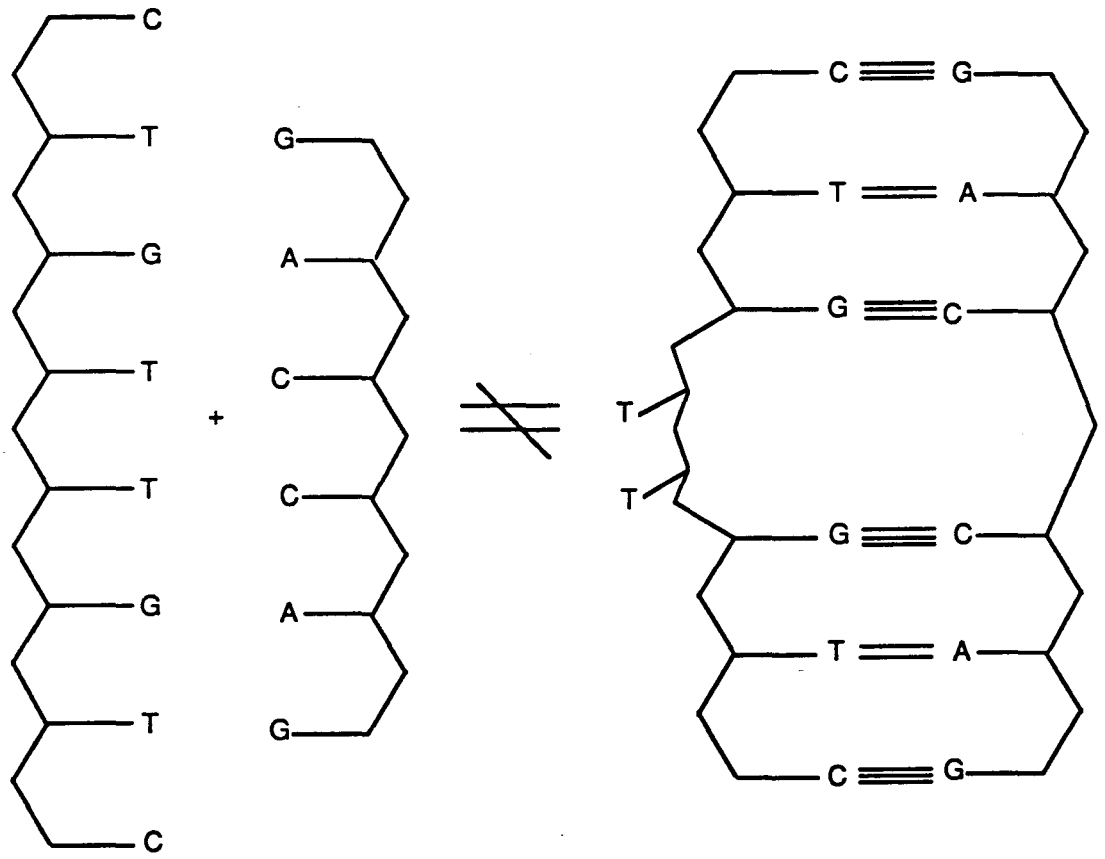


Figure 67

Schematic of possible base pairing in octamer-hexamer mixture. Duplex formation was not observed.

5.3.10 Variable temperature studies of the exchangeable imino protons in the mixture d(CTGTTGTC) + d(GACCAG).

The same sample used in the previous experiments was dissolved in a 90:10 mixture of H_2O:D_2O. The experiments conducted to examine the imino proton signals were started at -5°C. Even at this low temperature imino proton resonances were not detectable. This result supports the conclusions made from the variable temperature data of the non-exchangeable aromatic protons, that duplex formation between the octamer d(CTGTTGTC) and the partially complementary hexamer d(GACCAG) does not occur.

5.3.11 Conclusions to non-complementary mixing experiments.

In this set of mixing experiments we have examined combinations of oligomers which are only partially complementary to each other in their ability to form Watson-Crick type base pairs.

Two different sets of oligomer mixtures were studied which had involved the presence of one extra thymidine unit in the core of the duplex. In the mixing experiment involving the heptamer d(CTGTGTC) and the hexamer d(GACCAG), it was shown that a stable duplex was formed. Results from the variable temperature experiments indicated that the extra thymine base was "bulged out" of the double helix structure. Both the shape of the melting curves for the non-exchangeable aromatic protons along with the line widths of the imino protons as a function of the temperature indicated that the nature of the helix to coil transition was not cooperative. The overall T_m for the duplex in 1.0 M salt was 26.4°C. This value is 14.6°C less than the T_m obtained for the

complementary hexamer mixture (41°C) (section 5.2.1) and 21.5°C less than the T_m obtained for the complementary heptamer mixture at (47.9°C) (section 5.2.3), both of which were obtained in 0.1 M salt.

In the mixing experiment between the octamer d(CTGTTGTC) and the heptamer d(GACACAG), it was found that a stable duplex was formed between the two oligomers. Although this combination of oligomers contains one extra thymine base in the core of the duplex, as was the case in the previous example, it provides the central adenine on the heptamer strand with a choice of two thymine bases with which it may base pair. Results from both the non-exchangeable aromatic proton experiments and the exchangeable imino proton experiments suggest that the central adenine does not make the choice between the two thymine bases on the octamer. Instead, the adenine base exists in an rapid oscillating motion, sharing itself between the two central thymine bases on the octamer strand. It may be this interaction between the one adenine and the two thymine bases which holds the extra thymine within the duplex structure, unlike the bulged thymine observed in the previous case. The T_m values for this duplex are limited and yield an overall T_m of 29.0°C for the complex. Again, this value is considerably lower than that observed for the fully complementary hexamer mixture and heptamer mixture, by 12.0°C and 18.9°C respectively. The stability of octamer-heptamer mixture may be compared to that of the heptamer-hexamer mixture, both of which were found to possess six base pairs at minus five degrees. The T_m for the octamer-heptamer was found to be only 2.5°C higher than that of the heptamer-hexamer duplex. It would appear that the partial base pairing interaction between the lone, central adenine with the two thymine bases

in the octamer-heptamer mixture does help to stablise the duplex structure when compared to the heptamer-hexamer mixture. However, this small increase in T_m is not fully what would have been expected had a complete extra A-T base pair been formed.

One mixing experiment was carried out in which one strand of the partially complementary oligomers contained two extra thymidine units in the core. Results from the mixing experiments between the octamer d(CTGTTGTC) and the hexamer d(GACCAG) indicated that duplex formation between the two oligomers did not occur.

Chapter Six

6.1 Platinated deoxyoligomers and double helix formation.

The final section of research undertaken in this study involved deoxyoligomers which had been previously reacted with cis-$Pt(NH_3)_2Cl_2$ and were then mixed with their complementary and partially complementary oligomers. At the time this work was undertaken, a review of the mixing experiments involving deoxy oligomers which contained the cis-$Pt(NH_3)_2$[d(GpG)] chelate, indicated that platination disrupted the ability of the oligomer to participate in duplex formation (Caradonna et al., 1982; Girault et al., 1982b). It was initially believed, therefore, that the DNA-cisplatin complex was conformationally unable to form Watson-Crick type hydrogen bonds with a complementary oligomer strand. This finding was supported by several reseachers who, in turn suggested that this effect was the reason for the platinum compounds anti-tumor activity (Scovell and Collart, 1985). However, it is now clear that the examples which were initially examined were inappropriate with respect to sequence to arrive at this conclusion.

Several studies have reported on self-complementary sequences before and after reaction with cis-$Pt(NH_3)_2Cl_2$. The reaction of self-complementary sequences with the platinum compound would produce a duplex containing a platinum chelate on both strands. The short length of the oligomer strands examined and the nearness of the chelated regions

to each other in the proposed duplex, left little chance for double helix formation (Caradonna et al., 1982). Under these circumstances, the conclusions of the authors were correct. However, the duplex model systems studied did not permit any other result. More recent work has demonstrated that it is possible to form a duplex with short platinated deoxy oligomers. In these studies it has been shown that the adjacent guanine bases complexed with cis-$Pt(NH_3)_2Cl_2$ were still involved in normal Watson-Crick base pairing (den Hartog et al., 1984b; Van Hemelryck et al., 1984).

Work by den Hartog and coworkers (1985c) furthered their studies with a mixing experiment between the oligomers (A) d(TCTCGTGTCTC) and (B) d(GAGACACGAGA), inwhich (A) had been reacted with cis-$Pt(NH_3)_2Cl_2$. The platinated strand contained the chelated sequence d(GTG), which was the same type of platinum - oligomer sequence interaction examined in this work. The results observed in den Hartog's work indicated that only part of the chelated region of the oligomer was able to participate in duplex formation. Only one of the two platinated guanine bases formed a base pair, and the central thymine base was looped out of the duplex. The results were interpreted on the basis of an earlier study where they had shown that a rotation about a guanine glycosidic bond occurs when an internal base was present between the two guanosine moieties involved in the platinum chelate (den Hartog et al., 1983). The new conformation of the platinated 5' guanosine was syn with respect to its furanose ring and, as a result, removed the hydrogen bonding side of the guanine base from the core of the duplex. The findings of den Hartog's studies were

most helpful in determining which of the mixing experiments which should be carried out in this study.

The list of initial mixing experiments involving platinated oligomers has been given in Table 15. As discussed above, the reaction of cis-$Pt(NH_3)_2Cl_2$ with the three oligomers d(CTGGTC), d(CTGTGTC), and d(CTGTTGTC), would produce strands in which the chelated guanine bases would maintain a similar distance between each other, despite the differences in the number of intervening thymine base units. The structural restrictions in the chelate imposed by the four coordinate, square planar platinum atom should maintain this constant distance. In all three cases, therefore, the Pt-N(7) guanine bond distance would be approximately the same, as would the bond angle about the N(7) guanine-Pt-N(7) guanine atoms. This has been illustrated in Figure 36 (section 4.1.1). The structural similarity of the chelated region in the three oligomers was the basis for the design of the different mixing experiments outlined in Table 15.

The different combinations of oligomer mixing experiments involving platinated oligomers with their complementary strands and strands which do not contain the base complements to the loop region of the platinated strands could aid in addressing several of the questions regarding the structure of cis-$Pt(NH_3)_2$-DNA adducts. It has been shown that the reaction of large strands of DNA with cis-$Pt(NH_3)_2Cl_2$ results in an overall shortening of the DNA strand (Cohen et al. 1980, 1979). The deletion of the base units which would be complementary to the loop regions of the platinated oligomers would cause an overall shortening of

the duplex as well as a misreading of the platinated strand by the only partially complementary oligomer.

Relying on the structural integrity about the four coordinate, square planar platinum atom in the oligomer chelate accounts for only a small portion of the conformational affects which one should consider in the platinum-DNA adduct. Also of major importance, as reported by den Hartog et.al. (1983, 1985c), are changes in the DNA conformation, the major conformational change being the syn orientation of one of the two platinated guanine bases. This result was a determining factor in which of the mixing experiments in Table 15 were eventually undertaken.

6.2 Mixing experiments with the complementary oligomers

cis-$Pt(NH_3)_2$[d(CTGGTC)-N7(3),N7(4)] + d(GACCAG).

The first mixing experiment conducted with the platinated oligomers involved the platinated hexamer d(CTGGTC) with its hexamer complement d(GACCAG). The platinated oligomer used in this experiment has been characterized in an earlier section of this work, see section 4.2. The two oligomers were combined in a 1:1 ratio for an overall DNA concentration of 3.4 mM. The sample was dissolved in 0.1 M NaCl and 0.01 M sodium phosphate pH 7, and 0.5 mM EDTA. The results for the variable temperature study of the non-exchangeable aromatic protons are given in Table 36, and the data plotted on Figure 68. The proton assignments for the platinated oligomer have been designated with an asterix (*) preceding the assignment.

