Studies of the Use of Derivatised Polycations as Potential Drug Delivery Systems to DNA

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for the degree of

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in the

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STATEMENT

This thesis is submitted for the degree of Doctor of Philosophy and is entitled "Studies of the Use of Derivatised Polycations as Potential Drug Delivery Systems to DNA". It is based upon work carried out by the author in the Department of Chemistry at the University of Leicester during the period October 1986 to October 1989. All of the work described herein is original unless otherwise acknowledged in the text or by references. This work is not being presented for any other degree.

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.

ABSTRACT

Studies of the Use of Derivatised Polycations as Potential Drug Delivery Systems to DNA

The major target of ionising radiation has been determined as cellular DNA. Damage to DNA, as detected at 77K under conditions of direct damage by ESR, is localised on the bases thymine and guanine. This damage leads to single and double strand breaks, precursors of cell death and mutagenesis.

In an attempt to intercept the damage at the bases, before formation of strand breaks, the use of polycations as potential drug delivery systems to DNA has been examined.

Magnetic resonance techniques have been used to establish that polyamines used are present almost completely as polyammonium cations at pH 7 and to probe the interactions of a number of polycations with DNA.

Sodium-NMR was used to investigate the affinity of polyamines, polyaminothiols and transition-metal complexes for DNA, via sodium ion displacement from the DNA region. It was found that small metal complexes displace a greater number of sodium ions than polyamines of similar charge. Application of the counterion condensation theory led to a model of the counterions existing within a cylinder around the DNA of approximate radius 20Å.

The mode of interaction of polyammonium cations was studied using proton magnetic resonance. Linewidths, related to the transverse relaxation rate, give information on the motion of compounds close to DNA. Comparison of linewidths in the presence and absence of DNA revealed no significant broadening. This was interpreted as indicative of a loose, electrostatic interaction, not significantly hindering motion of the cations close to DNA, suggesting rapid motion of polyammonium ions along the DNA.

The radioprotection of DNA by various transition-metal complexes was studied using ESR. Certain compounds exhibited protection via electron transfer, resulting in a decreased radical yield.

Definition of Terms

Abbreviations are generally defined in the text but common ones and definitions of terms used are listed below.

Territorially bound cations are those that retain their full hydration sphere, characteristic of a pure aqueous environment, while moving in an unrestricted and random way around the DNA helix.

Discretely bound cations are those counterions in direct contact with one or more charged groups on the polyion, resulting in affected motion of the counterion.

The aminothiol prefixes WR and RW stand for Walter Reed (Army Institute, U.S.) and Richard Wheelhouse (Ph.D. Leicester, 1989) respectively.

Norspermidine	=	3,3'-diamino-N-methyldipropylamine	
diNOsar	=	dinitrosarcophagine	
diAMsar	=	diaminosarcophagine	
sarcophagine = 3,6,10,13,16,19-hexaazabicyclo[6.6.6]eicosa		3,6,10,13,16,19-hexaazabicyclo[6.6.6]eicosane	
cyclam	=	1,4,8,11-tetraazacyclotetradecane	
metronidazole	=	2-methyl-5-nitro-1-imidazoleethanol	
dien	=	diethylenetriamine	
trien	=	triethylenetetramine	
tetren = tetraethylenepentamine DSS = sodium 2,2-dimethyl-2-silapentane-5-sulpho		tetraethylenepentamine	
		sodium 2,2-dimethyl-2-silapentane-5-sulphonate	
NOE	=	nuclear Overhauser effect	
N/P	Ξ	number of counterions per DNA phosphate	
9S3	Ξ	1,4,7-trithiacyclononane	
16S4	=	1,5,9,13-tetrathiacyclohexadecane	

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B Publication

A

Chapter 1

Introduction

1.1 Introduction

The latter part of the twentieth century has seen a greater awareness of the potential dangers of ionising radiation and identification of the target site whose damage is primarily responsible for radiation-induced cell injury has been the subject of much investigation. It is now generally agreed that the principal target site in mammalian cells is DNA [1,2,3]. Radiation-induced changes in DNA have been related to replication errors, gene malfunction and chromosome aberration [4,5,6]. In order to understand the effects of ionising radiation on DNA a brief outline of the structure is given. A more extensive discussion may be found elsewhere [7].

1.2 The Structure of DNA

DNA is the nucleic acid responsible for storing the genetic information of the cell and is located in the nucleus. Ribonucleic acid (RNA) is responsible, in its messenger form, for the transcription of genetic information from the cell nucleus to the ribosomes (the site of protein synthesis) in the cytoplasm. The basic structure is of unbranched polymeric molecules consisting of repeating units, called nucleotides, of a nitrogenous purine or pyrimidine base, a five carbon (deoxy) ribose sugar and a phosphate group. Each nucleotide contains one of four different bases. Adenine, guanine and cytosine are the same in both DNA and RNA, whilst the fourth is thymine in DNA and uracil in RNA (Figure 1.1).

In both DNA and RNA the ribose sugars are in the D configuration. However, DNA has no hydroxyl group on the second carbon of the sugar ring and is hence termed 'deoxyribo' as opposed to 'ribo' in RNA.



Figure 1.1: The common nucleic acid bases.

The nucleotide unit is constructed by the joining of the sugar C1' with the N1 or N9 of the purine or pyrimidine base respectively via a glycosidic linkage. The phosphate group is linked to the sugar at either C3' or C5' and the nucleotides are linked together through 5'- and 3'- sugar-phosphate bonds forming a phosphodiester backbone[8](Figure 1.2a).

The structure of double-stranded DNA consists of two helical polynucleotide strands running in opposite directions constrained by hydrogenbonding between the bases. This structure, first proposed by Watson and Crick [9], has the distance between base pairs at 3.4Å and the repeat distance of the helix at 34Å. The structure has one narrow and one broad groove, called the minor and major grooves respectively(Figure 1.2b).



Figure 1.2: (a) The primary structure of DNA. (b) Schematic representation of B-DNA double helix.

The primary stabilising factor in the double-helical structure is hydrogen-bonding between the bases. The geometry of the structure only permits pairing between purine and pyrimidine bases and the structure of the bases is such that adenine forms two hydrogen bonds with thymine and guanine forms three hydrogen bonds with cytosine(Figure 1.3). Further stability is lent to the configuration by the overlapping π -orbitals of the bases as they 'stack' on top of each other in the helix.

DNA is stabilised in the cell nucleus by positively charged proteins called histones and exists as tightly folded DNA-histone (nucleohistone)





Figure 1.3: The Watson-Crick base pairs.

fibres called chromatin. Nucleosomes, the nucleohistone repeat unit, consist of an octamer of histones surrounded by 200 base pairs of helical DNA[10,11,12]. The octamer comprises two tetramers made up from four of the five histones: $(H2A)_2$, $(H2B)_2$, $(H3)_2$ and $(H4)_2$. One hundred and forty of the base pairs are tightly linked to the octamer; the remaining 60, termed linker DNA, join the nucleosome particles together. The fifth histone, H1, appears to be associated with the linker DNA and is involved with holding the nucleosomes together[13].

1.3 Radiation Damage to DNA

Radiation damage to DNA is described by two mechanisms, direct damage and indirect damage. Direct damage can be stated as that which is produced in the same molecule in which the primary process occurred. Indirect damage in fluid aqueous systems is caused by diffusable products of the radiolysis of water[14,15].

1.3.1 Indirect Damage

As water constitutes about 70 - 80% of the mass of the cell the majority of cellular primary ionisations are likely to occur in the water phase. The radiation chemistry of water has been extensively studied[16] and hydroxyl radicals appear to be of dominating importance, addition at the bases being the major reaction of these with DNA. Attack on the bases, and also on the sugar moiety, via abstraction from a C-H bond, frequently leads to strand breaks[14,15]. One proposed mechanism by which this may occur is given in Figure 1.4.

Strand breaks are thought to be of major importance in cell death and mutagenesis[17,18,19]. Single strand breaks (s.s.b.'s) can be repaired efficiently *in vivo* by a number of enzyme-dependent systems[20]. However, the extent to which double strand breaks (d.s.b.'s) can be similarly repaired is less clear, the probable diffusion of the two resulting fragments making repair more difficult[21].



Figure 1.4: β -elimination mechanism of the 4'-sugar radical leading to strand breaks in DNA.

1.3.2 Direct Damage

The direct mechanism of radiation damage results in the formation of electron-gain and electron-loss centres in the DNA itself. Whilst the probability of a distinct ionisation of DNA in dilute aqueous solution is low [22], high concentrations in the cell nucleus make it an important mechanism.

The system most commonly used to study direct damage is frozen aqueous solutions. The majority of water molecules are present as ice crystallites and the concentration of water remaining with the DNA is probably only enough to solvate the ionic moieties and residual non-hydrogen bonded groups [23,24,25]. When additives are included the ice crystallites tend to exclude these molecules which then become part of the DNA solvation phase, even though there may be no tendency for the molecules to bind to DNA[21]. Damage to the ice crystals will be localised in the ice matrix and thus will not affect the DNA.



Figure 1.5: Structures of the base centred radicals identified by ESR.

The Site of Electron Loss

Electron loss in frozen aqueous systems must occur indiscriminately from solvating water, phosphate units, sugar moieties and bases. Formation of radicals from solvating water would allow attack on DNA as they are in the same phase. However, studies have shown that OH^{\cdot} radical formation (from the initial $H_2O^{\cdot+}$ by proton transfer) is not normally important[26], electron transfer from DNA bases being more rapid under these conditions. Similarly, electron loss from phosphate units does not result in trapped $(RO)_2PO_2^{\cdot}$ radicals[27] and $ROR^{\cdot+}$ centres are not observed[28,29]. The only site of electron loss, as detected by ESR at 77K, is the guanine radical cation $G^{\cdot+}$ (I)[29,30,31,32]. The primary sites of electron loss must therefore undergo electron transfer allowing the 'hole' to migrate to the base.

The Site of Electron Gain

Electron gain is likely to occur at the phosphate units and the stacked bases. Whereas attachment at simple phosphate esters has been established and monitored[27], none has been detected in the DNA system, suggesting that electron transfer to the bases is more rapid[21].

Whereas the site of electron loss has been determined to be exclusively at guanine, the site of electron gain is still uncertain, despite the fact that this is an important part of the direct damage process.

It is generally accepted that the pyrimidine bases, more electron affinic than the purines, are the site of electron capture[33]. However, the similarity of the ESR spectra of the proposed electron gain species, $T^{\cdot-}$ (II) and $C^{\cdot-}$ (III) has made the absolute determination of concentrations of $T^{\cdot-}$ and $C^{\cdot-}$ problematical. Both species have been characterised using cytosine and thymine derivatives[34]. Studies using the self-complementary oligomer d(pApGpCpT) in 12M LiCl at 4K and the simulated spectra of the single bases has found cytosine to be the preferred site of electron capture[35].

Similar studies using H_2O and D_2O glasses[36] suggest that the species in H_2O glasses is a protonated form of cytosine, $C^{\cdot-}(H^+)$. The $C^6 - H$ coupling in $C^{\cdot-}(H^+)$ is almost identical to $C^{\cdot-}$ and thus a different mechanism from that which gives TH^{\cdot} from $T^{\cdot-}$ must be operating. The C-protonated 5-thymyl radical, TH^{\cdot} (IV), has been clearly identified from its characteristic 8 line ESR spectrum[20,37,38]. The most reasonable mechanism of protonation of $C^{\cdot-}$ is protonation of the $-NH_2$ group rather than the ring nitrogen (V). Prevention of rotation of the $-NH_3^+$ group by hydrogen bonding could result in one large coupling. However, in duplex DNA systems this method of protonation is thought to be less likely than protonation at the H(3) site.

The possibility remains of an electron transfer mechanism between cytosine and thymine before protonation of cytosine to V, even at low temperatures, through the stacked bases. The fact that IV is detected may be a consequence not of thymine being the most electron affinic but rather that it is irreversibly protonated at C6, fixing the unpaired electron. Further work on the site of electron capture is in progress[39].

The Generation of Strand Breaks

Studies have shown that under conditions where only $G^{\cdot+}$ and $T^{\cdot-}$ are detected, raising the temperature results in strand breaks [20,29]. As radicals generated in the ice phase are lost at temperatures well below the melting point the strand breaks must arise from the $G^{\cdot+}$ and $T^{\cdot-}$ centres. The $T^{\cdot-}$ radical gives TH^{\cdot} at 130–208K but these decay in the range 208–240K with no clear evidence for the generation of other trapped radicals[21]. There are no detectable intermediates for $G^{\cdot+}$ on annealing in neutral systems but it is probable that hydration to form GOH^{\cdot} radicals(VI) is the first stage and these are detected in alkaline systems[40].