Initial examination of the aromatic proton chemical shifts as a function of temperature, Figure 68, for the complementary hexamer

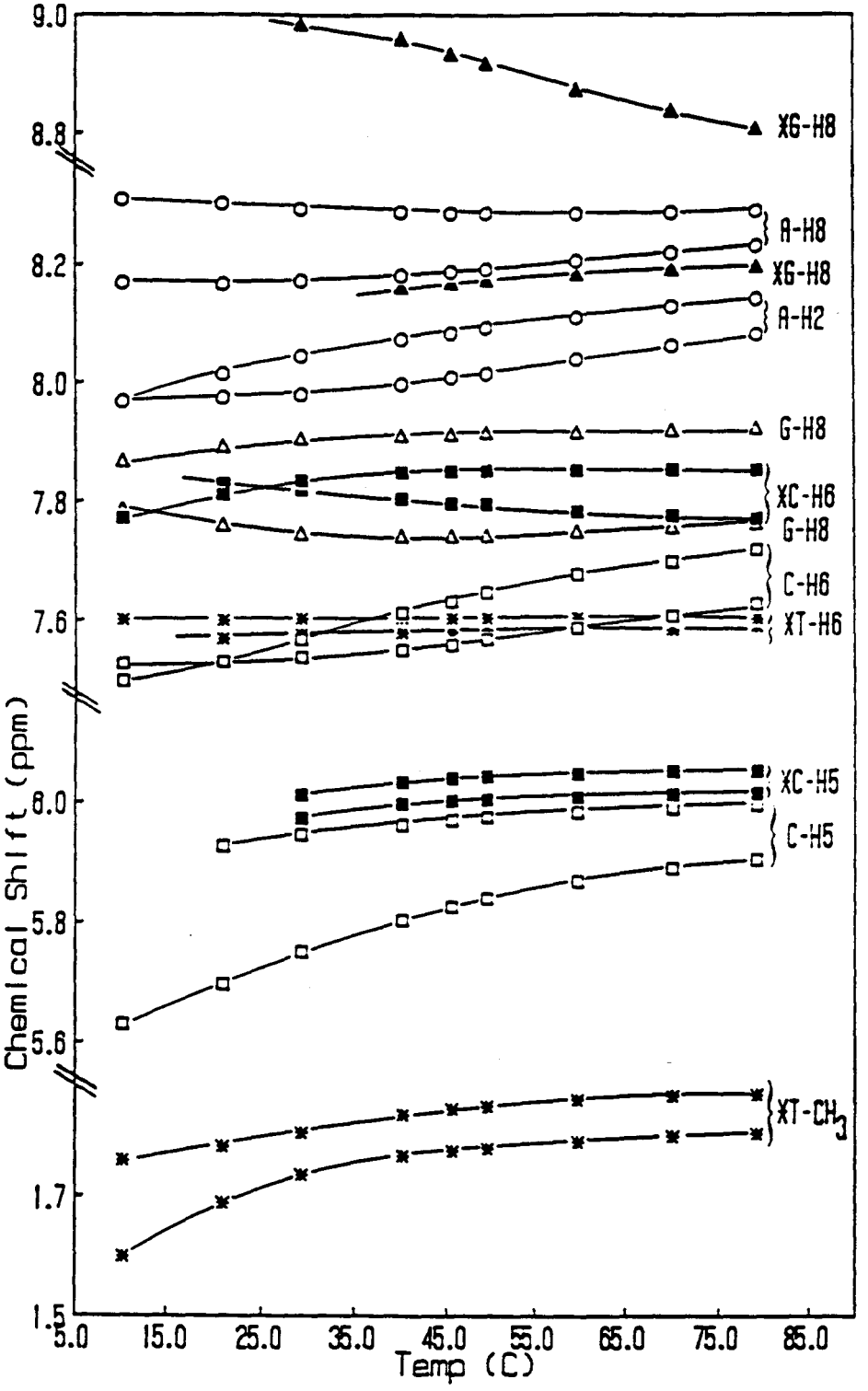


Figure 68

Chemical shift of the non-exchangeable aromatic and methyl protons as a function of temperature for the platinated complementary hexamer mixture <u>cis</u>-$Pt(NH_3)_2$[d(CTGGTC)-N7(3),N7(4)]* + d(GACCAG).

TABLE 36

Chemical shift as a function of temperature;
cis-$Pt(NH_3)_2$[d(CpTpGpGpTpG)-N7(3),N7(4)] + d(GpApCpCpApG),
(1-2-3-4-5-6){*} (7-8-9-10-11-12)
1.7 mM each strand, 0.1 M NaCl, 0.01 M phosphate, pH 7.

Proton	T(C)	Chemical Shift vs Temperature 79.2	70.0	59.8	49.5	45.5
*G-H8		8.805	8.836	8.871	8.917	8.932
A(11)-H8		8.280	8.278	8.277	8.279	8.279
A(8)-H8		8.221	8.210	8.198	8.186	8.181
*G-H8		8.186	8.182	8.175	8.165	8.161
A(11)-H2		8.131	8.118	8.103	8.085	8.078
A(8)-H2		8.071	8.052	8.032	8.009	8.002
G(12)-H8		7.912	7.911	7.909	7.908	7.907
*C-H6		7.844	7.845	7.846	7.846	7.845
*C-H6		7.763	7.769	7.777	7.789	7.792
G(7)-H8		7.752	7.747	7.741	7.736	7.735
C(9)-H6		7.710	7.692	7.670	7.641	7.627
C(10)-H6		7.618	7.601	7.582	7.562	7.554
*T-H6		7.594	7.597	7.597	7.597	7.597
*T-H6		7.581	7.578	7.580	7.579	7.575
*C-H5		6.051	6.049	6.045	6.041	6.037
*C-H5		6.012	6.010	6.007	6.002	5.999
C(10)-H5		5.993	5.988	5.981	5.973	5.968
C(9)-H5		5.903	5.887	5.867	5.838	5.824
*T-CH_3		1.867	1.864	1.858	1.848	1.844
*T-CH_3		1.803	1.797	1.790	1.779	1.776

Proton	T(C)	40.0	29.2	20.8	10.2
*G-H8		8.956	8.981	-----	-----
A(11)-H8		8.281	8.288	8.297	8.307
A(8)-H8		8.175	8.167	8.163	8.166
*G-H8		8.154	-----	-----	-----
A(11)-H2		8.066	8.039	8.010	7.966
A(8)-H2		7.991	7.975	7.971	7.966
G(12)-H8		7.905	7.899	7.888	7.865
*C-H6		7.843	7.831	7.808	7.771
*C-H6		7.799	7.816	7.828	-----
G(7)-H8		7.735	7.742	7.756	7.783
C(9)-H6		7.608	7.565	7.528	7.495
C(10)-H6		7.545	7.533	7.528	7.525
*T-H6		7.600	7.599	7.598	7.600
*T-H6		7.574	7.572	7.566	-----
*C-H5		6.032	6.010	-----	-----
*C-H5		5.995	5.970	-----	-----
C(10)-H5		5.961	5.943	5.924	-----
C(9)-H5		5.803	5.750	5.696	5.630
*T-CH_3		1.833	1.805	1.782	1.758
*T-CH_3		1.766	1.736	1.687	1.597

mixture showed little change in chemical shift over the entire temperature range, for the majority of the proton resonance signals. This result indicated that duplexing between the two strands had not occurred. A closer examination of the resonances which had been assigned to the bases on the platinated hexamer strand revealed that their behaviour paralleled the variable temperature data of the platinated strand on its own (see Figure 46, section 4.3.4). In the melting experiment involving just the platinated hexamer, many of the resonances where observed to broaden out below 25°C. This result had been interpreted as having arisen from an aggregation effect. In the mixing experiment involving the platinated hexamer with its normal hexamer complement it appears that, again, strand aggregation rather than duplex formation was the dominant physical process. As a result, before the two strands approached the T_m for the expected duplex, the platinated strand became involved in a self-aggregation process and was no longer available to its complementary hexamer for hydrogen bonding interaction.

In an attempt to decrease the aggregation affect of the platinated hexamer, the same mixing experiment was repeated at different salt concentrations. The NMR spectrum of the sample was recorded in a solution which did not contain sodium chloride, only the 0.01M phosphate buffer. This experiment was carried out to remove promotion of strand aggregation resulting from the ionic strength of the solution. The results from this experiment (not shown) were identical to those obtained for the experiment in 0.1 M sodium chloride.

The mixing experiment, involving the platinated hexamer and its normal complementary hexamer, was repeated using a higher salt

concentration, 1.0 M NaCl. These conditions were used to increase the T_m of the duplex so that strand duplexing might dominate over the aggregation problem. The results from this set of experiments (not shown) were again the same as those obtained for the 0.1 M salt sample, with aggregation negating any possibility of duplexing.

6.3 Variable temperature study of the exchangeable imino protons for cis-Pt(NH_3)$_2$[dCTGGTC)-N7(3),N7(4)] + d(GACCAG).

Mixing experiments between the platinated hexamer and its normal hexamer complement were also carried out to observe the effect of platination on the exchangeable imino protons involved in hydrogen bonding in duplex formation. The experiments were carried out at the three NaCl salt concentrations used for examination of the non-exchangeable aromatic protons: 1.0 M, 0.1 M, and no sodium chloride, all in 0.01 M sodium phosphate pH 7, and 0.5 mM EDTA.

In all three salt concentrations studied, imino proton signals were not observed, even at the lowest temperature examined, -5.0°C. This result is in agreement with the conclusions reached from the examination of the non-exchangeable aromatic protons, there was no duplex formation.

The variable temperature ^1H NMR results from the examination of the non-exchangeable aromatic protons and exchangeable imino protons for the mixing of the platinated hexamer and its normal hexamer complement were unexpected. Earlier studies by den Hartog *et al.* (1985a,1984b) and by Van Hemelryck *et al.* (1984), which examined similar platinated complementary nucleic acids mixtures, showed that duplex formation was possible for strands where one of the oligomers contained a

cis-$Pt(NH_3)_2$[-d(GpG)-] fragment. The oligomers used by den Hartog were d(TCTCGGTCTC) + d(GAGACCGAGA), and were able to observe imino proton signals for the cis-$Pt(NH_3)_2$[-d(GpG)-] : [-d(CpC)-] fragment up to 42°C. The sequences examined by Van Hememlryck were d(GATCCGGC) + d(CGCTAGGCCG), and were able to observe imino proton signals for the duplex up to 30°C. In each case the effect of platination on the stability of the duplex was a decrease in the T_m by approximately 10-15°C when compared with the normal parent duplex.

The T_m of the parent hexamer duplex, discussed in section 5.2.1, was 41°C. If our oligomers were to follow a similar trend as those reported in the literature, a T_m for the platinated duplex of approximately 26-31°C would have been expected. The cis-$Pt(NH_3)_2$[-d(GpG)-] : [-d(CpC)-] core region, in the proposed hexamer duplex, was bounded in both terminal directions by A-T base pairs. The platinum chelated region of the duplex examined in den Hartog's work was bounded by one G-C base pair and in Van Hemelryck's work both nearest neighbours to the platinum chelate were the more stable G-C base pair. This sequence difference may be one of the reasons why duplex formation with the platinated hexamer was not observed. If the formation of the duplex involving the platinated oligomer requires that the base pairs adjacent to the chelate region be a major contributor to aligning the chelated region for base pair formation, the A-T base pair may not provide the necessary stability. A second possibility why duplex formation was not observed is that the structure of platinated hexamer was not correctly interpreted, as outlined in section 4.3.4. However, the ^1H NMR results for platination of the hexamer are in agreement with similar examples recorded in the

literature (den Hartog et al. 1983; Caradonna et al. 1982; Girault et al. 1982b).

6.4 Variable temperature study of the non-exchangeable aromatic protons in the mixture cis-$Pt(NH_3)_2$[d(CTGTGTC)-N7(3),N7(5)] + dGACCAG.

Based on the results observed in the mixing of the platinated hexamer with its normal hexamer complement, as well as the results obtained by den Hartog et al. (1985c) it was concluded that the mixing of the platinated heptamer, cis-$Pt(NH_3)_2$[d(CTGTGTC)-N7(3),N7(5)], with its heptamer complement, d(GACACAG), would not lead to duplex formation. Den Hartog and coworkers had shown that the platinated oligomer containing an extra base between the complexed guanosine bases resulted in the rotation of one of the guanine bases to a syn conformation. The effect of the syn guanine base was a conformational loss of hydrogen bonding ability for this base. In the mixing of cis-$Pt(NH_3)_2$[d(TCTCGTGTCTC)-N7(5),N7(7)] + d(GAGACACGAGA), den Hartog et al. (1985c) not only lost one of the platinated G-C base pairs from the syn guanine base, but the bulged thymine base internal to the platinum chelate did not participate in hydrogen bond formation. Again, as was the case in their previous work, the platinated region of the duplex was bounded on either terminus by the more stable G-C base pair. Comparing these results with the possible duplex formation between the platinated heptamer cis-$Pt(NH_3)_2$[d(CTGTGTC)] and its complement, d(GACACAG), led to the following conclusions. Similar to the results observed by den Hartog et al.(1985c), the central A-T base pair would not form as well as one of the two G-C base pairs directly involved in the platinum chelate. Because of the internal

fraying effect which would result from the loss of two of the internal base pairs, the A-T base pair immediately adjacent to the syn guanine base would have little chance in forming stable hydrogen bonds. This assumption would leave only the terminal G-C base pair to hold together one end of the duplex. Such a possibility for base pair formation would be unlikely as single, isolated base pairs have not been observed in solution. Using this rationale, the mixing experiment between the platinated heptamer and its normal heptamer complement was not undertaken.

The study by den Hartog had shown that the thymine base located in the centre of the platinum chelate did not participate in base pair formation and, was probably looped out from the core of the duplex. Our research illustrated that the mixture of the non-platinated heptamer with the partially complementary hexamer, section 5.3.2, formed a stable duplex with the extra central thymine base bulged out from the core of the duplex. In this section of work the platinated heptamer, cis-$Pt(NH_3)_2$[d(CTGTGTC)], was combined with the partially complementary hexamer d(GACCAG). Reaction of the heptamer with the platinum compound would be expected to decrease the length of the heptamer causing it to be similar in length to a hexamer. Therefore, a combination of the platinated heptamer with the hexamer might be expected to form a duplex as stable, or perhaps more stable, than the non-platinated heptamer-hexamer mixture. However, this model does not take into account the effects of the syn conformation of one of the platinated guanine bases. This conformational change in the one guanine base would negate

the formation of one of the central G-C base pairs resulting in a helix containing the five remaining base pairs.

The platinated heptamer and the normal hexamer were combined in a 1:1 ratio for a total DNA concentration of 3.2 mM. The sample was dissolved in 400 ul of 0.1 M sodium chloride, 0.01 M sodium phosphate pH 7, and 0.5 mM EDTA. The variable temperature behaviour of the oligomer mixture was examined by monitoring the non-exchangeable aromatic base protons by NMR. The assignment of proton signals common to both strands were based on the previous examination of the strands individually before mixing. The results for the variable temperature studies have been listed in Table 37 and these data have been illustrated in Figure 69. The proton assignments for the platinated strand have been designated with an asterix (*) preceeding the assignment.