It is assumed that strand breaks arise from intramolecular H-atom abstraction by the base radical from the neighbouring sugar moiety. Strand breaks then occur by a similar mechanism to that proposed for conditions of indirect damage[14,15](Figure 1.6). Double strand breaks are presumed to occur from pairs of T^{--} and G^{++} on opposite strands[31]. The existence of such pairs within close proximity of each other would lead to rapid electron return, encouraged by large coulombic forces experienced by the pair.



Figure 1.6: Possible mechanism for the generation of strand breaks initiated by base radicals generated under conditions of direct damage. Pairs of radicals separated by more than 50-60Å, i.e. 16 base pairs, should behave as independent s.s.b.'s[41], but those within this separation, found on opposite strands, are expected to lead to d.s.b.'s on annealing (Figure 1.7).

1.4 The Protection of DNA

Research into the protection of living systems from the harmful effects of ionising radiation has been concentrated at the DNA-molecular level. The rôle of various small molecules acting as radical scavengers has been investigated, initially with respect to the hydroxyl radical, preventing their interaction with DNA and other macromolecules [42,43,44] and subsequently with radicals formed within the DNA molecule itself. These may be repaired [45,46,47,48,49,50], thus preventing strand breaks and other forms of damage.

It is expected that molecules with a high affinity for DNA will be the most effective at screening the DNA macromolecule from OH^{\cdot} radical attack and at repairing damage within the DNA. Thus, a number of polycations were studied as potential drug delivery systems to DNA. The basic class of compounds used was based upon the naturally occurring polyamines spermine and spermidine.

1.4.1 The Physiological Rôle of Polyamines

The rôle of polyamines in biological systems has been extensively studied although their full function is as yet unclear. Polyamines are present in essentially all living organisms at millimolar total intracellular concentrations[51]



Figure 1.7: Proposed mechanism for the formation of double strand breaks from pairs of radical cations and anions trapped close together.

[52]. Adequate concentrations of polyamines appear to be necessary for normal cell growth[53,54]. They are known to be involved in DNA, RNA and protein synthesis[55,56] and in the regulation of cell proliferative activity [55,57,58]. It has been shown that polyamines protect DNA against thermal denaturation[57], shear breakage [59] and radiation damage[60] and facilitate the transition of B-DNA to the Z-form[61].

At physiological pH ranges[62,63] spermine and spermidine exist as their polycations and thus a major factor in their activity is presumed to be the interaction between the cationic amino groups and the anionic phosphate backbone of DNA[64,65].

1.4.2 Study of Protonation Constants

There has been extensive work on the protonation constants of spermine and spermidine using a variety of techniques such as potentiometric titration [66], calorimetry[67] and proton, carbon-13 and nitrogen-15 NMR spectroscopy[68,69]. NMR is a particularly convenient method of study for the polyamines used in this work for which protonation data were not readily available.

The NMR Chemical Shift

Electrons shield a nucleus from the influence of the applied field B_o . The circulation of electrons about the nucleus generates a field aligned in such a way as to oppose the applied field[70].

$$B_{loc} = (1 - \sigma)B_o \tag{1.1}$$

where B_o , B_{loc} are the applied and local fields respectively. σ is the shielding constant, a small fraction usually listed in ppm. Shielding effects become more complicated for molecules, where the circulation of electrons is influenced by more than one positive centre, σ thus depending on the environment of the nucleus in the molecule.

The resonance condition governing NMR transitions is:

$$u = |\gamma/2\pi|B$$
 (1.2)

where

 ν = frequency of radiation causing transition

 $\gamma =$ magnetogyric ratio

Therefore, for nucleus j:

$$\nu_j = |\gamma/2\pi| B_o(1-\sigma_j) \tag{1.3}$$

 ν_j and σ_j refer to the resonance frequency (Larmor frequency) and shielding constant respectively for nucleus j. Variations in σ thus cause variations in resonance frequencies which gives rise to chemical shifts.

It can be seen from Equation 1.3 that resonance frequencies are proportional to the applied field B_o . Chemical shift differences are usually expressed as ppm. These are obtained by dividing through by ν_o , the operating frequency of the spectrometer ($\approx |\gamma B_o/2\pi|$). Thus, chemical shift differences are independent of spectrometer frequency and may be used as molecular characteristics.

The Effect of Protonation on Chemical Shift

Equation 1.3 may be rewritten:

$$\nu_j = \nu_o (1 - \sigma_j) \tag{1.4}$$

and chemical shift differences:

$$\Delta \nu / \nu_o = -\Delta \sigma \tag{1.5}$$

Therefore, in NMR spectra increased resonance frequency (to the left of the plot) expresses decreased shielding and *vice versa*.

To correct for variations in bulk magnetic susceptibility between samples, chemical shifts are usually measured relative to an internal reference (usually TMS), enabling direct comparison. Chemical shifts may be defined as:

$$\delta = 10^6 (\nu_{sample} - \nu_{TMS}) / \nu_{TMS} \text{ at } B_o$$
 (1.6)

and as shielding constants are small, the denominator may be replaced by the spectrometer operating frequency ν_o :

$$\delta = 10^6 (\sigma_{TMS} - \sigma_{sample}) \tag{1.7}$$

Circulation of electrons about nearby nuclei generates a field that can either oppose or reinforce the applied field at the proton. Groups adjacent to the proton that withdraw electron density by an inductive effect deshield the nucleus, whereas inductively electron-repelling groups cause shielding of the nucleus. On protonation of an amino group the withdrawal of electron density from the nitrogen nucleus and the resultant change in character of the nitrogen-carbon bond causes a deshielding of the proton nucleus causing a downfield chemical shift. The progress of a titration can thus be followed by monitoring the chemical shift of adjacent nuclei. Proton resonance shift studies appear to be more suitable than carbon-13 or nitrogen-15 studies[69,71] because distance effects from the more remote protonation sites are negligible.

1.5 Relaxation of Sodium Nuclei

The natural abundance of sodium-23 is 100% and its intrinsic NMR sensitivity is only a factor of 10 less than that of the proton. This makes it a very convenient and direct method for studying interactions between sodium and organic or bio-organic molecules[72]. The study of cation-DNA interactions using ^{23}Na -NMR depends upon the relaxation of the sodium ions and the effect this has on the linewidth of the sodium resonance[73,74,75,76].

Due to it having a spin number greater than 1/2 (I=3/2), sodium has an electric quadrupole moment. Distortion of the nucleus results in a nonspherical distribution of charge in the form of a prolate ellipsoidal distribution. An electric quadrupole interacts with an inhomogenous electric field, i.e. whenever the distribution of charges in the sodium coordination sphere is asymmetric. The quadrupole coupling constant, χ , expressed in terms of eQ, where e is the charge on the proton and Q is the electric quadrupole moment of the nucleus, is the product of this interaction.

$$\chi = (eQ.eq)/\hbar$$
 (1.8)

The ²³Na nucleus can assume (2I+1)=4 orientations in a magnetic field, B_o. In the absence of an electric field gradient at the nucleus, transitions between the energy states are degenerate, but this degeneracy is lifted by the quadrupolar interaction.

However, instead of the three resultant transitions being represented by three lines, the distance between each line being given by Equation 1.9,

$$dist = \frac{e^2 q Q}{\hbar} \cdot \frac{(3\cos^2 \theta - 1)}{4}$$
(1.9)

where θ is the angle between B_o and the direction of the field gradient q, in the liquid state θ fluctuates over all possible values due to fast reorientational motions. This results in the three lines collapsing to a single line having linewidth $\Delta \nu_{\frac{1}{2}}$, and the relaxation rate $R_i = T_i^{-1}$ (i=1,2) is given by:

$$R_i = \pi \Delta \nu_{\frac{1}{2}} = \frac{2\pi^2}{5} \cdot \left(\frac{e^2 qQ}{\hbar}\right)^2 \cdot \tau_c \tag{1.10}$$

where τ_c is a correlation time describing the reorientation of the quadrupolar interaction between the ²³Na nucleus and the local electric field gradient.

In the present study, electric field gradients arise from water dipoles solvating the sodium nucleus and the other charged species in solution, i.e. the DNA.

Sodium ions condensed close to the DNA helix have greatly enhanced relaxation rates, increasing from a value of about 18Hz for free $Na^+_{(aq)}$ to 170-220Hz for Na-DNA(aq) systems[77]. This enhanced relaxation results in substantially broadened lines in the ${}^{23}Na$ -NMR spectrum. Sodium ions are territorially bound to DNA, remaining non-localised, creating an ionatmosphere around the macromolecule[78] and it is assumed that the polyelectrolyte effects on relaxation rates and correlation times extend only to sodium ions within a certain radius of the immediately surrounding solution. Thus, ejection of sodium ions by competing counterions will affect the relaxation rate of these sodium ions and can be monitored by ${}^{23}Na$ -NMR spectroscopy.

1.5.1 The Two-State Model

The study of counterion binding to DNA uses the concept of the 'twostate' model[79,80,81]. This is an application of the counterion condensation model, developed by Manning[82,83,84,85] and considers two distinct environments for the sodium ions. "Territorially bound" cations are those that are close enough to the DNA to be influenced by its electric field gradient. They are not localised at specific sites on the DNA but are electrostatically held close to the DNA surface, whilst remaining almost fully hydrated[85]. "Free" cations are those that are far enough removed from the DNA so as to be essentially unaffected by its electric field.

1.5.2 Counterion Condensation

The local concentration of cations is not governed by the law of massaction, i.e. the local concentration does not tend to zero as the bulk concentration tends to zero. The local concentration is governed instead by the charge fraction of the polyelectrolyte. The charge fraction is itself independent of ionic strength and bulk concentration, depending only upon the valence of the counterion, N, and the axial charge density.

If b is the average axial charge spacing (along the helical axis for DNA) and the dimensionless parameter ξ is defined as proportional to the charge density then:

$$\xi = \frac{q^2}{\varepsilon k T b} \tag{1.11}$$

where;

- q = proton charge
- ε = relative permittivity of solvent
- k = Boltzmann's constant
- T = Kelvin temperature

Quantitatively, the physical observation is that the charge fraction is close to $(N\xi)^{-1}$. For DNA, the axial separation between phosphate pairs is 3.4Å, therefore b=1.7Å and in water at 25°C $\xi = 4.2$. The charge fraction of DNA in aqueous Na^+ is then 0.24, i.e. 76% of the phosphate charge is neutralised by sodium ions.

The operational definition of counterion condensation is therefore formulated as[85]

"the mode of binding of counterions of valence N to a polyelectrolyte is called condensation if, in an environment containing only the single counter ion species, the charge fraction of the polyelectrolyte equals the constant $(N\xi)^{-1}$ over a broad concentration range."



Figure 1.8: Schematic representation of the interaction of DNA with cationic drugs. a is the DNA cylinder width, R_M is the radius within which the counterions are said to be bound.

Thus, even when the polyelectrolyte is immersed in a 1:1 salt of vanishingly small concentration c_1 , the local concentration c_1^{loc} is approximately 1.2M[79], the majority of ions translating freely within approximately 30Å of the DNA surface[86,87,88].

$$c_1^{loc} = 10^3 \theta_1 V_p^{-1} = 24.3 (\xi b^3)^{-1} \tag{1.12}$$

where θ_1 =no. of associated counterions per fixed charge= $(1 - \xi^{-1})$ and V_p =volume of region surrounding the polyelectrolyte within which the cations are said to be bound.

The invariance of θ_1 , up to 0.1M, and the small deviations up to 1.0M, are given in Table 1.1. The calculated values demonstrate the theoretical

$c_1(M)$	$ heta_1$
0.0001	0.76
0.001	0.76
0.01	0.76
0.1	0.77
0.5	0.80
1.0	0.83

Table 1.1 Number of bound counterions per phosphate as a function of ionic strength for DNA (ξ =4.2).

invariance of θ_1 up to ionic strengths c_1 approaching c_1^{loc} . Experimental evidence reports no variation in the charge fraction up to $c_1 = 0.5M[79]$.

The ejection of sodium ions from the bound region by competing counterions (CC) (Figure 1.8) is governed by an equilibrium, the ejection of sodium tending to keep the charge fraction constant.

$$(CC)_f^{n+} + nNa_b^+ \rightleftharpoons (CC)_b^{n+} + nNa_f^+ \tag{1.13}$$

where b and f indicate bound and free ions respectively. The bound and free sodium ions are in rapid equilibrium themselves so the measured linewidths are weighted averages of the two states. Whilst the application of the counterion condensation model and the use of a two-state approximation for cations provides a useful frame of reference for the study of these systems the model must be, by definition, an oversimplified scheme, the regions of bound and free cations being arbitrary and approximate regions only, there being no actual radius beyond which sodium ions are suddenly no longer influenced by the DNA electric field gradient. However, for the purposes of this study, the model gives a good approximation.

1.6 Investigation of Potential Radioprotection Agents

In addition to the study of polyammonium cations as potential drug delivery systems, the use of non-intercalating transition-metal complexes was investigated. Mirroring the behaviour of polyamines, complexes should have a high affinity for DNA. The requirement of non-binding for the complexes, to facilitate possible repair over the length of the DNA helix has led to the study of complexes incorporating large macrocyclic ligands. Metal complex-DNA interactions that show anti-tumour activity involve specific binding to DNA[89,90], causing disruption of the local structure[91,92] and inhibiting repair and replication[93]. Complexes studied during the course of this study were chosen for possible redox activity with the DNA damage centres detected by ESR spectroscopy under conditions of direct damage[21].

Electron transfer in biological systems has been extensively reviewed [94,95,96,97] and there are well studied examples of long-distance electrontransfer involving metalloproteins[98,99,100] and cytochromes[101,102]. The idea of a redox agent capable of the restitution of these damage centres is an extension of these examples.

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Chapter 2

Experimental

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2.1 Preparative Details

2.1.1 Instrumentation

NMR spectra were obtained on a Bruker AM300 spectrometer operating at 300.15 MHz for protons and 75.47 MHz for carbon-13. All chemical shifts are reported relative to internal TMS or DSS. Spectra were recorded at 298K for proton and 300K for carbon. Infrared spectra were recorded on a Perkin Elmer Model 681 IR spectrophotometer using NaCl discs and run as Nujol mulls.

2.1.2 Syntheses

All chemicals were reagent grade unless otherwise stated. Commercial chemicals were used as supplied. Solvents used were distilled from either calcium hydride or activated molecular sieves and stored over the same. Microanalyses were performed by CHN Analysis Ltd. Leicester.

$RuCl_2(DMSO)_2(imidazole)_2$

4-nitroimidazole (Sigma), 2-Me-5-nitroimidazole (Aldrich) and metronidazole (2-methyl-5-nitro-1-imidazoleethanol, Sigma) variations of this were prepared by the following method[1] from the precursor $RuCl_2(DMSO)_4$ [2]. $RuCl_2(DMSO)_4$ (2.42g, 5mmol) and imidazole (12mmol) were refluxed in 70ml EtOH under N_2 for 4h. The solution was cooled and ether added until precipitation was complete. The solid was filtered, washed with acetone and ether and vacuum dried. Yields typically 70%. $RuCl_2(DMSO)_2(2 - Me - 5 - nitroimidazole)_2$

Anal. Calc. for $C_{12}H_{22}N_6O_6S_2Cl_2Ru$: C 21.51, H 3.97, N 15.05; found C 21.85, H 3.79, N 15.29. Infrared (cm^{-1}): 1155, 1105 (ν_{so}), 1575, 1530 (ν_{NO_2}).

 $RuCl_2(DMSO)_2(4 - nitroimidazole)_2$

Anal. Calc. for $C_{10}H_{18}N_6O_6Cl_2S_2Ru$: C 21.66, H 3.27, N 15.16; found: C 21.63, H 3.35, N 15.04. Infrared (cm^{-1}) : 1155, 1080 (ν_{SO}) , 1550, 1515 (ν_{NO_2}) .

 $RuCl_2(DMSO)_2(metronidazole)_2$

Anal. Calc. for $C_{16}H_{30}N_6O_6Cl_2S_2Ru$: C 28.66, H 4.51, N 12.54; found: C 28.85, H 4.41, N 12.58. Infrared (cm^{-1}): 1150, 1080 (ν_{SO}), 1555, 1510 (ν_{NO_2}).

$trans-[RuCl_2(cyclam)]Cl$

 $K_2[RuCl_5(OH_2)]$ (Johnson-Matthey 1.87g, 5mmol) and cyclam (Aldrich, 1.00g, 5mmol) were refluxed in 150ml MeOH for 3 days. The bulk of the solvent was evaporated and the solution cooled. The brown crystals formed were collected, washed with acetone and ether, and dried under vacuum. Yield 67%. Anal. Calc. for $C_{10}H_{24}N_4Cl_3Ru$: C 29.45, H 5.93, N 13.74; found: C 29.70, H 5.76, N 13.64. Infrared (cm^{-1}) : 795, 880, 890 $(CH_2 \text{ vibration characteristic of trans complexes [3]}).$ $Ru(1,5,9,13-tetrathiacyclohexadecane)Cl_2$

 $K_2[RuCl_5(OH_2)]$ (1.87g, 5mmol) and 1,5,9,13-tetrathiacyclohexadecane (Aldrich, 1.50g, 5mmol) were refluxed in 2-methoxyethanol (30ml) for 2 days[4]. A pink solid gradually formed which was filtered off, washed with ice-cold water, ether, and dried. Yield 65%. Anal. Calc. for $C_{12}H_{24}S_4Cl_2Ru$: C 30.76, H 5.16, S 27.37; found: C 30.72, H 5.11, S 27.06. Infrared (cm^{-1}) : 940, 925, 885, 870, 840, $(CH_2 \text{ vibration})$.

$[Ru(1,5,9,13-tetrathiacyclohexadecane)Cl_2](ClO_4)$

A solution of the previous compound (1g, 2mmol) in 10ml HCl (0.1M) was heated on a steam bath. $HClO_4$ (70%, $2cm^3$) was added and the solution heated for a further 2h. during which the solution turned deep red. The solution was filtered while hot. The crystals formed on cooling were filtered, washed with a little ice-cold water, ether and dried. Yield 70%. Anal. Calc. for $C_{12}H_{24}S_4Cl_3O_4Ru$: C 25.37, H 4.26, S 22.58; found: C 25.38, H 4.19, S 22.13. Infrared (cm^{-1}): 935, 910, 880, 865, 827 (CH_2 vibration).

1,4,7-Trithiacyclononane

Anhyd. cesium carbonate (Aldrich, 23.53g, 0.072mol) was suspended in anhyd. DMF (450ml) under N_2 . To the vigorously stirred solution, kept at 100°C, was added a solution of 2-mercaptoethyl sulphide (Aldrich, 10g, 0.065mol) and 1,2-dichloroethane (6.43g, 0.065mol) at a very slow rate[5]. After addition was complete, stirring was continued at 100°C for a further 12h. The solvent was removed under vacuum and the residual solid extracted with CH_2Cl_2 (3×150ml). The extract was washed with aq. NaOH (1M 2×100ml) and evaporated to dryness. The resultant solid was washed with water (3×50ml), dissolved in CH_2Cl_2 (150ml), dried over $MgSO_4$ and evaporated to dryness. The residue was placed in a sublimation apparatus and heated at 90°C under vacuum (0.5 mmHg). The product sublimed as white crystals. Yield 41%. ¹ $H(CDCl_3)$ δ : 3.14 (singlet); ¹³ C_{BB} δ : 35.02.

$[Ru(1,4,7,-trithiacyclononane)_2](ClO_4)_2$

 $RuCl_3.3H_2O$ (Aldrich, 0.25g, 0.96mmol) was dissolved in DMSO (15ml) at 200°C. The solution was concentrated to 10ml and cooled to room temperature. 1,4,7-trithiacyclononane (0.5g, 2.77mmol) was added and the solution stirred at 140°C for 3h. The precipitate formed on cooling was filtered, dissolved in water and recrystallised by the addition of $NaClO_4$ [6]. Yield 65%. Anal. Calc. for $C_{12}H_{24}S_6O_8Cl_2Ru$; C 21.81, H 3.66, S 29.12; found: C 21.65, H 3.62, S 29.34.

$[Co(diNOsar)]Cl_3$

(For an explanation of the notation used see Definition of Terms.)

To a solution of $[Co(en)_3]Cl_3$ (Aldrich, 50g, 0.145mol) in 1.51 water was added nitromethane (58.3g, 0.96mol) and the solution stirred until the nitromethane was fully dissolved. Aq. formaldehyde (40%, 370ml) and Na_2CO_3 (24.54g) were added and the solution stirred overnight, when the solution darkened. This was filtered and dissolved in hot HCl (3M) and crystallised by the addition of EtOH and cooling on ice. Yield 67%. ${}^1H(D_2O)$ δ : 2.92, 2.95, 3.31, 3.36, 3.52, 3.55, 3.85, 3.90 (consistent with superimposed doublet pairs [7,8]). ${}^{13}C_{BB}$ δ : 53.0, 56.6, 87.4. Infrared (cm^{-1}) ; 1380, 1560 (ν_{NO_2}) . Anal. Calc. for $C_{14}H_{36}N_8Cl_5Co$: C 31.15, H 5.60, N 20.77; found: C 30.82, H 5.66, N 20.62.

$[Co(diAMsar)]Cl_3$

 $[Co(diNOsar)]Cl_3$ (54.52g, 0.098mol) was dissolved in 21 water and the solution deoxygenated with N_2 . With vigorous stirring and the maintenance of a N_2 blanket, zinc dust (55g) was added, followed by cHCl (275ml) added dropwise. Stirring was continued for 1h after addition. When the reduction was complete, $30\% H_2O_2$ (60ml) was added to the reaction mixture, reoxidising the Co(II) to Co (III) and turning the mixture from green to its original orange. The solution was warmed on a steam bath for 20mins and then eluted onto a column of H^+ DOWEX 50WX2 cation exchange resin (Sigma). The column was washed with water (21) and 1M HCl (21) before elution with 3M HCl. The eluate volume was reduced until crystallisation commenced. Addition of EtOH completed the crystallisation. The complex was recrystallised from warm 1M HCl/EtOH. Yield 41%. ${}^1H(D_2O)$ δ :

2.90, 2.95, 3.49, 3.54 (each resonance exhibiting fine structure consistent with proposed structure[8]). ${}^{13}C_{BB} \delta$: 53.79, 57.48, 58.89. Infrared (cm^{-1}): 3400 (NH_3^+). Bands characteristic of NO_2 are absent. Anal. Calc. for $C_{14}H_{36}N_8Cl_5Co$: C 30.42, H 6.57, N 20.28; found: C 30.65, H 6.81, N 20.01.

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2.2 Determination of Protonation

Constants

2.2.1 Sample Preparation

Ethylene diamine, N,N-dimethylethylenediamine, N, N'-dimethylethylenediamine, N, N, N', N'-tetramethylethylenediamine, N-methyl-1,3-propanediamine, 3, 3'-diamine-N-methyldipropylamine, and 3, 3'-iminobispropylamine were purchased from Aldrich and were used as supplied. Water was doubly deionised through a Fisons FI-STREEM cartridge deioniser and Millipore Milli-Q system. $D_2O(99.8\%)$ was obtained from Goss Scientific Instruments Ltd.

Samples were prepared in a 15% D_2O/H_2O solution to allow internal locking on the deuterium signal. The concentration of amine was 0.01M. The pH was adjusted with aliquots of concentrated HCl so as to minimise volume change.

2.2.2 pH Measurements

pH measurements were made on a Corning Delta 240 Autocal pH meter equipped with a Delta 103 Extended Barrel Combination electrode. The meter was calibrated at pH 4.0, 7.0 and 10.0 prior to each set of measurements. Spectra were recorded at 298K. The pH measurements were uncorrected for the D_2O content of the solutions (an effect estimated at +0.04 pH units for 10% D_2O content[9]).

2.2.3 NMR Measurements

Proton spectra were obtained at 300.15 MHz on a Bruker AM300 spectrometer. Chemical shifts were measured relative to internal DSS. Spectra were accumulated in 5mm NMR tubes at 298K over typically 200 scans, with a sweep width of 4201.7 Hz and acquisition time 3.899 Hz. The resolution in digitisation was 0.256 Hz, corresponding to 8.5×10^{-4} p.p.m.

2.3 Sodium–23 Studies

2.3.1 Sample Preparation

Polyamines and polyaminothiols were used as their hydrochlorides. Compounds synthesised in this work were recrystallised and dried under vacuum before use. Commercial compounds were stored desiccated and used as supplied. Doubly deionised water was used throughout. D_2O was obtained from Goss. Calf-thymus DNA was purchased from BDH Ltd. and stored desiccated below $4^{\circ}C$.

Aliquots of a stock solution of additive were successively diluted to give a range of concentrations which, when DNA was added, gave a number of samples of differing additive to phosphate ratios. DNA phosphate concentrations were 0.06mmol, assuming a DNA monomer molecular weight of 337 gmol⁻¹, and the additive concentrations were such as to give a range of N/P ratios not exceeding 0.1. Samples were left for 48hrs at 5°C to form homogenous gels. The samples were then transferred into 5 mm NMR tubes using wide-tipped pipettes and any air removed by gentle centrifugation. Samples contained 15% D_2O to allow internal locking on the deuterium



Figure 2.1: A typical sodium-23 spectrum aquired over 10,000 scans at a Larmor frequency of 79.391 MHz.

signal. The sodium standard in the absence of DNA was taken from a 1ml sample of 1.3×10^{-3} mol NaCl in a 15% D_2O/H_2O solution.