The chemical shift versus temperature data on Figure 69 shows a similar trend to that observed for the mixture of the platinated hexamer with its normal hexamer complement. The non-exchangeable aromatic proton resonances show only slight changes in chemical shift as a function of temperature. As was observed for the platinated heptamer alone (section 4.3.5) there was line broadening of the resonances for the protons on the platinated strand below 45^{o}C. This line broadening effect did not recover to the lowest temperature examined, 0^{o}C. Again, paralleling the case for the platinated hexamer, aggregation of the platinated oligomer appeared to be a problem. A comparison of the variable temperature results for the platinated heptamer alone, section 4.3.5, Figure 47, with those in the mixture of the platinated heptamer with the partially complementary hexamer, reveals that the platinated oligomers exhibit the

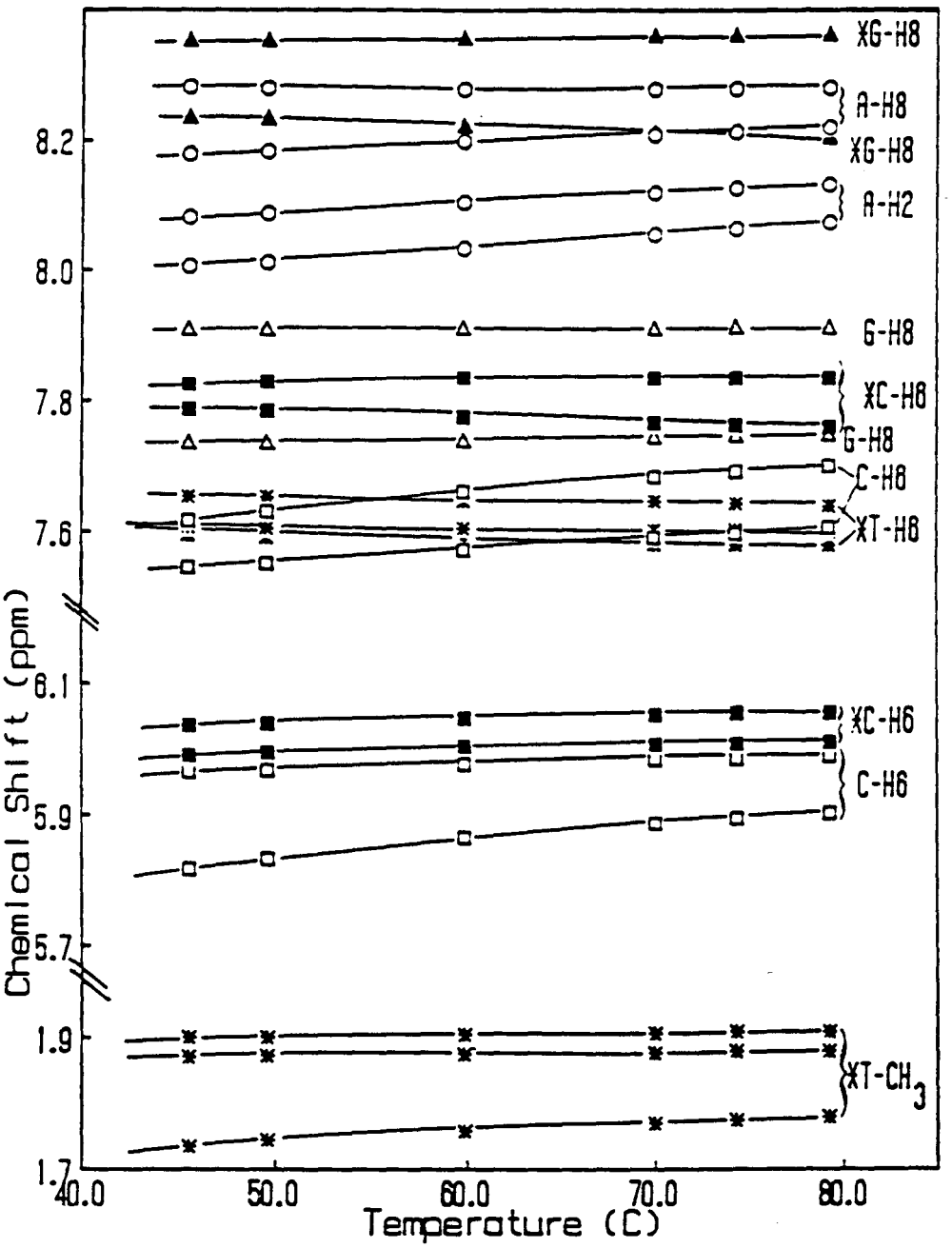


Figure 69

Chemical shift of the non-exchangeable aromatic and methyl protons as a function of temperature for the partially complementary mixture cis-$Pt(NH_3)_2$[d(CTGTGTC)-N7(3),N7(5)]* + d(GACCAG).

TABLE 37

Chemical shift as a function of temperature;
cis-$Pt(NH_3)_2$[d(CpTpGpTpGpTpC)-N7(3),N7(5)] + d(GpApCpCpApG)
(1-2-3-4-5-6-7){*} (8-9-10-11-12-13)
1.6 mM each strand, 0.1 M NaCl, 0.01 M phosphate, pH 7.

Chemical Shift vs Temperature (C)

Proton	T(C)	79.2	74.3	70.0	59.8	49.5	45.5
*G-H8		8.359	8.357	8.356	8.352	8.349	8.349
A(12)-H8		8.277	8.276	8.276	8.276	8.277	8.279
A(9)-H8		8.217	8.210	8.206	8.195	8.182	8.178
*G-H8		8.202	8.204	8.209	8.218	8.231	8.233
A(12)-H2		8.130	8.123	8.117	8.102	8.085	8.079
A(9)-H2		8.070	8.060	8.052	8.031	8.010	8.004
G(13)-H8		7.912	7.912	7.911	7.910	7.909	7.909
*C-H6		7.836	7.835	7.836	7.835	7.829	7.826
*C-H6		7.762	7.765	7.769	7.777	7.785	7.788
G(8)-H8		7.751	7.748	7.746	7.740	7.736	7.736
C(10)-H6		7.703	7.693	7.685	7.662	7.631	7.617
*T-H6		7.642	7.644	7.647	7.647	7.654	7.654
C(11)-H6		7.608	7.598	7.591	7.573	7.552	7.546
*T-H6		7.602	7.605	7.604	7.606	7.606	7.608
*T-H6		7.582	7.582	7.584	7.585	7.592	7.596
*C-H5		6.051	6.049	6.048	6.043	6.038	6.035
*C-H5		6.006	6.005	6.005	6.002	5.994	5.990
C(11)-H5		5.986	5.982	5.981	5.975	5.967	5.964
C(10)-H5		5.898	5.889	5.882	5.860	5.829	5.815
*T-CH_3		1.908	1.908	1.907	1.905	1.900	1.899
*T-CH_3		1.880	1.879	1.878	1.875	1.873	1.870
*T-CH_3		1.780	1.775	1.770	1.759	1.745	1.736

Lines broaden out below 45°C.

same temperature characteristics. These results indicate that duplexing between the two strands has not occurred.

The salt concentration for the mixing experiment was varied to establish if the aggregation problem was salt dependent. The variable temperature studies were repeated in a soution which contained no sodium chloride, the only contributor to the ionic strength being the 0.01 M sodium phosphate buffer. The results for this set of experiments (not given) were the same as those obtained in the 0.1 M sodium chloride. These data would suggest that the aggregation of the platinated oligomer at low temperature was not salt dependent. The experiments were again repeated in a solution 1.0 M in sodium chloride in an attempt to raise the T_m of the proposed duplex above, or within a temperature region where it might compete with the aggregation phenomenon. The results obtained for these experiments (not given) again indicated that no duplexing of the strands was occurring and that the major temperature effect was strand aggregation.

6.5 Variable temperature study of the exchangeable imino protons in the mixture cis-$Pt(NH_3)_2$[d(CTGTGTC)-N7(3),N7(5)] + dGACCAG.

The same oligomer sample used in the previous section was dissolved in a 90:10 H_2O:D_2O solution and examined for exchangeable imino protons resulting from base pair formation. The mixture was examined under three different salt concentrations, 1.0 M, 0.1 M, and no salt just 0.01 M sodium phosphate buffer, pH 7, EDTA 0.5 mM. The lowest temperature studied in the experiments was -5°C, however, imino protons were not

observed under these conditions. For all three salt concentrations, the same results were obtained.

The results obtained for the exchangeable imino protons are in agreement with the data collected from the variable temperature results for the non-exchangeable aromatic protons. For both sets of experiments, a base pairing interaction between the platinated heptamer and the partially complementary hexamer was not observed. The failure of the two strands to form a duplex has been attributed to the self-aggregation of the platinated oligomer. Changes in the ionic strength of the solution containing the oligomers did not alter the aggregation problem suggesting that the aggregate was not salt dependent.

6.6 Variable temperature study of the non-exchangeable aromatic protons in the mixture cis-$Pt(NH_3)_2$[d(CTGTTGTC)-N7(3),N7(6)] + d(GACACAG).

This was the last mixing experiment studied involving a platinated oligomer with a normal partially complementary oligomer. In this set of experiments, the previously characterized octamer, cis-$Pt(NH_3)_2$[d(CTGTTGTC)-N7(3),N7(6)] (see section 4.3.6) , was combined with the partially complementary heptamer d(GACACAG). The oligomers were combined in a 1:1 ratio for a overall DNA concentration of 2.6 mM. The sample was dissolved in 400 ul of 0.1 M sodium chloride, 0.01 M sodium phosphate pH 7, and 0.5 mM EDTA. The same oligomer combination, with the exception being that the octamer had not been complexed with cisplatin, was previously discussed above (section 5.3.5). In the case of the non-platinated mixture, the partially complementary oligomers were shown to form a stable duplex. The central adenine base of the heptamer was

shown to be in an oscillating base-pairing equilibrium between the two central thymine bases on the octamer. In the mixture involving the platinated octamer, a similar equilibrium would not be expected to exist. The two central thymine bases of the platinated octamer are bulged out, as a result of platinum complexation, and would be structurally removed from the hydrogen bonding region of the proposed duplex. The loss of the central thymine bases for hydrogen bonding negates the need for their base complements on the opposite strand. In this mixture only one of the thymine complements has been omitted from the non-platinated oligomer. With the two central thymines being bulged out of the octamer strand, the platinated guanines may not be as close together as in the cis-$Pt(NH_3)_2$[d(GpG)] complex and therefore, one of the thymine complements has been included in the centre of the heptamer, but strictly as a spacer.

The comparison of the 70°C H-8 protons chemical shifts of the platinated guanine on the octamer strand to similar signals on the platinated hexamer and heptamer (Figures 46 and 47), as well as to published results (den Hartog 1983), suggests that one of the platinated guanine bases in the octamer has taken up the syn conformation. NOE experiments were attempted on the platinated oligomer, however, the results obtained were insufficient to allow conformational conclusions to be made. Although this proposed conformational change has not been experimentally confirmed, the chemical shift data suggest this change in base orientation. As was the situation in the platinated heptamer-hexamer mixture (section 5.4.4), the syn guanine base can not participate in base pair formation with its complementary cytosine on the

opposite strand. The overall result of this conformational change is that the maximum number of expected base pairs for the platinated octamer-heptamer mixture is five.

The non-exchangeable aromatic protons on the platinated octamer and the partially complementary heptamer were examined over the temperature range of 10°C to 70°C. The data from the variable temperature experiments have been listed in Table 38 and these results plotted in Figure 70. Examination of the variable temperature plot shows only small changes in chemical shift of the various aromatic proton resonances as a function of temperature. Unlike the previous two mixing experiments, the aromatic proton signals for the platinated oligomer did not experience the extreme line broadening effects at low temperature, several of the signals being visible down to 10°C. These results parallel those observed for the variable temperature analysis of the platinated octamer alone (section 4.3.6, Figure 48), where no aggregation of the oligomer strand occurred. In the mixing experiment, the lack of temperature dependence reflected in the relatively straight lines for the majority of the resonances indicated little or no interaction between the platinated octamer strand and its partially complementary heptamer. A few of the proton resonances did exhibit minor chemical shift changes at the lower temperatures. This result may be the beginnings of an interstrand interaction or it may be the result of intrastrand stacking, i.e. the cytosine C-H5 signals.

In an effort to determine if the chemical shift changes observed in the 0.1 M salt solution were indicating initiation of duplex formation, the oligomer mixture was examined in a 1.0 M salt solution. The purpose

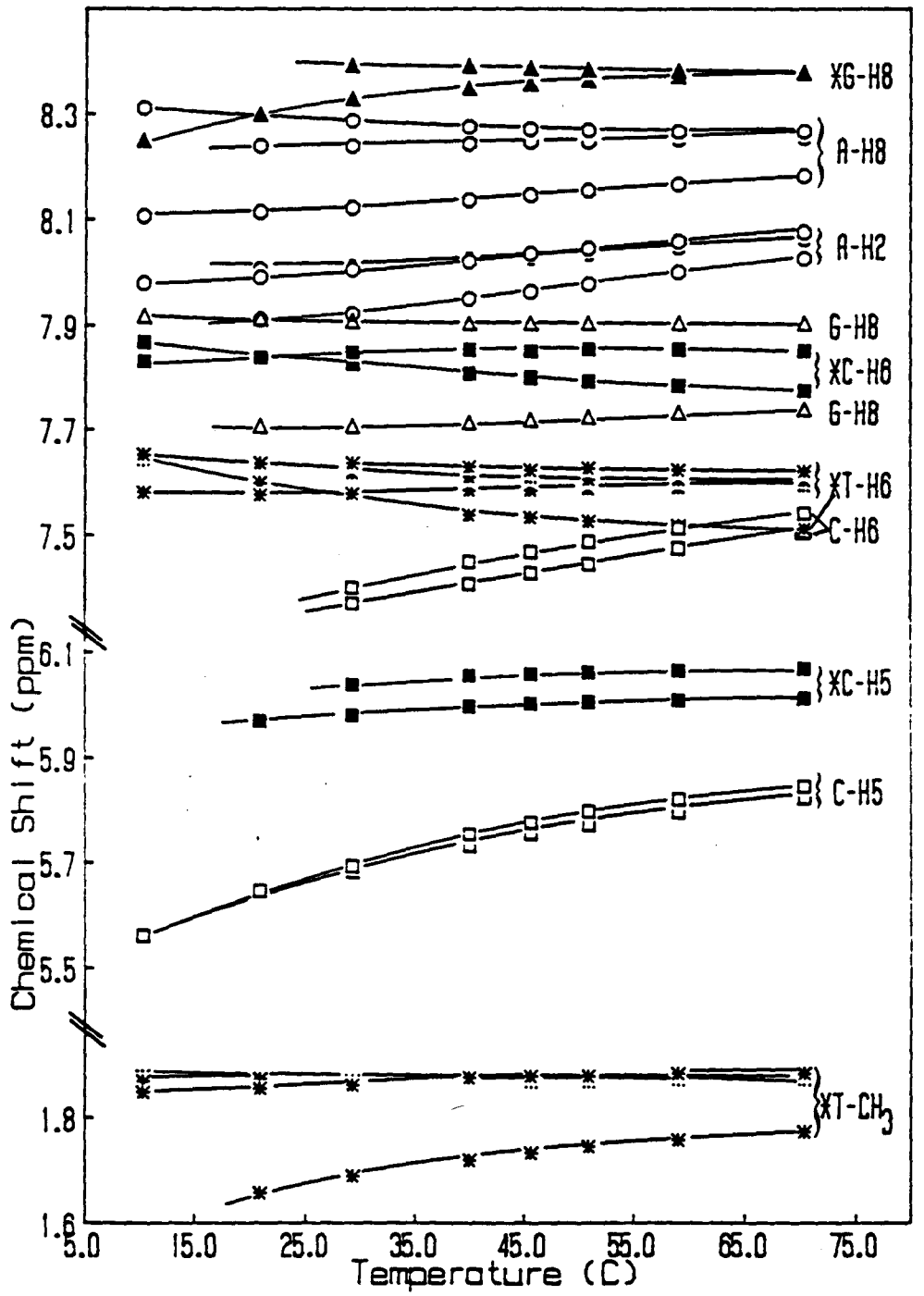


Figure 70

Chemical shift of the non-exchangeable aromatic and methyl protons as a function of temperature for the partially complementary mixture cis-$Pt(NH_3)_2$[d(CTGTTGTC)-N7(3),N7(6)]* + d(GACACAG).

TABLE 38

Chemical shift as a function of temperature;
cis-$Pt(NH_3)_2$[d(CpTpGpTpTpGpTpC)-N7(3),N7(6)]
(1-2-3-4-5-6-7-8){*}
+ d(GpApCpTpCpApG)
(9-10-11-12-13-14-15),
1.3 mM each strand, 0.1 M NaCl, 0.01 M phosphate, pH 7.

Chemical Shift vs Temperature (C)

Proton	T(C)	70.2	59.0	50.8	45.5
*G-H8		8.375	8.377	8.381	8.383
*G-H8		8.373	8.367	8.359	8.353
A(10)-H8		8.266	8.266	8.268	8.271
A(14)-H8		8.253	8.250	8.246	8.244
A(12)-H8		8.180	8.166	8.153	8.145
A(14)-H2		8.076	8.058	8.044	8.034
A(10)-H2		8.063	8.047	8.035	8.027
A(12)-H2		8.024	7.999	7.978	7.963
G(15)-H8		7.901	7.899	7.899	7.899
*C-H6		7.851	7.852	7.852	7.851
*C-H6		7.774	7.784	7.792	7.800
G(9)-H8		7.735	7.727	7.720	7.715
*T-H6		7.619	7.622	7.625	7.623
*T-H6		7.603	7.605	7.604	7.609
*T-H6		7.593	7.591	7.588	7.585
C(11)-H6		7.540	7.513	7.488	7.468
*T-H6		7.512	7.517	7.526	7.533
C(13)-H6		7.508	7.475	7.446	7.427
*C-H5		6.061	6.058	6.055	6.053
*C-H5		6.006	6.003	5.999	5.998
C(13)-H5		5.838	5.815	5.792	5.772
C(11)-H5		5.817	5.791	5.767	5.751
*T-CH_3		1.882	1.882	1.878	1.877
*T-CH_3		1.872	1.872	1.868	1.867
*T-CH_3		1.867	1.867	1.868	1.867
*T-CH_3		1.772	1.758	1.744	1.733

TABLE 38 (continued)

Chemical shift as a function of temperature;
cis-$Pt(NH_3)_2$[d(CpTpGpTpTpGpTpC)-N7(3),N7(6)]
(1-2-3-4-5-6-7-8){*}
+ d(GpApCpTpCpApG)
(9-10-11-12-13-14-15),
1.3 mM each strand, 0.1 M NaCl, 0.01 M phosphate, pH 7.

Chemical Shift vs Temperature (C)

Proton	T(C) 40.0	29.2	20.8	10.2
*G-H8	8.387	8.388	-----	-----
*G-H8	8.345	8.326	8.296	8.245
A(10)-H8	8.275	8.286	8.296	8.311
A(14)-H8	8.242	8.238	8.237	-----
A(12)-H8	8.136	8.121	8.113	8.104
A(14)-H2	8.021	8.006	7.989	7.979
A(10)-H2	8.024	8.011	8.005	-----
A(12)-H2	7.949	7.920	7.909	-----
G(15)-H8	7.900	7.904	7.909	7.914
*C-H6	7.852	7.848	7.838	7.830
*C-H6	7.807	7.824	7.842	7.868
G(9)-H8	7.710	7.702	7.702	-----
*T-H6	7.627	7.634	-----	-----
*T-H6	7.610	7.618	7.635	7.652
*T-H6	7.584	7.578	7.575	7.580
C(11)-H6	7.449	7.400	-----	-----
*T-H6	7.538	7.570	7.598	7.642
C(13)-H6	7.407	7.369	-----	-----
*C-H5	6.049	6.036	-----	-----
*C-H5	5.992	5.977	5.967	-----
C(13)-H5	5.751	5.691	5.644	5.560
C(11)-H5	5.728	5.681	5.638	5.560
*T-CH_3	1.875	1.861	1.855	1.847
*T-CH_3	1.867	1.867	1.872	1.868
*T-CH_3	1.867	1.867	1.872	1.877
*T-CH_3	1.721	1.690	1.658	-----

of the increased salt concentration in the oligomer mixture was to raise the T_m of the proposed duplex into a more practical temperature region. The results for the variable temperature experiments in 1.0 M salt (not shown) were similar to those found in the 0.1 M sample. This result suggests that the chemical shift changes observed for the oligomer mixture in the 0.1 M salt solution were not ionic strength dependent nor likely the result of duplex formation.

6.7 Variable temperature study of the exchangeable imino protons in the mixture cis-$Pt(NH_3)_2$[d(CTGTTGTC)-N7(3),N7(6)] + d(GACACAG).

The exchangeable imino protons involved in base pair formation for the mixture of the platinated octamer and the partially complementary heptamer were examined in both 0.1 M and 1.0M NaCl solutions. The samples for the NMR experiments were made up in a 90:10 H_2O:D_2O solution. The lowest temperature used to examine the samples was -5.0°C, no imino resonances were observed for the oligomer mixture at either salt concentration studied. This result is in agreement with the conclusions stated for the non-exchangeable aromatic protons, this being that duplex formation between the platinated octamer and the partially complementary heptamer had not occurred.

6.8 Conclusion to Mixing Experiments Involving Short DNA oligomers treated with cis-$Pt(NH_3)_2Cl_2$.

In this section we have examined the variable temperature effects, and the duplexing ability of short deoxyribonucleic acid oligomers, where one of the strands in the proposed duplex contained a cisplatin chelated

region. The three mixing experiments studied were, cis-$Pt(NH_3)_2$[d(CTGGTC)-N7(3),N7(4)] + d(GACCAG), cis-$Pt(NH_3)_2$[d(CTGTGTC)-N7(3),N7(5)] + d(GACCAG), and cis-$Pt(NH_3)_2$[d(CTGTTGTC)-N7(3),N7(6)] + d(GACACAG).

The results from the first two sets of mixing experiments indicated that the platinated oligomer was involved in an aggregation problem which prevented any duplex formation from occurring. Consequently, data from both the non-exchangeable aromatic protons as well as the exchangeable imino protons indicated that double helix formation did not occur between the two different strands in the above mixtures. For the third oligomer mixture involving the platinated octamer and its partially complementary heptamer, the aggregation problem with the platinated strand appeared negligible. However, the variable temperature experiments for the non-exchangeable aromatic protons and the exchangeable imino protons again indicated that duplex formation did not occur.

The lack of duplex formation for the complementary hexamers, cis-$Pt(NH_3)_2$[d(CTGGTC)-N7(3),N7(4)] and dGACCAG is not well understood in light of literature precedent for such double helical structures (den Hartog et al., 1984b; Van Hemelryck et al., 1984). The main explanation for the failure to form a duplex can be directed towards the actual strand sequences chosen. In the case of the complementary hexamer mixture, both of the platinum complexed guanine bases maintain the normal anti glycosyl torsion angle conformation allowing the two bases to participate in base pair formation. The neighbouring base pairs in both the 3′ and 5′ directions to the platinum chelate are A-T. If there is a structural need for assistance in the base pair formation of the central

cis-$Pt(NH_3)_2$[-d(GpG)-]:-d(CpC)- duplex core, i.e. stable neighbouring base pairs, the choice of A-T base pairs as immediate neighbours to the platinated region is poor. In the work by Van Hemelryck et al. (1984) the platinated region in the duplex was bounded in both the 3' and 5' directions by the more stable C-G base pairs. For the complementary hexamer system examined here, only the 5' and 3' terminal G-C base pairs remain to stablize the A-T base pairs which are immediately adjacent to the platinated region. Terminal base pairs already undergo base pair fraying as a result of normal end effects in the duplex. Two major changes should be made to the sequences before any definite conclusions can be made with regards to the affect of cisplatin on duplex stablity. The first change would be to insert the more stable G-C base pair as both the 3' and 5' nearest neighbours to the platinated region of the duplex. The second change in the oligomers used would be to extend the length of the sequences used the the studies, i.e. to repeat this experiment, the sequences would be cis-$Pt(NH_3)_2$[d(CTCGGCTC)-N7(4),N7(5)] and d(GAGCCGAG).

The aggregation problem involving the platinated strand is not well understood. Whether the aggregaton is the result of sequence length, an end effect, or sample preparation is not known and, as a result there are no suggestions to alleviate the problem. Further examination of the platinated oligomers by gel electrophoresis and size exclusion chromatography would aid in a better characterization of the strand.

The mixing experiments which involved the platinated oligomers containing one or two bases between the platinum complexed guanines presented an extra challenge towards duplex formation. It has been shown by den Hartog et al. (1985c) that one of the guanine bases involved in

the cis-$Pt(NH_3)_2$[-d(GpTpG)-] complex has rotated about its glycosidic bond and maintains a more syn type conformation. The GH-8 chemical shift values obtained for the platinated heptamer and octamer oligomers examined here are consistant with den Hartog's results and suggest a similar conformation. The major effect of this conformational change, with respect to base pair formation, is the removal of the hydrogen bonding region of this guanine base from the core of the helix. They have also shown that the thymine base central to the platinum chelate no longer participates in base pair formation. The loss of the central A-T base pair is a consequence of the thymine base being pushed out of the core of the helix as a result of platinum binding to the 3' and 5' neighbouring guanine bases. Overall, the effect of the formation of a cis-$Pt(NH_3)_2$[-d(GpTpG)-] complex within a strand is the loss of at least two base pairs when compared to the normal, parent duplex. With regards to the cis-$Pt(NH_3)_2$[d(CTGTGTC)] + d(GACCAG) mixture, this would mean that the maximum number of base pairs possible would be five. Again, as discussed above for the platinated hexamer mixture, the presence of the less stable A-T base pairs on either terminus of the platinated region of the proposed duplex may not be sufficient to stablise the altered core structure.

In the mixture involving the platinated octamer, cis-$Pt(NH_3)_2$[d(CTGTTGTC)], with the partially complementary heptamer d(GACACAG), NMR data again suggests that platination has resulted in one of the platinum bound guanine bases adopting a more syn-like conformation. The two thymine bases central to the platinated region of the octamer, have been forced out from the core of the helix and, like

the one syn-guanine base, are not structurally available for hydrogen bond formation. The end result of the conformational changes for the platinated octamer strand in the above mixing experiment is a maximum of five base pairs to hold the duplex together. As was discussed for the hexamer mixture involving the platinated hexamer with its fully complementary hexamer strand, the sequences used in these studies must be changed before any final conclusions can be made with regards to duplex formation between a platinated oligomer and a partially complementary strand. The above experiments should be repeated using sequences which contain the more stable C-G base pair immediately adjacent to the platinated region of the duplex. The strand containing one extra thymidine in the platinated region of the oligomer could be studied using cis-$Pt(NH_3)_2$[d(CTCGTGCTC)] along with its fully complementary strand d(GAGCACGAG), as well as the partially complementary d(GAGCCGAG). For the oligomer containing two thymidine units between the platinum complexed guanine bases, the oligomer cis-$Pt(NH_3)_2$[d(CTCGTTGCTC)] could be mixed with its normal complement, d(GAGCAACGAG), as well its partial complements d(GAGCACGAG) and d(GAGCCGAG).