2.3.2 NMR Measurements

Sodium-23 spectra were recorded on a Bruker AM300 spectrometer operating at a Larmor frequency of 79.391 MHz for sodium. The acquisition time was 0.205s and the spectra were accumulated over 10,000 scans for a total acquisition time of 34 mins. The sweep width was 1259.5 Hz and the spectrum was zero-filled to 16K data points to improve digitisation. Samples were spun at a rate of \approx 15 Hz to eliminate magnetic field inhomogeneities. Optimisation of resolution was achieved by adjustment of the shimming magnets such that the width of the HOD peak at half-height was less than 2 Hz. The temperature was maintained at 278 ± 0.5 K with the Bruker low-temperature unit, a stream of nitrogen gas being passed through the probe from a reservoir of liquid nitrogen. The samples were equilibrated at this temperature for 10 mins prior to acquisition.

2.4 Proton Studies

2.4.1 Sample Preparation

Calf-thymus DNA was obtained from BDH and stored desiccated below $4^{\circ}C$. Commercial compounds were stored desiccated and used as supplied. Compounds synthesised in this work were recrystallised and vacuum dried prior to use. Doubly deionised water was used for sample preparation. D_2O was obtained from Goss.

Samples were prepared by the addition of DNA to a solution of additive of known concentration. Samples were left at $4^{\circ}C$ for 48hrs to form homogenous gels and were transferred to 5mm NMR tubes using wide-tipped pipettes. Any air bubbles were removed by gentle centrifugation. DNA phosphate concentration was 0.12mmol, assuming a DNA monomer molecular weight of 337 gmol⁻¹. Additive concentration was 0.012mmol, giving an additive:phosphate ratio of 0.1. Samples contained 15% D_2O to allow internal locking on the deuterium signal.



Figure 2.2: Magnetisation vectors in the PRESAT.AU solvent suppression pulse sequence, $180^{\circ} - \tau - 90^{\circ}$.

2.4.2 NMR Measurements

Spectra were recorded on a Bruker AM300 spectrometer operating at 300.15 MHz over typically 200 scans. Suppression of the HOD peak (≈ 4.7 p.p.m.) was achieved by use of the PRESAT.AU microprogram. This involves selective excitation of the HOD resonance using a 180°(selective)- τ -90°(non-selective)-acquire pulse sequence.

The 180° pulse is applied selectively at the HOD resonance frequency and the delay time, τ , is sufficiently long to allow the signal to decay (Figure 2.2b). The application of the 90° pulse then gives no signal in the y-axis, the "null-point".

Other nuclei in the sample, with different relaxation times, T_1 , produce a signal as usual upon application of the 90° pulse. As the T_1 for the HOD proton is much longer than for protons in most organic molecules the large water signal can be easily eliminated[10].

The pulse power and time delay t_1 were determined by experiment so as to produce the greatest suppression of the HOD peak and least distortion of the remaining spectrum. Resolution was optimised by adjusting the shimming magnets such that the width of the HOD peak at half-height was less than 2Hz. The temperature was maintained at $298 \pm 0.5K$ by a flow of heated nitrogen gas around the sample.

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Chapter 3

Determination of Protonation

Constants by NMR

3.1 Introduction

This work concerns the use of NMR in the determination of protonation constants of di- and tri-amines. Both unsubstituted and N-methyl substituted amines were studied.

3.2 Previous Work

3.2.1 Initial Experiments

The technique of NMR has been used extensively to determine protonation constants and the mechanisms of protonation. In an early study, Grunwald *et al.*[1] studied the acid-base equilibrium:

$$R_3 N H^+_{(aq)} \rightleftharpoons R_3 N_{(aq)} + H^+_{(aq)}$$

$$(3.1)$$

where R=H or Me, and noted the characteristic S-curve when chemical shift was plotted against pH. The technique was found to be particularly useful in studying the ionisation of compounds with more than one ionisable group. Lowenstein and Roberts[2] followed the ionisation of citric acid(I) and found that the chemical shift of the methylene protons varied strongly with pH. The mechanism of protonation was determined from experiments with the mono-, di- and tri-esters of citric acid. It was found that the first and second ionisations occurred predominantly at the terminal carboxyl groups.

Sudmeier and Reilley[3] studied the protonation of EDTA(II) and derivatives with variations in the ethylene bridge, plotting the chemical shifts of the non-labile methylene protons against pH. This work noted trends

Amine	pK_1	pK_2	pK ₃	pK ₄	pK_5	Ref.
dien	10.18	9.14	4.25			3
dien ^a	9.94	9.13	4.34			32
dien ^b	9.98	9.21	4.61			32
trien	10.20	9.20	6.80	3.50		3
trienª	9.92	9.20	6.67	3.32		33
trien ^b	9.92	9.25	7.00	3.85		33
tetren	10.30	9.70	9.00	5.30	3.50	3
tetren	9.92	9.08	7.87	4.25	2.65	34

Table 3.1 Protonation constants of the polyamines dien, trien and tetren at $25^{\circ}C$. (a)0.1M KCl;(b)0.5M KCl.

such as:

- Chemical shifts increase as distance from functional groups decrease.
- Protonation shifts increase as distances from site of protonation decrease.
- Protonation shifts of amines increase in the order $1^{\circ} < 2^{\circ} < 3^{\circ}$.

This latter trend was in agreement with the earlier work by Grunwald $et \ al.[1]$.

The symmetrical triamines dien(III), trien(IV) and tetren(V) were studied as bridging alternatives in EDTA. Plots of methylene proton chemical shift against pH followed the protonation of each amino site allowing the determination of the protonation constants and sequence of protonation. The pK values taken from the graphs agree well with values calculated



IV $H_2N(CH_2)_2NH(CH_2)_2NH(CH_2)_2NH_2$

 $V \qquad \qquad H_2 N(CH_2)_2 NH(CH_2)_2 NH(CH_2)_2 NH(CH_2)_2 NH_2$

Figure 3.1: The structures of citric acid (I), EDTA (II), dien (III), trien (IV) and tetren (V).

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Figure 3.2: The proposed cyclic hydrogen bonded structure of ω -dimethylamino alkylamines.

from calorimetric studies[4,5](Table 3.1). Protonation was determined to start predominantly on the terminal amino groups.

Hine et al.[6] studied the basicities of individual amine groups in aqueous ω -dimethylamino alkylamines and concluded that for n > 3 there is evidence for a cyclic hydrogen-bonded structure for the monoprotonated form (VI). Subsequent work[7] found further evidence for this in both linear alkyl amines and cyclic systems.

3.2.2 Recent Experiments

As the instrumentation available improved more classes of compound were able to be studied. These have included tetracyclines[8], pyridines[9] and amino-acids[10], using not only proton-NMR but carbon-13[11] and nitrogen-15[12] also.

Recent work in this field has concentrated on the determination of protonation sequences rather than just the protonation constants. A study of both symmetrical and unsymmetrical triamines by Delfini *et al.*[13] used ${}^{1}H$ and ${}^{13}C$ NMR to determine the protonation sequences. The results led them to conclude that in all the amines studied protonation of the inner, secondary nitrogen occurs only after protonation of the two primary nitrogens is complete. For symmetrical triamines a fast tautomeric exchange between the primary nitrogens occurs. However, with unsymmetrical triamines, protonation of the nitrogen attached to the longer aliphatic chain starts first, protonation of the nitrogen attached to the shorter aliphatic chain starting before the former is complete.

Later studies by Kimberly and Goldstein[14] and Aikens *et al.*[15] have indicated that in monoprotonated spermidine the tautomeric equilibrium is not exclusively between the primary nitrogens but that the central, secondary nitrogen is protonated to a significant degree before protonation of the primary groups is complete.

These later studies by Aikens *et al.* used a further advance in NMR spectroscopy, that of 2-dimensional NMR[16]. The proton chemical shifts of the four methylene groups adjacent to the nitrogens in spermidine differ by only about 0.12ppm[17]. Thus, resolution by conventional ${}^{1}H$ -NMR, even at high fields, is impossible. The 2-dimensional spectrum can plot the ${}^{1}H$ chemical shift against the ${}^{13}C$ chemical shift to produce a 'map' of the spectrum. The normal ${}^{13}C$ spectrum of the four carbons of interest is well resolved and has been unambiguously assigned[15], thus each resonance can be identified and studied with ease.

3.3 Results

The dependence on pH of the chemical shift of protons alpha to base sites in polyamines is shown in Figures 3.3-3.9. The data have been normalised in each case. The limiting chemical shift values for each titration are given in Table 3.2.

Previous workers[17] for whom the study of protonation sequence was the main objective have quoted microscopic protonation constants, which describe the proton affinity of a particular base site in a molecule in which the previous protonations are located on specific base sites. Macroscopic constants describe the average proton affinity of all the molecules in which a given number of base sites have already been protonated and are thus used throughout this work.

The values determined in this work, measured by inspection, are consistently higher than most literature values (Table 3.3). The limits of accuracy reflect the uncertainty of the end-point of the titrations using this method.

3.4 Discussion

3.4.1 Substitution at Amine Sites

The protonation characteristics of amines, whether they be linear polyamines, as studied here, amino-acids[10,18], aminocarboxylate compounds [3,19], cyclic[11] or heterocyclic [9] amines, depend upon the substitution at the nitrogen centre. There is also a dependence on the separation of sites due to a base-weakening effect [17], the protonation of one site being affected by the protonation of a nearby site (see Table 3.4).

Aliphatic amines are more basic than ammonia due to the electronreleasing alkyl groups dispersing the positive charge of the substituted ammonium ion, stabilising it in a way that is not possible for the unsubstituted



protons of $H_2NCH_2CH_2NH_2$.



methyl protons of $MeHNCH_2CH_2NHMe$.







Figure 3.6: Proton chemical shift as a function of pH for the methylene and methyl protons of $Me_2NCH_2CH_2NMe_2$.



Figure 3.7: Proton chemical shift as a function of pH for the methyl protons (a), secondary nitrogen methylene protons (b) and primary nitrogen methylene protons (c) of $MeNHCH_2CH_2CH_2NH_2$.



er methylene protons (a) and the inner methylene protons (b) of $(H_2NCH_2CH_2CH_2)_2NH$.



Proton Site	Unprotonated	Protonated	$\Delta\delta$
	δ_1	δ_2	$(\delta_2-\delta_1)$
$H_2N - CH_2 - CH_2NH_2$	2.63	3.41	0.78
$MeHN - CH_2 - CH_2NHMe$	2.62	3.48	0.86
$Me - HNCH_2CH_2NHMe$	2.29	2.82	0.53
$Me_2N - CH_2 - CH_2NH_2$	2.36	3.53	1.17
$Me_2NCH_2 - CH_2 - NH_2$	2.68	3.51	0.83
$Me_2 - NCH_2CH_2NH_2$	2.17	2.99	0.82
$Me_2N - CH_2 - CH_2NMe_2$	2.42	3.64	1.22
$Me_2 - NCH_2CH_2NMe_2$	2.17	2.99	0.82
$MeHN - CH_2 - CH_2CH_2NH_2$	2.49	3.15	0.66
$MeHNCH_2CH_2 - CH_2 - NH_2$	2.59	3.19	0.60
$Me - HNCH_2CH_2CH_2NH_2$	2.27	2.77	0.50
$(H_2N - CH_2 - CH_2CH_2)_2NH$	2.59	3.13	0.54
$(H_2NCH_2CH_2-CH_2)_2-NH$	2.53	3.20	0.67
$(H_2N - CH_2 - CH_2CH_2)_2NMe$	2.59	3.13	0.54
$\left (H_2NCH_2CH_2 - CH_2)_2 - NMe \right $	2.39	3.33	0.94
$(H_2NCH_2CH_2CH_2)_2N - Me$	2.17	2.95	0.78

Table 3.2 Limiting chemical shifts for each protonation site in Figs. 3.3– 3.9 (δ_1, δ_2) and change in chemical shift upon protonation $(\Delta \delta)$ (in p.p.m. relative to D.S.S.).

Amine	No.	pK_1	pK ₂	pK_3	Ref.
$H_2N(CH_2)_2NH_2$	VII	$10.3{\pm}0.2$	8.0±0.2		This Work
		9.91	7.13		20
$MeHN(CH_2)_2NHMe$	VIII	10.7±0.2	7.7±0.2		This Work
		10.29	7.47		21
		9.88	7.01		22
$Me_2N(CH_2)_2NH_2$	IX	$10.2{\pm}0.2$	$7.5{\pm}0.2$		This Work
		9.85	6.81		23
		9.53	6.63		24
$Me_2N(CH_2)_2NMe_2$	Х	9.7±0.2	$6.5{\pm}0.2$		This Work
		9.20	5.93		25
$MeHN(CH_2)_3NH_2$	XI	$10.9{\pm}0.2$	$9.4{\pm}0.2$		This Work
		10.18	7.95		26
$H_2N(CH_2)_3NH(CH_2)_3NH_2$	XII	11.1 ± 0.2	$10.1{\pm}0.2$	$8.6{\pm}0.2$	This Work
		10.90	9.70	8.20	13
		10.65	9.57	7.72	27
$H_2N(CH_2)_3NMe(CH_2)_3NH_2$	XIII	10.6 ± 0.2	10.5 ± 0.2	$7.9{\pm}0.2$	This Work
		10.50	9.60	6.98	27
$H_2N(CH_2)_3NH(CH_2)_4NH_2$	XIV	11.02	10.02	8.57	17
		10.90	9.71	8.25	14

Table 3.3 Protonation constants for di- and triamines in water at $25^{\circ}C$.