As the sequences employed in these studies become longer the experimental techniques used to both obtain and purify the oligomers, as well as the NMR experiments used to examine duplex behaviour must change. With the increasing availability of solid phase DNA synthesis instruments, supplies of the desired sequences in quantities allowing for reasonable NMR experiment times, will be more readily at hand. Observation of all of the non-exchangeable aromatic protons in a duplex containing up to twenty bases would be very difficult, even utilizing 500

MHz spectrometers. Utilization of exchangeable imino protons studies in conjunction with more sophisticated NMR experiments, i.e. NOE studies, finer details of the structural changes resulting from platination could be determined. Other experimental techniques such as circular dichroism (CD) and ultimately, X-ray crystallography, would lead to an increased understanding of structural aberations resulting from the interaction of cis-$Pt(NH_3)_2Cl_2$ with nucleic acids.

Chapter Seven

Conclusions

The interaction of cis-$Pt(NH_3)_2Cl_2$ with short DNA oligomers has been examined under single stranded and duplexing situations. The oligomers were synthesized using a phosphotriester approach and varied in length from trimers to octamers. The specific sequences examined were designed to study the formation of the intrastrand cis-$Pt(NH_3)_2$[d(GpX$_n$pG)-N7(1),N7(n+1)] crosslink and it's effect on oligomer structure and duplexing ability. To aid in understanding the effect of cisplatin on duplex structure, model duplex systems of perfect helices composed of two complementary oligomers, and imperfect helices containing internal loops were investigated.

The reaction of cis-$Pt(NH_3)_2Cl_2$ with the DNA oligomers was monitored by UV and HPLC analysis. Product analysis indicated that only one major adduct was formed in each reaction. In order to characterize the site of platination, pH titration studies were carried out on the cisplatin-DNA adduct and followed by ^{1}H NMR. By monitoring the chemical shift changes of the various aromatic protons on the nucleobases as a function of pH, the guanine N7 position was shown to be the site of platinum attack. ICP platinum analysis of the adducts formed with d(GTG), d(GTTG), and d(GTTTG) indicated that a 1:1 platinum-DNA adduct had been isolated. These results, along with comparison to published data on other platinum-

DNA adducts established the adduct stuctures to be cis-$Pt(NH_3)_2[d(GpT_npG)-N7(1),N7(n+1)]$, for n=1,2, and 3.

Variable temperature ^1H NMR (70°C-0°C) was utilized to examine the effects of platination on the oligomer structure. Formation of the intrastrand platinum crosslink with the terminal guanine residues forces the oligomer ends together, looping-out the core thymine bases. A comparison of chemical shift values for the H-8 resonance of the platinated guanine bases with published data made it possible to predict the orientation about the guanosine glycosyl link. To relieve the structural strain brought about by formation of the loop complexes, one of the guanine bases appears to have flipped into a more syn-like conformation. NOE experiments were carried out on these molecules, however, the results were inconclusive for accurate prediction of base orientation. The overall deshielding of the thymine aromatic and methyl protons indicated the disruption of any intrastrand base stacking. The lack of chemical shift change as a function of temperature suggests that the loop structure was rigid. This result was confirmed with the examination of the exchangeable imino protons normally involved in base pairing. For adducts containing either two or three thymines in the loop region of the complex, the N(3)-H thymine imino proton was observed by NMR at low temperature. Detection of the imino proton signal showed the loop contained not only structural rigidity, but also the orientation of the nucleotides in the loop was such that solvent access to the thymines was restricted.

The formation of similar loop structures in longer synthetic deoxy oligomers allowed the examination of intrastrand stacking effects on

bases external to the platinum complexed region. Similar, to that observed for the thymine bases within the loop region in the shorter strands, bases external to the complex and immediately adjacent to the platinated guanines exhibited an overall deshielding. For the two platinated oligomers cis-$Pt(NH_3)_2$[d(CTGGTC)-N7(3),N7(4)] and cis-$Pt(NH_3)_2$[d(CTGTGTC)-N7(3),N7(5)], in temperatures below approximately 25°C the strands exist either, in a slow equilibrium between different conformational structures or, they undergo strand aggregation. The longer octamer strand cis-$Pt(NH_3)_2$[d(CTGTTGTC)-N7(3),N7(6)] did not experience this effect.

Model duplex studies on fully, and partially complementary oligomer mixtures provided information on the stability and duplex structure of these double helices before their interaction with cisplatin. The T_m values for the complementary hexamer, heptamer, and octamer mixtures increased with oligomer length, as would be expected. Observation of the duplex to coil transition showed that the helices did not unwind symmetrically from either end but rather exhibited a greater preference for fraying from one end more than the other. This indicates the importance of the direction of base stacking in stability of the duplex.

Mixing experiments were carried out on partially complementary oligomers containing extra thymine residues in the core of the duplex. The duplex to coil transition data indicated that the position of the extra thymine residue in the duplexed structure was dependant on the oligomer sequence.

For the heptamer-hexamer mixture d(CTGTGTC) + d(GACCAG), the central thymine was found to exist looped out of the base stacking arrangement in

the duplex core. This was not surprising considering the poor stacking nature of the thymine base. The remainder of the duplex structure more closely resembled that of the complementary hexamer duplex d(CTGGTC) + d(GACCAG).

In a similar, partially complementary oligomer mixture with d(CTGTTGTC) + d(GACACAG), again an extra thymine residue was present in the centre of the duplex. In this mixture, the central adenine base of the heptamer strand could choose between the two centre thymine bases of the octamer strand to form an A-T base pair. Results suggest that the adenine did not choose but rather, was shared, oscillating between the two thymine bases, holding them both inside the helix, base stacking arrangement.

The helix to coil transition for these two partially complementary mixtures differed from the perfect duplexes. In addition to the normal, terminal base pair fraying leading to duplex unwinding, the unpaired, extra bases in the core of the duplexes provided a new point for base pair instability. As well as unwinding from the external base pairs towards the core of the duplex, unwinding also occurred from the core outwards. This was reflected in the non-cooperative melting profiles of the imperfect duplexes, suggesting an almost all or nothing existence for the double helices formed. In an experiment between d(CTGTTGTC) and d(GACCAG), two thymines in the centre of the octamer strand were without a base pairing complement on the hexamer strand, duplex formation did not occur.

Understanding the stability and helix to coil transition characteristics of the perfect and imperfect duplexes provided a

foundation on which the effect of the cisplatin-DNA complex on duplex stability could be bulit. Mixing experiments between the platinated hexamer, cis-$Pt(NH_3)_2$[d(CTGGTC)-N7(3),N7(4)] and its normal complement, d(GACCAG), did not result in duplex formation. The platinated oligomer was dominated by the same strand aggregation or slow conformational change observed in variable temperature results of the platinated strand alone. Similar results were observed with mixing experiments involving the platinated heptamer, cis-$Pt(NH_3)_2$[d(CTGTGTC)-N7(3),N7(5)]. The platinated octamer, cis-$Pt(NH_3)_2$[d(CTGTTGTC)-N7(3),N7(6)], did not exhibit the aggregation or conformational change effects, however, duplex formation with the partially complementary oligomer d(GACACAG) was not observed.

Addendum

Investigators continue in their efforts to elucidate the biological mechanism of cis-DDP as an anti-tumor compound. The generally accepted view that celluar DNA is the main target of the drug remains unaltered. The pathway by which cis-DDP is specifically delivered to the target molecule is as yet unknown. Other possible reaction sites for the platinum compound are blood proteins and peptides, RNA, and cell wall components (Reedijk, 1987). Is cis-DDP, in some way, able to select between tumor and normal cells or, are tumor cells more susceptible to the drug as a result of rapid replication, and perhaps an inefficient repair mechanism for the platinum-DNA lesion? These are just a few of the unanswered questions in the study of platinum-DNA interactions.

Several groups are presently examining reaction kinetics of DNA with cis-DDP and several of its analogues (Kleinwachter et al. 1988; Millar et al. 1988; Eastman and Barry, 1987; Schaller et al. 1987). The initial bimolecular reacton between DNA and the aquated platinum compound is the rapid formation of a transient, monofunctionally bound adduct. This initial reaction would appear to cause localized disruption of base pairing resulting in single stranded regions in the DNA (Kleinwachter et al. 1988). Both the biologically active cis and the inactive trans-DDP compounds form similar monofunctional complexes, at similar rates, with DNA. The remaining chloride ligand on the adduct hydrolyzes in preparation for the formation of the complete, bifunctional platinum-DNA

adduct. The reaction paths of the cis and the trans isomers diverge at this stage (Schaller et al. 1987).

For the cis isomer, the formation of the bifunctional, intrastrand crosslink is accompanied by a structural rearrangement of the duplex. The observed rearrangement has been attributed to the reannealing base pairs, which were melted upon initial monofunctional binding (Kleinwachter et al. 1988), as well as a kinking of the helix axis at the site of platination (Millar et al. 1988). When the ratio of the platinum drug to DNA base pairs was low (<0.01), only the rearranged duplex structure was observed. As the platinum to base pair ratio was increased, there was a progressive disruption of the duplex structure. The rate of formation of the bifunctional adduct was significantly enhanced if the experiment was carried out with single stranded DNA.

The geometry of the monofunctionally bound trans isomer does not allow the formation of 1,2-intrastrand crosslinks, especially if the adduct was formed on duplexed DNA. As with the cis isomer, formation of the monofunctional adduct resulted in localized disruption of the DNA duplex. The structural changes in the DNA, upon further reaction of the monofunctional trans adduct, did not indicate kinking of the helix or reannealing of the duplex, although less duplex disruption was reported with the trans isomer when compared with the results from the cis complex (Millar et al. 1988). The geometrical constraints imposed by the square planar platinum atom negate intrastrand reaction. As a result, hydrolysis of the remaining chloride ligand on the trans isomer opens the adduct to reaction with nucleophiles in the surrounding medium i.e. the formation of DNA-platinum-protein crosslinked complexes (Eastman and

Barry 1987). These types of adducts may not be as stable as the intrastrand DNA-platinum crosslinks because the trans conformation of the ligands may labilize the DNA from the platinum as demonstrated through attempts to trap the trans monofunctional adducts using thiourea.

The existance of the monofunctional adducts has been further demonstrated with the formation of ternary complexes between cis-DDP, DNA, and ethidium bromide (Malinge et al. 1987). Ethidium bromide is an intercalator which reversibly binds to the duplexed form of nucleic acids. Ethidium intercaltes between the base pairs in a duplex with the exocyclic amino groups of the heterocycle projecting into the major groove of the duplex. The position of the primary amino groups on the intercalator with respect to guanine N7 allows the formation of the platinum-G(N7) monofunctional adduct followed by reaction with the intercaltor to form the ethidium-Pt-DNA ternary complex. There is little reaction between ethidium and cis-DDP in the absence of duplexed DNA, and the ternary complex was not observed when the trans isomer was used (Malinge et al. 1987; Sundquist et al. 1988).

The effect of cis and trans-Pt(DNA) complexes on the activity of DNA polymerase has been examined by Bernges and Holler (1988). This enzyme may act as a synthetase and carry out DNA synthesis, or act as an exonuclease repair enzyme. Interaction of the enzyme with DNA treated with trans-DDP showed that the polymerase retained its exonuclease repair activity. However, the DNA synthesis activity of the enzyme was lost. The interaction of the enzyme with DNA containing monoadducts, formed with either the cis or trans isomer, again showed the retention of exonuclease activity with the nucleotide-monoadducts eventually excised

from the strand. The formation of bifunctional adducts with the cis-DDP compound resulted in the complete loss of enzyme activity. This led the authors to suggest that DNA synthesis was stopped when a platinated site on the DNA was encountered. At low platinum/base pair ratios, the loss of activity was attributed to the formation of kinks in the duplex which result in a decrease in the binding affinity of the platinated DNA for the active site on the enzyme. At higher cis-DDP/base pair ratios, there was melting of the template and the primer, and this led to complete loss of synthesis ability. Both of these conclusions are in agreement with the previously mentioned mechanistic studies.

The importance of cis-DDP as a therapeutic agent has directed a major effort towards improving the platinum drug. Changes in the leaving groups as well as the amine ligands, in addition to the oxidation state of the platinum atom have been examined (Pasini and Zunino, 1987). Physiologically, the substitution of different ligands on cis-DDP may: increase the solubility of the compound, target the drug for a particular organ using a tissue specific carrier molecule as a ligand, or, complexed with other cytotoxic drugs as ligands obtain a combined chemotherapeutic effect.

Investigations into the exchangeable ligands on the platinum have indicated that the reactivity of the leaving groups must not be too labile, i.e. nitrato, as they may be displaced before the drug reaches the target molecule (Razaka et al. 1987). The increased reactivity not only decreases the pharmacological effect but may also increase the toxicity of the drug.

The non-exchangeable amine ligands appear to have several roles. The ability of the amine ligand to form hydrogen bonds with either the nucleobases or the phophate backbone, is important in the activity of the drug. Platinum compounds containing ammonia and primary amines are the most active while tertiary amines are inactive (Razaka et al. 1987).

Although the majority of the platinum drugs under investigation are Pt(II), there are a few Pt(IV) complexes being studied. It is believed that the Pt(IV) compounds are reduced to Pt(II) after administration and only then do they become active (Pasini and Zunino, 1987).

Monoclonal antibodies have been isolated which specifically recognize the Pt-DNA lesion created when adjacent purine bases complex with the cis oriented platinum compound (Sundquist et al. 1987). The antibody binds to both d(GpG) and d(ApG) platinated sites, as well as to adducts which are formed when bulky amine groups are used as non-leaving group ligands. These results led the authors to suggest that the antibody recognizes the conformational structure resulting from the 1,2-bifunctional platinum binding to duplexed DNA. The antibody will be useful in the testing of new platinum analogues in that the monoclonal will be able to determine if structurally similar DNA conformations are formed with various adducts and this, in turn, may be equated with biological activity.

Modeling Studies

Molecular mechanics modeling has been used in several studies to further elucidate the structures of the various adducts formed through the interaction between cis-DDP and DNA. Modeled structures reveal subtleties in the platinum-DNA complex which may aid in the explanation

of experimental data. Taking advantage of this increased structural understanding, results may be used in the design of alternative platinum analogues. Models of the trimer duplex d(GCC):d(CGG)* with cis-platinum complexes containing different amine ligands have been examined (Hambley 1987). Results indicated that the bulk of the amine ligand did not affect the binding interaction of the platinum compound, however, the conformation of the amine ligand and the effect of conformation on the hydrogen bonding ability of the ligand were important. For the duplex containing the cis-DDP moiety, the structure was stablized through the formation of two hydrogen bonds. One hydrogen bond between an ammine ligand and the 5'-phosphate on the 5' platinated guanine, and the second between the second ammine ligand and the O(6) site on the 3' platinated guanine. This result may be used to explain the structure-activity relationship where the activity of the platinum drug decreases in the order: NH_3 > NH_2R > NHR_2, and drugs containing tertiary amine ligands being inactive. This decrease in activity correlates directly with the decrease in hydrogen bonding ability of the amine ligand (Hambley 1987).

An examination into the binding of cis-DDP to d(ApG) sequences, but not d(GpA), by molecular mechanics modeling furthers the information gained in the previous study. In the d(ApG) structure, the ammine ligand of the platinum moiety is again able to hydrogen bond to the O(6) site of the 3' platinated guanine (Hambley 1988a). For the d(GpA) complex, the 3'purine base no longer carries an oxygen on the C(6) position but rather contains an NH_2 group. The presence of the adenine N(6) amino group produces an unfavourable steric interaction with the cis-DDP ammine ligand which destablizes the structure in comparison with the cis-

DDP[d(ApG)] complex. This interaction between the ammine ligand and the group at the 6-position purine was also shown to be the major factor in determining the stucture of bis(amine)bis(purine)platinum(II) models (Hambley 1988b). The barrier to rotation between the head-to-head and the head-to-tail isomers was shown to be primarily the result of the interaction between the amine ligand and the group occupying the 6-position of the purine. An oxygen at the 6-position produced a favourable interaction and allowed for easy rotation of the purine bases, while an amino group at the 6-position of the purine produced an unfavourable interaction which ultimately hindered rotation of the purine bases. These results suggest the importance of the amine ligand on the platinum drug and that its interaction with DNA may strongly influence the activity of the drug.

The work of Kozelka and co-workers (Kozelka et al. 1987) has modeled the structures of both single stranded and duplexed oligomers after reaction with cis-$[Pt(NH_3)_2]^{+2}$. Models of cis-DDP with an A-DNA structure illustrated how the difference in helix structure may alter the interaction of the platinum drug. The major groove of the A-helix is much deeper and narrower than that of the B-helix and as a result would hinder the platinum interaction with the guanine N(7) site. In the B-DNA structures, there was a disruption of the base-stacking interactions between the 5'-platinated guanine in a cis-$Pt(NH_3)_2$[d(GpG)] complex and its 5'-nearest neighbour. The furanose ring of the 5'-platinated guanine was shown to be most stable when the sugar pucker was in the N-type, C(3')-endo conformation. This repuckering of the furanose ring was shown to stablize the structural strain resulting from platination primarily by

shortening the hydrogen bond formed between an ammine ligand and the phosphate group 5′ to the complex. For stuctures using single stranded oligomers, a larger degree of flexiblity within the platinated complex was seen. Again, the structures were shown to be stablized by a hydrogen bond between the ammine ligand and the phosphate group 5′ to the platinated complex. A second hydrogen bond was also observed between the second ammine ligand and the O(6) atom on the 3′-platinated guanine. The same hydrogen bonds were observed in modeling studies discussed in the Hambley's work (1987, 1988a,b).

Synthetic Oligonucleotide Studies

Studies which involve the use of short synthetic oligonucleotides with both cis and trans-DDP remain of interest as researchers continue in their attempts to further elucidate the mechanism and results of platinum-DNA interactions.

The reaction of cis-DDP with d(pGpGpG) provided the opportunity for the formation of three types of structures: cis-Pt[d(pGpGpG)-N7(1),N7(2)], cis-Pt[d(pGpGpG)-N7(2),N7(3)], cis-Pt[d(pGpGpG)-N7(1),N7(3)] (van der Veer et al. 1987). The major product isolated in this work was the N7(1),N7(2) structure with a minor amount of the N7(2),N7(3) complex. For both complexes, the basic structures were determined by NMR and revealed that the furanose ring on the 5′-platinated guanine was in the C3′-endo conformation. There was no indication for the formation of a complex containing an intervening nucleotide, i.e. cis-Pt[d(GpXpG)-N7(1),N7(3)].

An intrastrand N7(1),N7(3) cross-link has been formed between the biologically inactive trans-DDP isomer and d(GpCpG) (Gibson and Lippard,

1987). The furanose ring on the 5'-platinated guanine was shown to be in the N-type, C(3')-endo conformation, similar to that in cis-Pt[d(-GpG-)] structures. This is contrary to the results obtained for d(GpXpG) structures formed with cis-DDP, where all of the furanose rings in the complex remain in the normal S-type, (C2')-endo conformation (den Hartog et al. 1983). In the trans complex, the majority of the protons were more desheilded than those in the cis complex, indicating even further intrastrand destacking in the trans-Pt[d(GpXpG)] structures.

Results for the crystal structure of cis-$Pt(NH_3)_2$[d(CpGpG)-N7(2),N7(3)] have been reported (Admiraal et al. 1987). The crystal structure is complex in that it contains three crystallographically independent molecules in the unit cell. There are many intermolecular interactions in the crystal which alter the conformation of the molecule with respect to its solution structure (den Hartog et al. 1983). Two intramolecular hydrogen bonds involving the ammine ligands were found. One ammine ligand forms a hydrogen bond with a phosphate group and the second ammine with a guanine O(6) site. Both of these interactions have been predicted in the previously discussed modeling studies (Hambley 1987, 1988a,b; Kozelka et al. 1987). Intramolecular stacking interactions between the cytosine(1) and the platinated guanine(2) were not present. This result was inconclusive, however, and most likely reflects the many intermolecular stacking and hydrogen bonding interactions resulting from crystal packing.

The platinated adducts of d(ApGpGpCpCpT) with both cis (Caradonna and Lippard 1988) and trans-DDP (Lepre et al. 1987) isomers have been characterized. The reactions with the platinum isomers were carried out

under conditions such that the self-complementary oligomer was duplexed. For the trans-DDP reaction, product analysis indicated that several products were formed during the reaction. These included monofunctional platinum-DNA adducts and interstrand platinum cross-linked DNA complexes, with the major product being the intrastrand bifunctional complex of trans-$Pt(NH_3)_2$[d(AGGCCT)-N7-A(1),N7-G(3)]. Approximately 40% of the starting oligomer was recovered unreacted. In the cis-DDP reaction, only one major product was isolated from the reaction, cis-$Pt(NH_3)_2$[d(AGGCCT)-N7-G(2),N7-G(3)], with no evidence for the formation of the possible N7-A(1),N7-G(2) adduct.

Characterization of the trans adduct revealed that the furanose ring of the platinated A(1) moiety had adopted a more N-type, C(3')-endo conformation, while the remaining furanose rings in the oligomer were in the S-type B-DNA conformation of C(2')-endo (Lepre et al. 1987). This repuckering of sugar conformation has been observed with cis-DDP 1,2-intrastrand cross-link adducts and with trans-Pt[d(GpCpG)-N7(1),N7(3)] (Gibson and Lippard 1987), but was not observed with cis-DDP 1,3-instrastrand complexes (Caradonna and Lippard, 1988).

Formation of cis-$Pt(NH_3)_2$[d(AGGCCT)-N7-G(2),N7-G(3)] from the self-complementary hexamer duplex resulted in a single stranded platinated adduct which would not participate in duplex formation even after unreacted d(AGGCCT) was added to the system. The platinated species appear to have undergone aggregation below 35°C as indicated by severe line broadening in the variable temperature ^1H NMR. The platinated oligomer did not exhibit single stranded base stacking with the decrease in temperature but rather remained destacked over the entire temperature

range. The furanose ring of the 5'-platinated guanine, G(2), was in a C(3')-endo conformation, while the remaining nucleotides retained the C(2')-endo conformation.

^{31}P NMR has been used for the investigation of platinum-DNA complexes and characterization of the hydrogen-bonding relationship between the platinum atom and backbone phosphate groups as a function of phosphorous chemical shift. The phosphate group involved in the phosphodiester linkage between the platinated guanines in a cis-Pt[d(GpG)] type adduct was shown to be deshielded with respect to the remaining phosphate groups on the oligomer (Spellmeyer Fouts et al. 1987). This result was shown to occur only with cis bifunctional complexes, and only when the adduct was formed with adjacent guanines. The exact chemical shift of the intervening phosphate was shown to have a dependancy on the bulk, and hydrogen bonding ability of the non-leaving amine ligand on the platinum, as well as the presence of either a phosphate or nucleotide 5' to the platinum complex (Spellmeyer Fouts et al. 1987,1988). The observed change in phosphorous chemical shift has been attributed to an unwinding of the backbone, resulting in a lengthening of this particular phosphodiester linkage. In addition to conformational changes induced by platinum binding, a further backbone elongation is suggested to result from hydrogen bonding between an amine ligand and the phosphate group 5' to the cis-Pt[d(GpG)] complex. When either the amine ligand was bulky or unable to participate in hydrogen bonding, or the cis-Pt[d(GpG)] complex did not have a 5'-phosphate present, the deshielding of the intervening phosphate group was not as strong. These results are consistant with the presence of a hydrogen

bond between the amine ligand and the phosphate group which is 5′ to the platinated region of the oligomer and, are in agreement with the predicted existance of such a hydrogen bond by molecular modeling calculations (Kolzeka et al. 1987, Hambley 1987) and crystal structure data (Sherman et al. 1985).

References

Aboul-ela, F., Koh, D., Tinoco, I. (1985) Nucleic Acids Res. 13, 48.

Adamaik, R.W., Barciszewska, M.Z., Biala, E., Grzeskowiak, K., Kierzek, R., Krazewski, A., Markiewicz, M.T., Wiewiorwski, A. (1976) Nucleic Acids Res. 3, 3397.

Admiraal,G., van der Veer,J.L., de Graaff,R.A.G., den Hartog,J.H.J., Reedijk,J., (1987) J. Am. Chem. Soc. 109, 592.

Aggarwal, S.K., Whitehouse, M.W., Ramachandran, C. (1980) in "Cisplatin, Current Status and New Developments", pp. 79-111, Presrayko, A.W., Crooke, S.T., Carter, S.K., Eds., Academic Press, New York.

Aggarwal, S.K. (1979) J. Cell Biol. 1758.

Alix, A.J.P., Bernard, L., Manfait, M., Ganguli, P.K., Theophanides, T. (1981) Inorg. Chim. Acta 55, 147.

Alkema, D, Hader, P.A., Bell, R.A., Neilson, T. (1982) Biochemistry 21, 2109.

Alkema, D, Bell, R.A., Hader, P.A., Neilson, T. (1981) J. Am. Chem. Soc. 103, 2866.

Altona, C. (1982) Recl. Trav. Chim. Pas-Bas 101, 413.

Altona, C., Sundaralingam, M. (1972) J. Am. Chem. Soc. 94, 8205.

Arnold, F.H., Wolk, S., Cruz, P., Tinoco, I (1987) Biochemistry 26, 4068.

Ball, C.R., Roberts, J.J. (1970) Chem.-Biol. Interact. 2, 321.

Bell, R.A., Everett, J.R., Hughes, D.W., Coddington, J.M., Alkema, D, Hader, P.A., Neilson, T. (1985) J. Biolmol. Struc. Dyn. 2, 693.

Bell, R.A., Everett, J.R., Hughes, D.W., Alkema, D., Hader, P., Neilson, T., Romaniuk, P. (1981) Biopolymers 20, 1383.

Bernges,F., Holler,E. (1988) Biochemistry 27, 6398.

Beyerle-Pfnur, R., Jauorski, S., Lippert, B., Schollhorn, H., Thewalt, U. (1985) Inorg. Chim. Acta 107, 217.

Borer, P.N., Kan, L.S., Ts'o, P.O.P. (1975) Biochemistry 14, 4847.

Bowler, B.E., Lippard, S.J. (1986) Biochemistry 25, 3031.

Braddock, P.D., Connors, T.A., Jones, M., Khokhar, A.R., Melzack, D.H., Tobe, M.L. (1975) Chem.-Biol. Interact. 11, 145.

Breslauer, K.J., Frank, R., Blocker, H., Marky, L.A. (1986) Proc. Natl. Acad. Sci. USA 83, 3746.

Brouwer, J., van de Putte, P., Fichtinger-Schepman, A.M.J., Reedijk, J. (1981) Proc. Natl. Acad. Sci. USA 78, 7010.

Bruckner, H.W., Cohen, C.C., Deppe, G., Kabakow, B., Wallach, R.C., Greenspan, E.M., Gusberg, S.B., Holland, I.F. (1977) J. Clin. Hematol. Oncol. 7, 619.

Byrd, A.R., Summers, M.F., Zon, G., Fouts, C.S., Marzilli, L.G. (1986) J. Am. Chem. Soc. 108, 504.

Cantor, C.R., Schimmel, P.R. (1980a) in "Biophysical Chemistry, Part III; The behaviour of biological macromolecules", p.1182, W.H. Freeman and Co., New York.

Cantor, C.R., Schimmel, P.R. (1980b), ibid, p.1154.

Caradonna,J.P., Lippard,S.J. (1988) Inorg. Chem. 27, 1454.

Caradonna, J.P., Lippard, S.J., Gait, M.J., Singh, M. (1982) J. Am. Chem. Soc. 104, 5793.

Catlin, J.C., Cramer, F. (1973) J. Org. Chem. 38, 245.