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ammonium ion. This being the case it should be expected that further N-alkyl substitution, e.g. methyl or ethyl, and an increased length of alkyl group between amino groups in di- or tri-amines should increase the stability of the protonated form of the amine and thus increase the pK_a . However, as can be seen from Table 3.3, both values determined from this work and from previous studies give an order of basicity for the amines VII to X of:

 $1^o<2^o>3^o$

This order is consistent with the order determined by Arnett *et al.* [28] for alkylamines in aqueous solution. It is presumed that the inductive effect for tertiary amines is outweighed by a steric barrier to protonation.

Hine and Li[7] noted the generalisation that:

"primary amine groups attached to saturated carbon are ordinarily significantly more basic than their N,N-dimethyl derivatives in aqueous solution"

The values for VII and IX support this trend. Hine *et al.* report acidity constants for $H_2N - (CH_2)_3 - NH_2$ of 10.16 and 8.17 and for $Me_2N - (CH_2)_3 - NH_2$ of 9.91 and 7.67[6]. The values measured in this work for $H_2N - (CH_2)_2 - NH_2$ (10.3, 8.0) and $Me_2N - (CH_2)_2 - NH_2$ (10.2, 7.5) are consistent with Hine's results on moving from an unsubstituted to an N,N-dimethyl derivative.

3.4.2 Separation of Amine Sites

As the separation of amine groups within a molecule increases, the basicity of each increases steadily[29,30], as shown in Table 3.4. However, in
Amine	pK_1	pK_2
$H_2N - (CH_2)_2 - NH_2$	9.98	7.28
$H_2N - (CH_2)_3 - NH_2$	10.55	8.88
$H_2N - (CH_2)_4 - NH_2$	10.80	9.63
$H_2N - (CH_2)_5 - NH_2$	10.92	10.05
$H_2N - (CH_2)_6 - NH_2$	11.02	10.24

Table 3.4 Protonation constants of diamine series in KNO_3 at $25^{\circ}C[29]$.

monoamines the basicity of the corresponding n-alkylamines does not increase with chain length but shows only a small fluctuation about a fixed value[31]. Thus, the addition of a methylene group appears to have a negligible effect on basicity. The increasing value of second pK_a for the series of diamines in Table 3.4 must therefore be related to the increased separation of the second amine group from the centre being studied. This is consistent with the base-weakening effect of an electron-withdrawing $-NH_2$ group.

This effect can be seen with the values for the second protonation of the diamines studied. The value for XI is over one pK unit higher than any of the diamines VII to X. Also, on moving from ethylenediamines to propane-1,3-diamines, the N-methyl protons experience only a slight effect from protonation of the other amino group. As the methylene protons adjacent to the N-methyl nitrogen still experience a small effect from the other nitrogen the methyl results give a false value for the second protonation. Whereas in Fig. 3.5 the the curves are coincident, in Fig. 3.7 the curves are coincident for the first protonation (N-methyl) but diverge significantly

Amine	$\Delta \delta$
$H_2NCH_2CH_2NH_2$	0.78
MeHNCH ₂ CH ₂ NHMe	0.86
$Me_2NCH_2CH_2NMe$	1.22

Table 3.5 Change in chemical shift of α -methylene protons upon protonation.

on addition of the second proton. The change in chemical shift upon protonation (Table 3.5) reflects a trend noted by Sudmeier and Reilley[3] and Grunwald[1]. They observed that protonation shifts of methylene protons adjacent to amines increase in the order:

 $1^o < 2^o < 3^o$

Values measured in this work are given in Table 3.5. These and other values for secondary and tertiary amines are in agreement with Sudmeier and Reilley.

3.4.3 Protonation of Triamines

The triamines, of particular interest in this study, exhibit protonation constants markedly more basic than the diamines. The base-weakening effect, of major importance in VII-X, appears to have lost a significant part of its effect with the amino groups separated by 3 methylene groups, as seen with XI.

Spermidine (XIV), which has been extensively studied [13,14,15,17] gives pK values of about 11 and 10 for the first two protonations. The first proton is added at the primary amino group attached to the longer alkyl chain. The difference in values between XII and XIV for addition of the third proton is presumably dependent upon the increased separation afforded by the extra methylene group.

The polyamines XII and XIII are able to be studied using conventional 1-dimensional proton NMR because of the symmetry of the molecules. The assignment of resonances to particular methylene groups and the monitoring of these throughout the titration is a much simpler task than for the unsymmetrical spermidine molecule.

For XIII, the shape of curve 'c' in Figure 3.9 would appear to be an average of the first and second protonations, presumably due to the symmetry of the molecule. A similar effect is observed for XII[13](Figure 3.8).

The value of 7.9 for addition of the third proton in XIII is over half a pK unit less than that for either XII or XIV. This is due to the reduced basicity of tertiary amines, a steric effect, and in the case of XIV, also due, in part, to the increased proximity of the third amino group.

However, for the purposes of this work, the crucial result is that, at pH 7, the polyamines are effectively fully protonated.

3.4.4 Application to Aminothiols

The complexity of the NMR spectra of the polyaminothiols used in this study (Figure 3.10) makes the determination of protonation constants difficult, as is the case for spermidine. However, the aminothiols can be regarded as being composed of polyamine subunits, the thiol groups approximating to methyl groups as they remain unprotonated within the pH range considered here[35] Thus the protonation constants already determined may be applied with reasonable confidence to these compounds.

XV
$$H_3N^+(CH_2)_3NH_2^+(CH_2)_2SH$$

XVI $H_3N^+(CH_2)_3NH^+(CH_3)(CH_2)_3NH_2^+(CH_2)_2SH$

XVII
$$H_3N^+(CH_2)_3NH^+(CH_3)(CH_2)_3NH_2^+(CH_2)_2SCH_2CONH$$

XVIII $H_3N^+(CH_2)_2SH$

XIX
$$H_3N^+(CH_2)_2SS(CH_2)_2NH_3^+$$

Figure 3.10: The aminothiols used in this work, WR1065 (XV), RW222 (XVI), the nitroxide derivative used in ESR studies (XVII), cysteamine (XVIII) and cystamine (XIX).

The aminothiol RW222 (XVI) and its nitroxide derivative used in ESR studies (XVII) are composed of a norspermidine subunit (XIII). The three amino groups are thus expected to have pK values similar to those of XIII. The substitution of one of the primary amines with the thiol chain is expected to increase the basicity, according to the order determined by Arnett *et al.*[28].

Similarly, WR1065 (XV) is composed of a 1,3-dipropylamine subunit (XI). Again, the thiol chain is expected to increase the basicity of the adjacent amino group.

The thiolamines cysteamine (XVIII) and cystamine (XIX) can be con-

sidered as analogous to ethylenediamine (VII). The protonation constant of XVIII is expected to have approximately the same value as the first protonation constant of VII (≈ 10). Cystamine, consisting of two ethylenediamine subunits, may have lower protonation constants than expected from analogy with cysteamine. The electron deficient disulphide bond may hinder protonation of the amino groups. This is not expected to be a large effect however.

The application of data to the protonation of these thiolamines suggests that, as with the polyamines, polyaminothiols are effectively fully protonated at pH 7.

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Chapter 4

Sodium-23 NMR Studies of Cation-DNA Interactions

4.1 Introduction

This work concerns the interpretation of linewidths in ${}^{23}Na$ -NMR spectra of DNA-additive systems. A rigorous treatment of the technique is not attempted here but a more detailed explanation is given in the appendix.

4.2 Previous Work

4.2.1 Initial Experiments

Jardetsky and Wertz[1] were among the first to use sodium NMR, studying the complexation of $Na^+_{(aq)}$ by organic chelating ligands. The observed line broadenings correlated with stability constants as measured for the complexes by potentiometric titration[2]. Other groups entering the field studied topics such as ion-pairing of $Na^+_{(aq)}$ with a number of simple anions[3,4] and $Na^+_{(aq)}$ binding to simple carbohydrates[5] and polysaccharides[6].

The applicability of ²³Na-NMR to the study of counterion binding to polyions was recognised in studies with soluble RNA[7], polyacrylates[8,9], polyphosphate [10] and a number of other systems[11,12,13]. These studies used the relaxation rates of $Na^+_{(aq)}$ nuclei to probe the environment of the counterions around the polyion.

Reuben, Shporer and Gabbay[14], in a study of Na–DNA, determined the relaxation rate of $Na^+_{(aq)}$ as about 170—220 Hz. This compares with a value of about 18 Hz for free $Na^+_{(aq)}$. If the sodium ions were bound tightly to the slowly reorienting DNA the expected relaxation rate would be of the order of 15,000 Hz.

Similar studies with parvalbumin[15], a calcium binding protein, have

determined that upon replacement of one $Ca^{2+}_{(aq)}$ with one $Na^+_{(aq)}$ the conformation is maintained and the protein-cation complex reorients as a single moiety.

4.2.2 DNA Binding Studies

Subsequent studies have concentrated largely on the relative binding affinities of counterions for DNA[16,17,18,19,20,21].

Anderson, Record and Hart[16] explained their results for the binding of $Na^+_{(aq)}$, $Et_4N^+_{(aq)}$ and $Bu_4N^+_{(aq)}$ in terms of Manning's model of counterion condensation[22,23,24]. This is in contrast to Reuben *et al.*[14] who proposed a mass-action model for sodium binding to DNA. There is a vast body of experimental findings in accordance with Manning's model[25] and this appears to be a more favourable analysis than that of a mass-action model or the Poisson-Boltzmann treatment[8,14].

Bleam, Anderson and Record[17] performed a series of competition experiments and the preference for binding to DNA was found to be:

 $Mg^{2+}_{(aq)} > NH^+_{4(aq)} > Cs^+_{(aq)} > K^+_{(aq)} > Li^+_{(aq)} > Na^+_{(aq)} > Bu_4N^+_{(aq)}$

This series suggests, very generally, that increasing binding affinity is correlated with decreasing hydrated radius of the ion. This is attributed to the strength of electrostatic interaction between the polyion and hydrated counterions.

Further studies by Anderson and Record [26,27] investigated sodium relaxation in solutions of DNA under conditions of changing temperature and sodium concentration. The plot of linewidth against temperature exhibited hysteresis above $60^{\circ}C$, caused by denaturation of the double-helix. Under low-salt conditions renaturation is kinetically blocked [28] and thus

I

$$H_3N^+(CH_2)_3NH_2^+(CH_2)_4NH_2^+(CH_2)_3NH_3^+$$

 II
 $H_3N^+(CH_2)_3NH_2^+(CH_2)_4NH_3^+$

 III
 $H_3N^+(CH_2)_4NH_3^+$

 IV
 $H_3N^+(CH_2)_3NH^+(CH_3)(CH_2)_3NH_3^+$

 V
 $H_3N^+(CH_2)_2SH$

 VI
 $H_3N^+(CH_2)_2SS(CH_2)_2NH_3^+$

 VII
 $H_3N^+(CH_2)_3NH^+(CH_3)(CH_2)_3NH_2^+(CH_2)_2SH$

 VII
 $H_3N^+(CH_2)_3NH_2^+(CH_2)_2SH$

 IX
 $H_3N^+(CH_2)_3NH_2^+(CH_2)_2SS(CH_2)_2NH_2^+(CH_2)_3NH_3^+$

Figure 4.1: The protonated forms of the polyamines Spermine (I), Spermidine (II), Putrescine (III), N-methyl-1,3-propanediamine (norspermidine) (IV) and the thiolamines Cysteamine (V), Cystamine (VI), RW222 (VII), WR1065 (VIII) and WR33278 (IX). linewidths measured after denaturation and cooling are significantly smaller than those measured prior to denaturation.

Burton, Forsén and Reimarsson[29], in 1981, studied the interaction of a range of polyamines, based upon spermine (I), spermidine (II) and putrescine (III). Within each group of amines the separation of two amino groups was increased by the introduction of successive methylene groups. Spermine and spermidine were found to be the most effective at expelling sodium from DNA, whilst putrescine was found to exhibit very little binding. Their results are consistent with polyamine-DNA models[30,31,32,33] that depend upon a favourable geometrical fit between the polyamine amino groups and the DNA phosphate groups (This is discussed below).

4.2.3 Recent Work

Braunlin, Anderson and Record[34] determined the change in linewidth of $Na^+_{(aq)}$ during titrations of DNA with the polyamines putrescine and spermidine and the inorganic cations $Mg^{2+}_{(aq)}$ and $Co(NH_3)^{3+}_{6(aq)}$. At a given extent of titration, the polyamine binding produced a smaller decrease in the linewidth than did the binding of the inorganic ion of the same charge.

The aminothiol cysteamine(V) was studied by Vasilescu and Mallet [35] for the interaction of its protonated form with DNA. The linewidth was seen to decrease with increasing cysteamine up to a saturation point corresponding to a cysteamine concentration of 3/4 phosphate concentration.

Most recently Anderson and Record[36] have reported the interaction of the N-methylated polyamine analogue hexamethonium, Hex^{2+} , with DNA. $(Hex^{2+} = (CH_3)_3N^+ - (CH_2)_6 - N^+(CH_3)_3)$

In conjunction with ^{15}N -NMR relaxation data obtained for the methy-

lated nitrogen atoms, unaffected by pH changes, it was concluded that:

- the association of one Hex^{2+} molecule displaces 1.7—2.0 sodium ions from the vicinity of the DNA.
- cation accumulation near DNA neutralises approximately half of the phosphate charge at all points in the titration.
- the displacement of $Na^+_{(aq)}$ by Hex^{2+} is of the same order of magnitude as that by other divalent cations such as $Mg^{2+}_{(aq)}$ and putrescine.

These results suggest that the interaction between Hex^{2+} and DNA is primarily electrostatic in nature and delocalised in character.

4.3 **Results and Discussion**

4.3.1 Introduction

The technique of sodium-NMR, used in this context, measures the relative binding affinities of cations compared with that of the sodium ion. To allow a comparison of results, a quantity used by Braunlin *et al.* [34] has been used in this work. This quantity is related to the ratio of the total amount of sodium ions displaced to the total amount of counterion bound, at any stage of the titration.

4.3.2 Derivation of r^o/n^o

If the total amount of sodium present is N, the observed relaxation rate, R, is:

$$NR = N_B R_B + N_F R_F \tag{4.1}$$

where the subscripts B and F denote bound and free ions respectively. If P is the total amount of DNA phosphate in solution then the fraction of polyion charge compensated by association of sodium is:

$$r^{o} = N_{B}/P \tag{4.2}$$

Combining Equations 4.1 and 4.2, and expressing the independent variable as a ratio of concentrations:

$$R = R_F + r^o (R_B - R_F) [P] / [Na]$$
(4.3)

On addition of a second counterion M^{z+} , a quantity, n, is defined as the ratio of the total number of Na^+ ions displaced to the total number of M^{z+} ions bound at any stage of the titration. This is expressed in Equation 4.4.

$$n \equiv (r^{o}[P] - [Na])/[M^{z+}]_{B}$$
 (4.4)

n may be interpreted as the stoichiometric coefficient in Equation 4.5.

$$nNa_B^+ + M_F^{z+} \rightleftharpoons nNa_F^+ + M_B^{z+} \tag{4.5}$$

Calculations based on the counterion condensation and Poisson-Boltzmann models indicate that $n \leq z[26]$. Combining Equations 4.3 and 4.4 gives an expression for the Na^+ relaxation rate in terms of $[M^{z+}]$:

$$R = R_F + (r^o - np_M[M^{z+}]/[P])(R_B - R_F)[P]/[Na]$$
(4.6)

where $p_M \equiv [M^{z+}]_B/[M^{z+}]$.

During titrations the quantity [P]/[Na] remains constant. Thus, changes in the slope of the titration curve must be due to changes in the quantity $np_M(R_B - R_F)$. Over the concentration ranges studied, $[M^{z+}]_B \gg$ $[M^{z+}]_F$ therefore $p_m \approx 1$. The slope can thus be represented by $n(R_B -$ $R_F)[P]/[Na]$. Over the concentration range used in this study ($0 \leq [M^{z+}]/[P] \leq 0.1$) the titration is represented by a straight line, thus n is constant at its initial value, n^o , as R_B remains constant by assumption. The absolute value of the initial slope is therefore given by $n^o(R_B - R_F)[P]/[Na]$. The difference between the Na^+ linewidth in the DNA solution before addition of M^{z+} ions and the Na^+ linewidth in a blank solution containing no DNA is therefore:

$$R_o - R_F = r^o (R_B - R_F) [P] / [Na]$$
(4.7)

The ratio of Equation 4.7 to the initial slope gives the quantity r^o/n^o . The association of sodium with DNA conforms to the counterion condensation model [16,26] and thus r^{o} , the number of sodium ions per DNA phosphate in the absence of competing counterions, is constant. The counterion condensation model treats the DNA as an array of charges of infinite length. A dependence of r^{o} on strand length has been determined for polyphosphates [37] and more recently polyuridylic acid and polycytidylic acid 38. This dependence on chain length is thought to be a result of the increasing influence of end-group effects as the chain length decreases[39]. However, as these effects only become apparent at very low polymer lengths the assumption of the effective independence of r^o in double-stranded DNA is considered justified. The value of r^o/n^o therefore depends on n^o , the ratio of the total amount of Na^+ ions displaced to the total amount of M^{z+} ions bound at any stage in the titration. The greater the value of z, the more Na^+ ions displaced and thus the greater the value of n^o . The counterions most effective at displacing sodium from the region of the DNA will therefore have the smallest values of r^o/n^o .

4.3.3 The Relaxation Rate of Bound Sodium

A recent study of mono- and bis-intercalation in DNA by Eggert *et al.*[40] has suggested that the study of intercalation in DNA using sodium-NMR is complicated by the fact that a large proportion of the decrease in sodium linewidth is due to the reduction in the relaxation rate of bound sodium ions caused by intercalation.

The process of intercalation results in an extension of the DNA helix and thus an increase in the average interphosphate distance. This, coupled with a hindrance of internal DNA motion and the neutralisation of anionic phosphate charges by cationic charges on the intercalator, reduces the charge density of the DNA and thus the field gradients at the bound sodium. This lowers the relaxation rate of the bound sodium.

Whilst these effects appear reasonable for the intercalators studied, Eggert also suggests that the addition of non-intercalating cations, e.g. $Mg_{(aq)}^{2+}$, affects the relaxation rate of bound sodium at all values of added magnesium. Manning's CC model[22,23,24,25] gives the maximum charge fraction for DNA in the presence of $Na_{(aq)}^+$ and $Mg_{(aq)}^{2+}$ as 0.24 and 0.12 (i.e. 76% and 88% charge neutralisation) respectively. In a titration of Na-DNA with $Mg_{(aq)}^{2+}$ it is therefore expected that the degree of neutralisation will increase during the titration from 76% to 88%. This increased neutralisation is not expected to increase the average interphosphate distance significantly (as compared with an average increase of 2.7Å per intercalation site). As internal DNA motion is unaffected at the concentrations of cations used in this study, the assumption of a constant relaxation rate for bound sodium is considered valid.

4.3.4 Assumption of the "Fast-Exchange" Condition

Previous studies with sodium-NMR have determined that at fields of 200 MHz (for ¹*H*), corresponding to a Larmor frequency for sodium, ω_{Na} , of 52.9 MHz the limit of extreme narrowing is no longer valid [26]. Sodium resonances are thus no longer true Lorentzian but are a combination of two Lorentzians, corresponding to the fast and slow components of the (spin=3/2) quadrupolar relaxation mechanism. Thus, $\Delta \nu_{\frac{1}{2}}$ is not a direct measure of the relaxation rate.

However, Nordenskiöld et al. [27] have shown that the fast exchange limit is applicable to sodium ions in synthetic polynucleotides over a temperature range of $0 - 50^{\circ}C$ at a field of 200 MHz. Whilst sodium resonances may exhibit deviations from true Lorentzian lineshape the temperature dependence of $\Delta \nu_{\frac{1}{2}}$ is an accurate qualitative indicator of the fast exchange condition. Under conditions of fast exchange, the relaxation rates are proportional to the correlation time τ_c (see Introduction). Since τ_c decreases with increasing temperature the relaxation rate also decreases. Bleam etal.[26] have demonstrated the negligible temperature dependence of r^{o} , the fraction of sodium ions bound. It is reasonable therefore, to assume that the observed temperature dependence of $\Delta \nu_{\frac{1}{2}}$ is reflecting the temperature dependence of the relaxation rate. Figure 4.2 shows the temperature dependence of a number of sodium systems. The inverse proportionality of the linewidth, and therefore relaxation rate, to temperature demonstrates the validity of applying the fast exchange condition to ^{23}Na -DNA systems in addition to the polynucleotide systems.

In light of this, measured linewidths have been used in this work, as an



Figure 4.2: Temperature dependence of the ²³Na linewidth in solutions of aqueous sodium (+) ([Na]=0.1M), $d(GC)_n$ (\triangle) ([P]=2.6mM, [Na]/[P]=1.5), Br- $d(GC)_n$ (\Box) ([P]=3.2mM, [Na]/[P]=1.4)[26] and NaDNA (×) ([P]=0.06mM, [Na]/[P]=0.95).

accurate reflection of sodium relaxation, without any attempt to deconvolute the spectra into fast and slow components.

4.3.5 Effect of Ionic Strength on Counterion Binding

The interaction of spermidine with DNA was studied by equilibrium dialysis over a wide range of salt concentrations [41]. The results indicated that at moderate salt concentrations (0.3M NaCl, 0.002M MgCl₂) there was little detectable binding of spermidine. The association constant for spermidine–DNA interactions was essentially unchanged below 10mM NaCl but decreased as the NaCl concentration was increased above this. Thus, excess inorganic ions were excluded from the samples during the titrations. The use of the hydrochloride salts of amines and aminothiols also ensured that the sodium ions free in solution were those displaced from the region of DNA by the counterions. The change in sodium resonance was therefore a more accurate reflection of the change in counterion binding, unaffected as it was by a contribution from the bulk sodium resonance which would have decreased the range of change in the linewidth.

As stated above, the quantity n can be treated as the stoichiometric coefficient in the equilibrium between bound sodium and free counterion (Equation 4.5).

A further consequence of the absence of any added sodium is that the equilibrium is not artificially reversed in favour of bound Na^+ . The low concentrations of counterions used ($0 \leq [M^{z+}]/[P] \leq 0.1$) also keeps the equilibrium in favour of bound counterions. Under the conditions used during the titrations, the binding constants of amines are of the order of $10^3 M^{-1}[42]$. The similarity of the results for amines and metal complexes suggests that the assumption of almost complete binding, for both inorganic and organic counterions, is justified.

4.3.6 Radial Distribution of Counterions

The definitions introduced by the two-state model, territorially bound and free counterions, are distance effects, implying only radial localisation for the bound counterions.

LeBret and Zimm[43] have estimated the radius R_M of the cylinder containing the fraction of condensed counterions

$$R_M \simeq (2ar_D)^{\frac{1}{2}} exp \left[\frac{\xi - 2}{2(\xi - 1)}
ight]$$
 (4.8)

where a is the radius of the DNA cylinder (9.7Å), ξ is the DNA charge parameter (4.2) and r_D is the Debye-Hückel screening radius of the electrolyte in which the DNA is dissolved.

Values of r_D were estimated from

$$r_D^2 = \frac{\varepsilon RT}{2\rho L^2 e^2 I} \tag{4.9}$$

where ε = relative permittivity, R = gas constant, ρ = density of solvent, L = Avagadro constant, e = protonic charge and I = ionic strength (I = $\frac{1}{2}(m_+z_+^2 + m_-z_-^2)$ where m = molality and z = charge)[44].

Values for r_D were calculated for concentrations equivalent to N/P = 0.1 and assuming molarity \equiv molality. There is no distinction made between classes of ion, i.e. spermidine and $[Co(NH_3)_6]^{3+}$ have the same value of r_D .

The values in Table 4.1 were calculated under the following conditions: a = 9.7Å, $\xi = 4.2$, $\rho = 10^3$ kgm⁻³, $m = 5.94 \times 10^{-3}$ molkg⁻¹.

Counterion	r_D (Å)	R_M (Å)
M^+X^-	39.5	3 9.1
M ²⁺ 2X ⁻	22.8	29.7
M ³⁺ 3X ⁻	16.1	24.9
$M^{4+}4X^{-}$	12.5	21.9
M ⁵⁺ 5X ⁻	10.2	19.9

Table 4.1 The radius of the cylinder surrounding DNA, R_M , within which counterions are said to be bound and the Debye-Hückel screening length, r_D , from which they were calculated.

Counterions translating freely within these radii are thus considered territorially bound, whilst there is rapid exchange between ions in the bound and free regions.

4.3.7 Results

The sodium linewidth in a solution of DNA before addition of competing counterions, the DNA blank, was determined for each titration. The linewidth for free sodium was determined to be 11.2 Hz at 278K for 0.06mmol NaCl.

The difference between the value for the DNA blank and the value for free sodium gives $r^o(R_B - R_F)[P]/[Na]$, according to Equation 4.7. The value of $n^o(R_B - R_F)[P]/[Na]$, the value of the initial slope of a plot of sodium linewidth against N/P ($[M^{z+}]/[P]$), was determined for each competing counterion and the value of r^o/n^o calculated.

The variation of linewidth with N/P for the different counterions is





Figure 4.4 Linewidth measurement as a function of N/P for the titration of Na-DNA with cystamine.