Chottard, J.C., Girault, J.P., Chottard, G., Lallemand, J.Y., Mansuy, D. (1980) J. Am. Chem. Soc. 102, 5565.

Chottard, J.C., Girault, J.P., Chottard, G., Lallemand, J.Y., Mansuy, D. (1980b) Nouv. J. Chim. 2, 551.

Chu, Y.G., Tinoco, I. (1983) Biopolymers 22, 1235.

Cleare, M.J. (1977) J. Clin. Hematol. Oncol. 7, 562.

Clore, G.M., Kimber, B.J., Gronenborn (1983) J. Mag. Res. 54, 170.

Cohen, G.L., Ledner, J.A., Bauer, W.R., Ushay, H.M., Carvana, C., Lippard, S.J. (1980) J. Am. Chem. Soc. 102, 2487.

Cohen, G.L., Bauer, W.R., Barton, J.K., Lippard, S.J. (1979) Science 203, 1014.

Connors, T.A., Jones, M., Ross, W.C.J., Braddock, P.D., Khokhar, A.R., Tobe, M.L. (1972) Chem.-Biol. Interact. 5, 415.

Dhara, S.C. (1970) Indian J. Chem. 8, 193.

den Hartog, J.H.J., Altona, C., van Boom, J.H., van der Marel, G.A., Haasnoot, C.A.G., Reedijk, J. (1985a) J. Biomol. Struct. Dyn. 2, 1137.

den Hartog, J.H.J., Altona, C., van der Marel, G.A., Reedijk, J. (1985b) Eur. J. Biochem. 147, 371.

den Hartog, J.H.J., Altona, C., van den Elst, H., van der Marel, G.A., Reedijk, J. (1985c) Inorg. Chem. 24, 986.

den Hartog, J.H.J., Altona, C., van Boom, J.H., Reedijk, J. (1984a) FEBS Lett. 176, 393.

den Hartog, J.H.J., Altona, C., van Boom, J.H., van der Marel, G.A., Haasnoot, C.A.G., Reedijk, J. (1984b) J. Am. Chem. Soc. 106, 1528.

den Hartog, J.H.J., Salm, M.L., Reedijk, J. (1984c) Inorg. Chem. 23, 2001.

den Hartog, J.H.J., van den Elst, H., Reedijk, J. (1984d) J. Inorg. Biochem. 21, 83.

den Hartog, J.H.J., Altona, C., van Boom, J.H., Marcelis, A.T.M., van der Marel, G.A., Rinkel, L.J., Wille-Hazeleger, G., Reedijk. J. (1983) Eur. J. Biochem. 134, 485.

den Hartog, J.H.J., Altona, C., Chottard, J.C., Girault, J.P., Lallemand, J.Y., de Leeuw, F.A.A.M., Marcelis, A.T.M., Reedijk, J. (1982) Nucl. Acids Res. 10, 4715.

Dijt, F.J., Canters, G.W., den Hartog, J.H.J., Marcelis, A.T.M., Reedijk, J. (1984) J. Am. Chem. Soc. 106, 3644.

Donohue, J., Trueblood, K.N. (1960) J. Mol. Biol. 2, 363.

Eapen, S., Green, M., Ismail, I.M. (1985) J. Inorg. Biochem. 24, 233.

Early, T.A., Olmsted, J., Kearns, D.R., Lezius, A.G. (1978) Nucl. Acids Res. 5, 1955.

Eastman, A., Barry, M.A. (1987) Biochemistry 26, 3303.

Eastman, A. (1986) Biochemistry 25, 3912.

Eastman, A. (1985) Biochemistry 24, 5027.

Eastman, A. (1983) Biochemistry 22, 3927.

Einhorn, L.H., Furnas, B.E., Powell, N. (1976) Proc. Am. Soc. Clin. Oncol. 17, 240.

England, T.E., Neilson, T. (1977) Can. J. Chem. 55, 365.

England, T.E., Neilson, T. (1976) Can. J. Chem. 54, 1714.

Evans, D.H., Morgan, A.R. (1982) J. Mol. Biol. 160, 117.

Faggiani, R., Lippert, B., Lock, C.J.L., Sperinzini, R.A. (1981a) J. Am. Chem. Soc. 103, 1111.

Faggiani, R., Lock, C.J.L., Pollock, R.J., Rosenberg, B., Turner, G. (1981b) Inorg. Chem. 20, 804.

Fazakerley, G.V., Hermann,D., Guschlbauer, W. (1980) Biopolymers 19, 1299.

Feigon, J., Denny, W.A., Leupin, W., Kearns, D.R. (1983) Biochemistry 22, 5930.

Fichtinger-Schepman, A.M.J., van der Veer, J.L., den Hartog, J.H.J., Lohman, P.H.M., Reedijk, J. (1985) Biochemistry 24, 707.

Fichtinger-Schepman, A,M.J., Lohman, P.H.M., Reedijk, J. (1982) Nucl. Acids Res. 10, 5345.

Filipski, J., Kohn, K.W., Bonner, W.M. (1980) Chem.-Biol. Interact. 32, 321.

Fink, T.R., Crothers, D.M. (1972) J. Mol. Biol. 66, 1.

Foster, P.L., Eisenstadt, E., Miller, J.H. (1983) Proc. Natl. Acad. Sci. USA 80, 2695.

Ganguli, P.K., Theophanides, T. (1981) Inorg. Chim. Acta 55, L43.

Ganguli, P.K., Theophanides, T. (1979) Eur. J. Biochem. 101, 377.

Gerchman, L.L., Ludlum, D.B. (1973) Biochim. Biophys. Acta 308, 310.

Gibson,D., Lippard,S.J. (1987) Inorg. Chem. 26, 2275.

Giessner-Prettre, C., Pullman, B. (1976a) Biopolymers 15, 2277.

Giessner-Prettre, C., Pullman, B. (1976b) Biochem. Biophys. Res. Commun. 70, 578.

Girault, J.P., Chottard, G., Lallemand, J.Y., Huguenin, F., Chottard, J.C. (1984a) J. Am. Chem. Soc. 106, 7227.

Girault, J.P., Chottard, J.C., Neumann, J.M., Tran-Dinh, S., Huynh-Dinh, T., Ingolen, J. (1984b) Nouv. J. Chim. 8, 7.

Girault, J.P., Chottard, G., Lallemand, J.Y., Chottard, J.C. (1982a) Biochemistry 21, 1352.

Girault, J.P., Chottard, G., Guittet, E.R., Lallemand, J.Y., Huynh-Dinh, T., Ingolen, J. (1982b) Biochem. Biophys. Res. Commun. 109, 1157.

Goodgame, D.M.L., Jeeves, I., Phillips, F.L., Skapski, A.C. (1975) Biochim. Biophys. Acta 378, 153.

Gralla, J., Crothers, D.M. (1973) J. Mol. Biol. 78, 301.

Greene, R.F., Chatterji, D.C., Hiranaka, P.K., Gallelli, J.F. (1979) Am. J. Hosp. Pharm. 36, 38.

Gronenborn, A.M., Clore, G.M. (1985) Prog. Nucl. Magn. Reson. Spectrosc. 17, 1.

Haasnoot, C.A.G., de Bruin, S.H., Berendsen, R.G., Janssen, H.G.J.M., Binnendijk, T.J.J., Hilbers, C.W., van der Marel, G.A., van Boom, J.H. (1983) J. Biomolec. Struct. Dyn. 1, 115.

Haasnoot, C.A.G., den Hartog, J.H.J., de Rooij, J.F.M., van Boom, J.H., Altona, C. (1980) Nucl. Acids Res. 8, 169.

Haasnoot, C.A.G., den Hartog, J.H.J., de Rooij, J.F.M., van Boom, J.H., Altona, C. (1979) Nature 281, 235.

Hadjiliadis, N., Theophanides, T. (1976) Inorgan. Chim. Acta 16, 77.

Hambley, T.W. (1988a) J. Chem. Soc., Chem. Commun., 221.

Hambley, T.W. (1988b) Inorg. Chem. 27, 1073.

Hambley, T.W. (1987) Inorg. Chim. Acta. 137, 15.

Harder, H.C., Smith, R.G., Leroy, A.F. (1976) Cancer Res. 36, 3821.

Heyl, B.L., Shinozuka, K., Miller, S.K., van der Veer, D.G., Marzilli, L.G. (1985) Inorg. Chem. 24, 661.

Hilbers, C.W., Haasnoot, C.A.G., de Bruin, S.H., Joordenx, J.J.M., van der Marel, G.A., van Boom, J.M. (1985) Biochimie 67, 685.

Hilbers, C.W., Patel, D.J. (1975) Biochemistry 14, 2656.

Hitchcock, A.P., Lock, C.J.L., Pratt, W.M.C., Lippert, B. (1983) in "Platinum, Gold, and Other Metal Chemotheraputic Agents", p. 209, ACS Symposium Series 209, Lippard, S.J., Ed., Amer. Chem. Soc., Washington, D.C..

Horacek, P., Drobnick, J. (1971) Biochem. Biophys. Acta 254, 341.

Howle, J.A., Gale, G.R. (1970) Biochem. Pharmacol. 19, 2757.

Inagaki, K., Kidani, Y. (1986) Inorg. Chem. 25, 3.

Inagaki, K., Kidani, Y. (1985) Inorg. Chim. Acta 106, 187.

Inagaki, K., Kasuya, K., Kidani, Y. (1984) Chem. Lett. 171.

Izatt, R.M., Christensen, J.J., Rytting, J.H. (1971) Chem. Rev. 71, 439.

Jenkins, B.G., Wartell, R.M., Alderfer, J.L. (1986) Biopolymers 25, 823.

Johnson, N.P., Mazard, A.M., Escalier, J., Macquet, J.P. (1985) J. Am. Chem. Soc. 107, 6376.

Johnson, N.P., Macquet, J.P., Wiebers, J.L., Monsarrat, B. (1982) Nucl. Acids Res. 10, 5255.

Johnson, N.P., Hoeschele, J.D., Rahn, R.O. (1980) Chem.-Biol. Interact. 30, 151.

Jones, R.A. (1984) in "Oligonucleotide Synthesis: a practical approach", pp. 23-34, Gait, M.J., Ed., IRL Press, Oxford.

Jones, S.S., Rayner, B., Reese, C.B., Ubasawa, A., Ubasawa, M. (1980) Tetrahedron 36, 3075.

Kan, L.S., Cheng, D.M., Jayaraman, C.K., Leutzinger, E.E., Miller, P.S., Ts'o, P.O.P. (1982) Biochemistry 21, 6723.

Kataguri, M., Ikatura, K., Narang, S.A. (1974) J. Am. Chem. Soc. Chem. Comm. 355.

Kelman, A.D., Peresie, H.J., Stone, P.J. (1977) J. Clin. Hematol. Oncol. 7, 440.

Kistenmacher, T.J., Wilkowski, K., deCastro, B., Chiang, C.C., Marzilli, L.G. (1979) Biochem. Biophys. Res. Comm. 91, 1521.

Khorana, H.G. (1978) Bioinorganic Chem. 7, 351 (and references therein).

Kleinwachter, V., Vrana, O., Brabec, V., Johnson, N.P. (1988) Studia Biophys. 123, 85.

Kong, P.C., Theophanides, T. (1975) Bioinorg. Chem. 5, 51.

Kozelka, J., Archer, S., Petsko, G.A., Lippard, S.J., Quigley, G.J. (1987) Biopolymers 26, 1245.

Kozelka, J., Petsko, G.A., Quigley, G.J., Lippard, S.J. (1986) Inorg. Chem. 25, 1075.

Kozelka, J., Petsko, G.A., Lippard, S.J., Quigley, G.J. (1985) J. Am. Chem. Soc. 107, 4079.

Krakoff, I.H., Lippman, A.J. (1974) Recent Results Cancer Res. 48, 183.

Laoui, A., Kozelka, J., Chottard, J.-C. (1988) Inorg. Chem. 27, 2751.

Le Goff, E. (1964) J. Org. Chem. 29, 2048.

Lepre, C.A., Strothkamp, K.G., Lippard, S.J. (1987) Biochemistry 26, 5651.

Lippard, S.J. (1983) "Platinum, Gold and Other Metal Chemotheraputic Agents", ACS Symposium Series 209, Lippard, S.J., Ed., American Chemical Society, Washington, D.C..

Lippert, B., Schubert, U. (1981) Inorg. Chim. Acta 56, 15.

Lock, C.J.L., Peresie, H.J., Rosenberg, B., Turner, G. (1978) J. Am. Chem. Soc. 100, 371.

Lock, C.J.L., Sperinzini, R.A., Powell, J. (1976a) Can. J. Chem. 54, 53.

Lock, C.J.L., Sperinzini, R.A., Turner, G., Powell, J. (1976b) J. Am. Chem. Soc. 98, 7865.

Loveless, A. (1969) Nature 233, 206.

Macquet, J.P., Butour, J.L., Johnson, N.P. (1983) in "Platinum, Gold, and Other Metal Chemotheraputic Agents", pp. 75-100, ACS Symposium Series 209, Lippard, S.J., Ed., Amer. Chem. Soc., Washington, D.C..

Macquet, J.P., Butour, J.L. (1978) Bichimie 60, 901.

Macquet, J.P., Theophanides, T. (1975) Bioinorg. Chem. 5, 59.

Malinge, J.M., Schwartz, A., Leng, M. (1987) Nucl. Acids Res. 15, 1779.

Malinge, J.M., Ptak, M., Leng, M. (1984) Nucl. Acids Res. 12, 5767.

Mansy, S., Rosenberg, B., Thoomson, A.J. (1973) J. Am. Chem. Soc. 95, 1633.

Marcelis, A.T.M., Erkelens, C., Reedijk, J. (1984) Inorg. Chim. Acta 91, 129.