Figure 4.6 Linewidth measurement as a function of N/P for the titration of Na-DNA with norspermidine.





Figure 4.8 Linewidth measurement as a function of N/P for the titration of Na-DNA with RW222.





Figure 4.10 Linewidth measurement as a function of N/P for the titration of Na-DNA with RuCl₂(DMSO)₂(4-nitro-imidazole)₂.





Figure 4.12 Linewidth measurement as a function of N/P for the titration of Na-DNA with [Co(diAMsarH₂)]Cl₅.

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Figure 4.13 Linewidth measurement as a function of N/P for the titration of Na-DNA with $[Ru(9S3)_2](ClO_4)_2$.

Competing Counterion	r^o/n^o	zr^o/n^o
$RuCl_2(DMSO)_2(4-nitroimidazole)_2$	0.49 ± 0.06	0.98 ± 0.12
cysteamine	0.42 ± 0.09	0.42 ± 0.09
$[Ru(9S3)_2](ClO_4)_2$	0.41 ± 0.05	0.82 ± 0.10
$RuCl_2(DMSO)_2(metronidazole)_2$	0.39 ± 0.05	0.78 ± 0.10
putrescine[34]	0.35 ± 0.05	0.70 ± 0.10
putrescine	0.32 ± 0.03	0.62 ± 0.06
WR1065	0.31 ± 0.02	0.62 ± 0.04
$MgCl_2[26]$	0.27 ± 0.04	0.54 ± 0.08
cystamine	0.25 ± 0.03	0.50 ± 0.06
spermidine	0.25 ± 0.03	0.75 ± 0.09
spermidine[34]	0.24 ± 0.01	0.74 ± 0.03
norspermidine	0.22 ± 0.01	0.66 ± 0.03
RW222	0.20 ± 0.02	0.60 ± 0.06
$[Co(NH_3)_6]^{3+}[34]$	0.20 ± 0.01	0.60 ± 0.03
$[Co(diAMsarH_2)]Cl_5$	0.18 ± 0.03	0.90 ± 0.15

Table 4.2 Values of r^o/n^o and zr^o/n^o calculated for competing counterions indicating the extent of binding to DNA.

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shown in Figures 4.3-4.13. The quantity N/P represents the ratio of molecules of competing counterion to DNA phosphate. The results of the titrations are given in Table 4.2. In addition to the values of r^{o}/n^{o} , the quantity zr^{o}/n^{o} is given. This allows comparison between counterions of different charge. Whilst in this study the value of r^{o}/n^{o} , the direct measure of sodium displacement and counterion binding, is the quantity of primary importance, the values of zr^{o}/n^{o} nevertheless yield information on the efficacy of different groups of counterions for DNA binding. It has been demonstrated[25] that the value of r^{o}/n^{o} is insensitive to temperature change in the range $6^{o} - 33^{o}C$, as expected with the exchange equilibrium being governed primarily by electrostatic interactions.

4.3.8 Discussion

The data in Table 4.2 reveal some interesting trends. Generally, metal ions and small complexes displace a greater number of sodium ions than polyamines at a given stage of titration. This is presumably due to the higher charge density of the inorganic ions relative to the polyamines. As the metal complex or small ion approaches, the sodium ions experience the full charge. The overall polyamine charge however, consists of unit charges localised on the amino groups. thus, as the polyamine molecule approaches the DNA, the sodium ions experience only the effect of the nearest amino group, each charge being separated by at least two methylene groups.

This can be seen from the values of zr^o/n^o . This normalises the r^o/n^o values with respect to their charge and the different types of counterions separate into groups.

A further group in this classification is metal ions with large ligands.



Figure 4.14: Structures of the ruthenium complexes $\operatorname{RuCl}_2(DMSO)_2($ metronidazole)₂ (a), $\operatorname{RuCl}_2(DMSO)_2($ 4-nitroimidazole)₂ (b) (S=sulphur bonded DMSO[45]) and $[\operatorname{Ru}(9S3)_2]^{2+}$ (c).

Complexes used in this study have either Ru^{2+} or Co^{3+} centres. In both cases the large ligands effectively screen the metal centre preventing close approach of the complex to the DNA (Figure 4.14). The sodium ions thus experience a greatly diminished effect and are not as readily displaced from the DNA. While the cobalt sarcophagine complex, $[Co(diAMsar)]Cl_3$, might have been expected to fall into this category, the value of r^o/n^o $=0.18 \pm 0.03$ shows the effect of external positive charges. In this case, the two amino groups capping the sarcophagine cage promote the close association of the complex with DNA.

However, the zr^{o}/n^{o} values of the complex and spermidine suggest that the tripositive cobalt ion is being screened by the encapsulating sarcophagine ligand to an approximately univalent effect (Figure 4.15).

4.3.9 Interaction of Aminothiols with DNA

Results from Smoluk et al.[46] and Jellum[47] indicate only a weak interaction of cysteamine with DNA, suggesting that the minimum requirement for DNA binding is the di-cation. This would appear to indicate a "pseudochelate effect". The proximity of the molecule to DNA, caused by the binding of the first amino group, greatly increases the probability of the second amino group binding, and presumably increases the lifetime of the DNA-polyamine complex. Whilst this effect would keep the molecules territorially bound for a greater length of time it is not expected that the molecules are in close contact with the DNA, e.g. not directly H-bonded to the phosphates. The diameter of the B-form of helical DNA is 19.4Å[48] and the surface charge density is low. It is therefore likely that the association of counterions with DNA produces little perturbation of the primary



Figure 4.15: Comparison of the structures of $[Co(diAMsar)]Cl_3$ and spermidine illustrating the screening of the cobalt ion by the sarcophagine ligand.

solvent sphere of either the counterion or DNA[16].

The low value of r^{o}/n^{o} for cysteamine recorded here may be due to partial oxidation[46]. The cysteamine oxidising to cystamine would increase the amount of binding to DNA, by providing the di-cation.

The data in Table 4.2 suggest that the thiolamines WR1065 (VIII) and RW222 (VII) are slightly more effective at displacing sodium than are the corresponding amines putrescine (III) and norspermidine (IV). This is consistent with the observation of Smoluk *et al.*[46] who, in a comparison of their results with earlier results of Braunlin *et al.*[42], found that the thiolamines WR33278 and WR1065 bind more strongly than corresponding polyamines of the same net charge, in equilibrium dialysis experiments. This increased binding is presumably due to the significant charge fraction on the sulphydryl group[49] which would increase the size of the binding site over the molecule.

4.3.10 Differential Binding of Polyamines to DNA

Burton et al. [29] report that polyamines of the same charge exhibit different binding characteristics, varying with the separation of the amino groups, and that very little binding of putrescine to DNA at pH 8.25 was observed. However, these phenomena became apparent only at values of N/P approaching 0.5 (0.2 for spermine). At the values used in this study ($0 \le N/P \le 0.1$) all the amines studied by Burton exhibit the expected sharp reduction in sodium linewidth characteristic of strong binding. At higher concentrations of amine Burton suggests that the DNA undergoes a conformational change and that this causes the anomalous binding behaviour. This conformation change is to be expected from the CC model[22,23,24,25]. This states that the number of associated univalent cations per DNA phosphate, n, is equal to 0.76. In the absence of competing polyamines, $Mg^{2+}_{(aq)}$ (n = 0.88) does not precipitate DNA. However, the nature of polyamine binding, the pseudo-chelate effect, means that with increased concentrations of amine the maximum charge fraction postulated by Manning is exceeded and the DNA must either change conformation or precipitate out due to the diminished repulsions between phosphates. The tetravalent polyamines, spermine and WR33278, thus cause precipitation at lower concentrations than do lower valent polyamines [29,46]. At the concentration range used in this study this effect is absent and is therefore of no interest in the work presented here.

The study of cation binding using sodium-NMR gives no information on

preferential binding sites, if any, nor on the mode of binding. However, the territorial binding of these cations, through the counterion condensation theory and the sodium-NMR results is well established.

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Chapter 5

Proton NMR Studies of Cation–DNA Interactions

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5.1 Introduction

This work studies the interaction of polyamines and other cations with DNA by analysis of the proton magnetic resonance linewidths of the additives in aqueous solution in the presence and absence of DNA.

5.2 Previous Work

5.2.1 Initial Experiments

The use of ¹H-NMR in the study of the interaction of small molecules with macromolecules was initiated in the early 1960's by Jardetsky[1,2]. Studies of penicillin G with bovine serum albumin (BSA)[3,4] noted differential broadening of peaks arising from protons in different parts of the penicillin molecule. The broadenings were attributed to specific interactions of parts of the penicillin molecule with the BSA. These interactions were found to be specific to BSA insofar as no differential broadenings were observed in the presence of ribonuclease.

Similar studies were carried out with oxytetracycline [2] and sulphonamides[5].

Gabbay[6,7,8] used 'reporter' molecules designed specifically to interact with polyions to probe the interaction specificity of nucleic acids. Reporter molecules of the type (I) and (II) were studied by ¹H-NMR in the absence and presence of DNA.

At $30^{\circ}C$ the spectrum of II was found to be identical in the presence and absence of DNA. This was interpreted as rapid tumbling of the molecule in the DNA complex, indicative of an external electrostatic bind-



II $(CH_3)_3 N^+ (CH_2)_3 N^+ (CH_3)_3 \cdot 2Br^-$

Ι

Figure 5.1: 'Reporter' molecules used as probes for the interaction specificity of nucleic acids.

ing mechanism[9,10,11]. Reporter molecule I at $30^{\circ}C$ exhibited differential broadening. Resonances from the polyammonium side chain showed negligible broadening, whereas the ring methyl proton signal was considerably broadened. This behaviour was explained in terms of restricted motion of the 4-nitroaniline ring intercalated into the DNA helix. The polyammonium side chain still retains a significant degree of rotational freedom. As the temperature of the sample is raised, the width of the signal due to the ring methyl protons sharpens and narrows as the reporter molecule gains more rotational freedom. Eventually, as the temperature is raised to the denaturation point of DNA, the spectra of the reporter molecule in the absence and presence of DNA become identical as the molecule regains its motional freedom.



Figure 5.2: The intercalating molecules ethidium (III) and propidium (IV).

5.2.2 Recent Studies

Later studies of complexes between polyamines and nucleic acids used both 1 H and 13 C-NMR. Bunce and Kong[12] studied the interaction between spermidine and adenosine monophosphate at high concentration (0.5M) and found evidence for complexation between the two. They proposed a strong complex with two amino groups of spermidine participating in hydrogen-bonding with the phosphate moiety and the third amino group interacting with the N7 of adenine, a position that has been reported to be susceptible to binding by cations[13]. At a concentration of 0.006M there was no evidence of complexation.

¹³C-NMR was also used to study putrescine distribution in Escherichia Coli[14]. Broadening of the putrescine methylene signal was attributed to restriction in the tumbling of the polyamine molecules when bound to macromolecules in the cells.



V

Figure 5.3: The probe molecule for stereospecific and molecular twist requirements used by Wilson *et al.* $R=CH_3$ or H.

Wilson et al.[15,16] studied intercalator-DNA interactions by the analysis of imino proton resonance shifts and widths. Temperature studies indicated that the change from slow exchange to fast exchange takes place at approximately $60^{\circ}C$ for ethidium (III) and approximately $70^{\circ}C$ for propidium (IV).

Stereospecific and molecular twist requirements for intercalation were investigated with the unfused aromatic molecule (V)[17]. It was found that when R=Me the twist induced in the molecule was such that full intercalation of the molecule with DNA was prevented. With R=H, however, the addition of DNA to a solution of V resulted in considerable line broadening of the aromatic proton signals, indicating that V was fully intercalated with the DNA.

Wemmer *et al.*[18] studied the binding of spermine with the self-complementary DNA oligomer d(C-G-C-G-A-A-T-T-C-G-C-G). In the spermine-DNA complex there was no broadening of the spermine resonances and very weak, positive nuclear Overhauser effects were observed, indicating that the spermine molecule retains a significant degree of rotational freedom within the complex. This independent mobility of the spermine molecule was expected to arise from rapid diffusion along the duplex between specific binding sites, or from delocalised interactions with no discrete binding sites.

5.3 The Origin of Proton Linewidths

The study of linewidths depends upon the characteristics of molecular tumbling in solution. If the rate of molecular tumbling of molecules in solution is lower than their Larmor frequencies, ω_0 , then the transverse relaxation time, T_2 , is considerably reduced, leading to substantial line-broadening of the proton resonance.