Marcelis, A.T.M., den Hartog, J.H.J., van der Marel, G.A., Wille, G., Reedijk, J. (1983a) Eur. J. Biochem. 135, 343.

Marcelis, A.T.M., Reedijk, J. (1983b) Recl. Trav. Chim. Pays-Bas 102, 121.

Marcelis, A.T.M., den Hartog, J.H.J., Reedijk, J. (1982) J. Am. Chem. Soc. 104, 2664.

Marky, L.A., Blumenfeld, K.S., Kozlowski, S., Breslauer, K.J. (1983) Biopolymers 22, 1247.

Martin, R.B. (1985) Acc. Chem. Res. 18, 32.

Marzilli, L.G., Reily, M.D., Heyl, B.L., McMurray, C.T., Wilson, W.D. (1984) FEBS Lett. 176, 389.

Marzilli, L.G., Chalilpoyil, P. (1980a) J. Am. Chem. Soc. 102, 873.

Marzilli, L.G., Chalilpoyil, P., Chiang, C.C., Kistenmacher, T.J. (1980b) J. Am. Chem. Soc. 102, 2480.

Millard, M.M., Macquet, J.P., Theophanides, T. (1975) Biochem. Biophys. Acta 402, 166.

Millar, D.P., Ho, K.M., Aroney, M.J. (1988) Biochemistry 27, 8599.

Miller, K.J., Taylor, E.R., Basch, H., Krauss, M., Stevens, W.J. (1985) J. Biomol. Struct. Dyn. 2, 1157.

Miller, S.K., Marzilli, L.G. (1985) Inorg. Chem. 24, 2421.

Morden, K.M., Chu, G.Y., Martin, F.H., Tinoco, I. (1983) Biochemistry 22, 5557.

Munchausen, L.L., Rahn, R.O. (1975) Biochem. Biophys. Acta 414, 242.

Narang, S.A., Jacob, T.M., Khorana, H.G. (1965) J. Am. Chem. Soc. 87, 2988.

Neilson, T., Werstiuk, E. (1971a) Can. J. Chem. 49, 493.

Neilson, T., Werstiuk, E. (1971b) Can. J. Chem. 49, 3004.

Neilson, T. (1969) Chem. Comm. 1139.

Neumann, J.M., Tran-Dinh, S., Girault, J.P., Chottard, J.C., Huynh-Dinh, T. (1984) Eur. J. Biochem. 141, 465.

Okamoto, K., Behnam, V., Theophanides, T. (1985) Inorg. Chim. Acta 108, 237.

Ornstein, R.L., Fresco, J.R. (1983a) Biopolymers 22, 1979.

Ornstein, R.L., Fresco, J.R. (1983b) Biopolymers 22, 2001.

Otter, B.A., Fox, J.J. (1973) in "Synthetic Procedures in Nucleic Acid Chemistry", Vol. 1, p.285, Zorbach, W.W., Ed.; J. Wiley & Sons Inc., Toronto.

Pardi, A., Morden, K.M., Patel, D.J., Tinoco, I. (1982) Biochemistry 21, 6567.

Pascoe, J.M., Roberts, J.J. (1974a) Biochem. Pharmacol. 23, 1345.

Pascoe, J.M., Roberts, J.J. (1974b) Biochem. Pharmacol. 23, 1359.

Pasini, A., Zunino, F. (1987) Angew. Chem. Int. Ed. Eng. 26, 615.

Pasini, A., Bersanetti, E. (1985) Inorg. Chim. Acta 107, 259.

Patel, D.J., Shapiro, L., Hare, D. (1986) Biopolymers 25, 693.

Patel, D.J., Kozlowski, S.A., Hare, D.R., Reid, B., Ikuta, S., Lander, N., Itakura, K. (1985a) Biochemistry 24, 926.

Patel, D.J., Kozlowski, S.A., Weiss, M., Bhatt, R. (1985b) Biochemistry 24, 936.

Patel, D.J., Kozlowski, S.A., Ikuta, S., Itakura, K. (1984a) Biochemistry 23, 3207.

Patel, D.J., Kozlowski, S.A., Ikuta, S., Itakura, K. (1984b) Biochemistry 23, 3218.

Patel, D.J., Kozlowski, S.A., Ikuta, S., Itakura, K. (1984c) Fed. Proc., Fed. Am. Soc. Exp. Biol. 43, 2663.

Patel, D.J., Pardi, A., Itakura, K. (1982a) Science 216, 581.

Patel, D.J., Kozlowski, S.A., Marky, L.A., Rice, J.A., Broka, C., Dallas, J., Itakura, K., Breslauer, K.J. (1982b) Biochemistry 21, 437.

Patel, D.J., Kozlowski, S.A., Marky, L.A., Rice, J.A., Broka, C., Itakura, K., Breslauer, K.J. (1982c) Biochemistry 21, 445.

Patel, D.J. (1976) Bioplymers 15, 533.

Patel, D.J., Hilbers, C.W. (1975a) Biochemistry 14, 2651.

Patel, D.J., Tonelli, A.E. (1975b) Biochemistry 14, 3990.

Polissiou, M., Viet, M.T.P., St.Jacques, M., Theophanides, T. (1985) Inorg. Chim. Acta 107, 203.

Raudaschl-Sieber, G., Marzilli, L.G., Lippert, B., Shinozuka, K. (1985a) Inorg. Chem. 24, 989.

Raudaschl-Sieber, G., Lippert, B. (1985b) Inorg. Chem. 24, 2426.

Raudaschl-Sieber, G., Schollhorn, H., Thewalt, U., Lippert, B. (1985c) J. Am. Chem. Soc. 107, 3591.

Razaka, H., Wimmer, F., Wimmer, S., Villani, G., Johnson, J.P. (1987) Chem. Biol. Interact. 61, 265.

Reedijk, J. (1987) Pure & Appl. Chem., 59, 181.

Reedijk, J. (1984) in "Platinum Coordination Complexes in Cancer Chemotherapy". pp. 3-8, Hacker, M.P., Douple, E.B., Krakoff, I.F., Eds., M. Nijhoff, Boston.

Reese, C.B. (1978) Tetrahedron 34, 3143 (and references therein).

Reese, C.B., Titmas, R.C., Yau, L. (1978) Tetrahedron, 2727.

Reily, M.D., Marzilli, L.G. (1985) J. Am. Chem. Soc. 107, 4916.

Revert, B., Malinge, J.M., Delain, E., Le Bret, M., Leng, M. (1984) Nucl. Acids Res. 12, 8349.

Richmond, R.C., Powers, E.L. (1976) Radiat. Res. 68, 251.

Roberts, J.J., Pera, M.F. (1983) in "Platinum, Gold, and Other Metal Chemotherapeutic Agents". pp. 2-26, ACS Symposium Series 209, Lippard, S.J., Ed., Amer. Chem. Soc., Washington, D.C..

Roberts, J.J., Thomson, A.J. (1979) Prog. Nucl. Acid Res. Mol. Biol. 22, 71.

Roberts, J.J., Brent, T.P., Crathorn, A.R. (1971) Eur. J. Cancer 7, 515.

Romaniuk, P.J., Hughes, D.W., Gregoire, R.J., Bell, R.A., Neilson, T. (1979) Biochemistry 18, 5109.

Roos, I.A.G. (1977) Chem.-Biol. Interact. 16, 39.

Rosenberg, B. (1978) Biochimie 60, 859.

Rosenberg, B. (1977) in "Inorganic and Nutritional Aspects of Cancer", pp. 129-150, Schrauzer, G.N., Ed., Plenum Press, New York.

Rosenberg, B., van Camp, L., Trosko, J.E., Mansour, V.H. (1969) Nature 222, 385.

Rosenberg, B., van Camp, L., Krigas, T. (1965) Nature 205, 698.

Roy, S., Sklenar, V., Appella, E., Cohen, J.S. (1987) Biopolymers 26, 2041.

Saenger, W. (1984a) in "Principles of Nucleic Acid Structure", p.253, Cantor C.R., Ed., Springer-Verlag, New York.

Saenger, W. (1984b) ibid, p.145.

Saenger, W. (1984c) ibid, p.148.

Saenger, W. (1984d) ibid, p.203.

Saenger, W. (1984e) ibid, p.108.

Sarma, R.H. (1980) in "Nucleic Acid Geometry and Dynamics", p.1, Sarma, R.H., Ed., Permagon Press, Elmsford, N.Y..

Schaller, W., Hermine, R., Holler, E. (1987) Biochemistry 26, 943.

Schaller, H., Weiman, G., Lerch, B., Khorana, H.G. (1963) J. Am. Chem. Soc. 85, 3821.

Scovell, W.M., Collart, F. (1985) Nucl. Acids Res. 13, 2881.

Scovell, W.M., Capponi, V.J. (1984) Biochem. Biophys. Res. Comm. 124, 367.

Segal, E., Le Pecq, J.B. (1985) Cancer Res. 45, 492.

Segal, E., Girault, J.P., Muzard, G., Chottard, G., Chottard, J.C., Le Pecq, B. (1984) Biopolymers 23, 1623.

Sherman, S.E., Gibson, D., Wang, A.H.-J., Lippard, S.J. (1985) Science 230, 412.

Shooter, K.V., Howse, R., Merrifield, R.K., Robins, A.B. (1972) Chem-.Biol. Interact. 5, 289.

Sinex, F., Stone, P.J. (1974) in "Recent results in Cancer Chemotherapy", Connors, T.A., Roberts, J.J., Eds., Springer, New York.

Smith, M., Rammler, D.H., Goldberg, I.H., Khorana, H.G. (1962) J. Am. Chem. Soc. 84, 430.

Sood, A.K., Narang, S.A. (1977) Nucleic Acids Res. 4, 2757.

Spassovska, N.H., Pelova, R.G., Wolowiec, S., Jezowska-Trzebiatowska, B. (1985) Inorg. Chim. Acta 106, 171.

Spellmeyer Fouts, C., Reily, M.D., Marzilli, L.G. (1987) Inorg. Chim. Acta. 137, 1.

Spellmeyer Fouts, C., Marzilli, L.G., Byrd, R.A., Summers, M.F., Zon, G., Shinouzuka, K. (1988) Inorg. Chem. 27, 366.

Srivastava, R.C., Froehlich, J., Eichhorn, G.L. (1978) Biochimie 60, 879.

Stawinski, J., Hozumi, T.A., Narang, S.A., Bahl, C.P., Wu, R. (1977) Nucleic Acids Res. 4, 353.

Stone, P.J., Kelman, A.D., Sinex, F.M. (1974) Nature 251, 736.

Sundaralingam, M. (1973) in "The Jerusalem Symposia on Quantum Chemistry and Biochemistry", Vol.5, p.417, Bergmann, E.D., Pullman, B., Eds., The Isreal Academy of Sciences and Humanities, Jerusalem.

Sundquist, W.I., Bancroft, D.P., Chassot, L., Lippard, S.J. (1988) J. Am. Chem. Soc. 110, 8559.

Sundquist, W.I., Lippard, S.J., Stollar, B.D. (1987) Proc. Natl. Acad. Sci. USA 84, 8225.

Sundquist, W.I., Lippard, S.J., Stollar, B.D. (1986) Biochemistry 25, 1520.

Tamburro, A.M., Celotti, L., Furlan, D., Guantieri, V. (1977) Chem.-Biol. Interact. 16, 1.

Tibanyenda, N., DeBruin, S.H., Haasnoot, C.A.G., Van Der Marel, G.A., Van Boom, J.H., Hilbers, C.W. (1984) Eur. J. Biochem. 139, 19.

Tinoco, I., Uhlenbeck, O.C., Levine, M.D. (1971) Nature 230, 362.

Tran-Dinh, S., Neumann, J.M., Taboury, J., Huynh-Dinh, T., Renous, S., Genissel, B., Ingolen, J. (1983) Eur. J. Biochem. 133, 579.

Tullius, T.D., Ushay, H.M., Merkel, C.M., Caradonna, J.P., Lippard, S.J. (1983) in "Platinum, Gold, and Other Metal Chemotherapeutic Agents". pp. 51-74, ACS Symposium Series 209, Lippard, S.J., Ed., Amer. Chem. Soc., Washington, D.C..

Tullius, T.D., Lippard, S.J. (1982) Proc. Natl. Acad. Sci. USA 79, 3489.

Tullius, T.D., Lippard, S.J. (1981) J. Am. Chem. Soc. 103, 4620.

van der Veer, J.L., van der Marel, G.A., van den Elst, H., Reedijk, J. (1987) Inorg. Chem. 26, 2272.

Van Hemelryck, B., Guittet, E., Chottard, G., Girault, J.P., Huynh-Dinh, T., Lallemand, J.Y., Igolen, J., Chottard, J.C. (1984) J. Am. Chem. Soc. 106, 3037.

Visentin, D. (1984) M. Sc. Thesis, McMaster University, Hamilton, Ontario, Canada.

Wallace, H.J., Higby, D.J. (1974) Recent Results Cancer Res. 48, 167.

Watson, J.D., Crick, G.H.C. (1953) Nature 171, 737.

Weiss, M.A., Patel, D.J., Sauer, R.T., Karplus, M. (1984) Nucleic Acids Res. 12, 4035.

Wemmer, D.E., Chou, S.H., Hare, D.R., Reid, B.R. (1985a) Nucl. Acids Res. 13, 3755.

Wemmer, D.E., Benight, A.S. (1985b) Nucl. Acids Res. 13, 8611.

Wiltshaw, E., Carr, B. (1974) Cancer Res. 48, 178.

Wing, R.M., Pjura, P., Drew, H.R., Dickerson, R.E. (1984) EMBO J. 3, 1201.

Woodson, S.A., Crothers, D.M. (1988) Biochemistry 27, 436.