5.3.1 Experimental Effects

The lineshape predicted by the Bloch equations[19] is Lorentzian and would be observed in an ideal experiment if relaxation of the transverse components of the spin $(M_x \text{ and } M_y)$ were exactly exponential with a single value of T_2 . However, experimentally the lines are not exactly exponential and may be unsymmetrical. This is because the theoretical width of the lines, $(\pi T_2)^{-1}$, is so small that the width is due to instrumental effects, e.g. the variation of B_o , the operating magnetic field, by a few parts per million over the area of the sample. The lineshape is an indication of field inhomogeneity over the sample and the effect of this, called inhomogeneity broadening, is due to the signal being a composite of lines with slightly different Larmor frequencies. The effects of field inhomogeneities can be minimised by spinning the sample at a frequency of about 15 Hz which averages the inhomogeneities. The variation in B_o is minimised by the use of "shimming magnets". These are conducting loops carrying small, variable currents producing magnetic fields which can be adjusted to compensate for field gradients in B_o .

The total observed linewidth, $\Delta \nu_{\frac{1}{2}}$, is used to define an effective relaxation time, T_2^* , even though the lineshape is not exactly Lorentzian.

$$\Delta \nu_{\frac{1}{2}} = (\pi T_2^*)^{-1} \tag{5.1}$$

Minimisation of inhomogeneity broadening by the use of shimming magnets and spinning of the sample means that the major factor in linewidth effects is changes in the observed relaxation time T_2^* .

5.4 The Viscosity Broadening Effect

Upon moving from an aqueous system to an aqueous-DNA system there is a concomitant increase in the viscosity of the system. The resultant decrease in molecular motion results in increased correlation times for the molecules in solution. This leads to a "viscosity broadening" of the proton resonances[19].

To compensate for this effect the reported linewidths are corrected using a factor derived from the linewidth of DSS. The negative charge on the molecule precludes any interaction between it and DNA and ensures that, as the DSS remains in the bulk solution, any change in the linewidth is due to the increase in viscosity alone.

The comparison of actual linewidths and corrected linewidths thus gives an approximate guide to interactions between the added compound and DNA[14]. (DSS = $(CH_3)_3 SiCH_2 CH_2 CH_2 SO_3^- Na^+$)

5.5 Results

5.5.1 Predicted Linewidths

The linewidths of proton resonances in the absence and presence of DNA are given in Table 5.1. The predicted linewidths are the linewidths of the specified resonances in the absence of DNA multiplied by a factor

$$\frac{\Delta\nu_{\frac{1}{2}}(DSS+DNA)}{\Delta\nu_{\frac{1}{2}}(DSS)}$$

which corresponds to the increase in the DSS resonance linewidth (defined as $\delta = 0$ p.p.m.).

The predicted values are those expected in the absence of any DNA interaction. Differences between the predicted values and the experimental values of the linewidths in the presence of DNA give an indication of the mode of interaction.

The value of the above ratio was determined experimentally as 3.13 ± 0.63 Hz, i.e. the width of the DSS resonance tripled upon addition of DNA.

5.5.2 Linewidth Measurements

The use of proton linewidths as a measure of relaxation rate offers the advantages of natural abundance and sensitivity of proton over carbon-13 NMR. However, difficulties are encountered when dealing with resonances showing a multiplicity greater than the singlet state. It is often impractica-

Compound	Linewidth $\Delta \nu_{\frac{1}{2}}$ (Hz)		
	DNA absent	DNA present	Predicted
2-Me-5-NO2-imidazole			
(methyl)	$0.92{\pm}0.02$	$2.59{\pm}0.02$	$2.88{\pm}0.55$
metronidazole			
(methyl)	$0.86{\pm}0.02$	$3.16{\pm}0.02$	$2.69{\pm}0.52$
$RuCl_2(DMSO)_2(2-Me-5-NO_2-Im)_2$			
(DMSO methyl)	$1.04{\pm}0.02$	$1.79{\pm}0.02$	$3.26{\pm}0.62$
(imidazole methyl)	$1.14{\pm}0.02$	$3.65{\pm}0.02$	$3.57{\pm}0.68$
norspermidine			
(methyl)	$3.27{\pm}0.02$	$4.03{\pm}0.02$	$10.24{\pm}1.95$
RW222			
(methyl)	$0.92{\pm}0.02$	$2.63{\pm}0.02$	$2.86{\pm}1.21$
WR1065			
$CH_2-NH(\delta 3.12)$	$4.12{\pm}0.02$	$4.80{\pm}0.02$	$12.89{\pm}2.60$
$H_2N-CH_2(\delta 3.19)$	$4.28{\pm}0.02$	$5.76{\pm}0.02$	$13.39{\pm}2.70$

Table 5.1 Measured linewidths in the absence and presence of DNA and the predicted linewidths derived from the linewidth in the absence of DNA and the increase in the DSS linewidth upon addition of DNA.

ble to get accurate linewidth measurements from triplets or quartets. This study has thus concentrated on compounds where the presence of a methyl moiety permits the convenient measurement of linewidths. Where linewidths measured from triplets are given they are measured from the central peak of the triplet and may be less accurate than those measured from singlets.

5.6 Comparison with Previous Results

5.6.1 Restricted Molecular Motions

Previous results[10] shown in Table 5.2 indicate that protons with restricted mobility show linewidths a factor of ten greater than those of the compound in the absence of any binding. Whilst these results were not corrected for viscosity effects the increases are of such an order as to be indicative of greatly reduced motion.

Results for the intercalator ethidium bromide (III) indicate that at an N/P ratio of 0.1 (i.e. an intercalation of one ethidium every five base pairs) the ethidium aromatic resonances are broadened beyond resolution and are indistinguishable from baseline noise (Figure 5.4).

The aliphatic resonances (Figure 5.5) exhibit a small broadening in addition to that attributable to the viscosity increase. This is consistent with the observations of Gabbay *et al.*[10] (Table 5.2). The ethyl side chain is restricted in its motion by the process of intercalation but retains relatively unhindered rotational motion through the N—C bond. The difference in linewidths given for the reporter molecules (1) and (2) are attributed to the formation of hydrogen bonds between the side-chain of (1) and DNA.

System	Temperature	Linewidth $\Delta \nu_{\frac{1}{2}}$ (Hz)	
	(°C)	a-CH3	b-CH ₃
(1)	25	2.0	2.2
(1)+DNA	25	SIBLN	SIBLN
(1)+DNA	33	12	>20
(1)+DNA	51	7	>20
(1)+DNA	88	2	3
(2)	25	2	2.5
(2)+DNA	25	18	SIBLN
(2)+DNA	51	5	>20
(2)+DNA	88	2	2

$$NO_{2}$$

$$CH_{3} (b) (a)$$

$$NH - (CH_{2})_{2} - N^{+}(CH_{3})_{2}R.Br^{-1}$$
(1) R=H
(2) R=CH_{3}

Table 5.2 Linewidths at half-height of proton resonances for the DNA bound molecules (1) and (2) at various temperatures (SIBLN = signal indistinguishable from baseline noise).



(0.12mmol DNA phosphate, 0.012mmol ethidium bromide).



Figure 5.5 Part of the aliphatic region of the 300 MHz ¹H spectrum of ethidium bromide in the presence (a) and absence (b) of DNA (0.12mmol DNA phosphate, 0.012mmol ethidium bromide).

VI
$$H_3N^+(CH_2)_3NH_2^+(CH_2)_3NH_3^+$$

VII $H_3N^+(CH_2)_3NH_2^+(CH_2)_4NH_3^+$

VIII
$$H_3N^+(CH_2)_3NH^+(CH_3)(CH_2)_3NH$$

Figure 5.6: The protonated forms of the polyamines used to study the amplification of Bleomycin-mediated degradation of DNA.

The enforced proximity and reduction in mobility caused by intercalation of the 4-nitroaniline ring is expected to promote the formation of hydrogen bonds. However, this is not taken as being applicable to the case of polyamines and aminothiols, where the formation of hydrogen bonds causing reduced mobility is considered unlikely (see below).

Similar results were obtained with a study of the interaction of polyamine derivatives with DNA with respect to the bleomycin-mediated degradation of DNA[20]. Binding of polyamine derivatives induced changes in the DNA structure which enhanced the bleomycin degradation. Both aliphatic and aromatic groups exhibited amplification. Considerably broadened aromatic resonances were observed whereas aliphatic resonances were not significantly broadened.

5.6.2 Structural Activity of Polyamines

Polyamines and derivatives that gave the greatest amplification of bleomycin degradation[20], and thus the greatest binding, were the triamines VI, VII and VIII. Compound VII exhibited an activity twice that of VI, attributed to structural differences governing the activity of amines. However, the experiments were conducted at compound to base pair ratios of unity. Similar studies of polyamine binding to DNA at these ratios have observed similar structural differential binding[21] and it is suggested that only at high concentrations of polyamines do these structural characteristics become evident. At the ratio used in this study (0.1 compound to phosphate) it is assumed that the different polyamines exhibit similar binding characteristics, and that there is no conformational change associated with these concentrations.

Compounds VII and VIII exhibit similar amplifications (4.84 and 5.00 respectively). VIII is a dication at pH 7.0[20], the amine adjacent to the aromatic moiety having a pK<5[22]. The introduction of an intercalating moiety thus appears to have an effect similar to the addition of an extra amino group on the interaction of these molecules with DNA.

5.7 Interaction of Inorganic Complexes with DNA

The radiosensitising ruthenium compound $\operatorname{RuCl_2(DMSO)_2(2-Me-5-nitro-imidazole)_2}$ in solution has been found to lose a single chloride ligand[23]. Whilst this is expected to aid the DNA binding properties of the complex,



Figure 5.7: Structures of the imidazoles 2-Me-5-nitroimidazole (IX) and metronidazole (X).

through a ruthenium-base interaction [24,25] the results given do not indicate any strong interaction, as the binding of the complex would result in restricted rotation of the DMSO and imidazole ligands and lead to broadened resonances for the methyl protons. Results from Chapter 4 support this with the absence of any appreciable displacement of sodium from the region of DNA.

Studies of $[Ru(NH_3)_5Cl]Cl_2$ binding to yeast transfer RNA[26] indicate that the ruthenium binds to guanine in non-helical regions and that there is no detectable binding to Watson-Crick base pairs in the helical regions. Whilst the structure of DNA is different from that of RNA and the more open form of B-DNA would presumably allow closer approach of the $[Ru(NH_3)_5(OH_2)]^{3+}$ moiety it is expected that the bulk of the $RuCl_2(DMSO)_2(2$ -Me-5-nitroimidazole)_2 would prohibit any close association of the complex with the bases through the major groove[27,28].

There is no binding suggested from the linewidth studies of 2-Me-5-



Figure 5.8 Part of the 300 MHz ¹H spectrum of $\operatorname{RuCl_2(DMSO)_2(2-Me-5-nitroimidazole)_2}$ in the presence (a) and absence (b) of DNA (arom H $\delta 8.00$, DMSO methyl $\delta 2.72$, imidazole methyl $\delta 2.42$, 0.12mmol DNA phosphate, 0.012mmol metronidazole).



0.12mmol DNA phosphate, 0.012mmol 2-Me-5-nitroimidazole).



Figure 5.10 Part of the 300 MHz ¹H spectrum of metronidazole in the presence (a) and absence (b) of DNA (arom H δ 8.05, methyl δ 2.50, 0.12mmol DNA phosphate, 0.012mmol metronidazole).

nitroimidazole (IX) and metronidazole (X). The absence of any charged groups on these compounds would limit the strength of any possible interactions. The reduced activity of free imidazoles relative to ruthenium complexes incorporating imidazole ligands[23] is suggested as being due to an increase in reduction potential of the ligand[29,30] and the increased proximity to DNA[31] caused by the charge on the metal centre.

5.8 The Delocalised Nature of Polyamine Binding

5.8.1 Nuclear Overhauser Effect Spectroscopy

The results for the polyamines and aminothiols are consistent with those of Gabbay *et al.*[9,11] and Wemmer *et al.*[18]. Both observed no changes in linewidth in polyamine resonances upon addition of DNA (or DNA oligomers). Wemmer also used NOE spectroscopy to investigate possible interaction mechanisms. In spermine:DNA systems small positive NOE's were observed, in contrast to larger positive NOE's for internal methylene protons in free spermine, and strong negative NOE's within the DNA oligomer. If spermine were bound in a tight defined manner to the oligomer, negative NOE's should have been observed, the spermine tumbling with the oligomer correlation time. Even if the lifetime of the oligomer:spermine complex is short compared with the irradiation time, 2 ms, the NOE's will be weighted averages of those arising from the bound and free forms. The concentration of free spermine is very small[32], therefore cancellation of negative NOE's in the complex by positive NOE's of free spermine cannot XI $H_3N^+(CH_2)_3NH^+(CH_3)(CH_2)_3NH_3^+$

XII
$$H_3N^+(CH_2)_3NH^+(CH_3)(CH_2)_3NH_2^+(CH_2)_2SH$$

XIII $H_3N^+(CH_2)_3NH_2^+(CH_2)_2SH$

XIV
$$H_3N^+(CH_2)_3NH^+(CH_3)(CH_2)_3NH_2^+(CH_2)_2SCH_2CONH$$

Figure 5.11: The protonated forms of the polyamines, norspermidine (XI), RW222 (XII), WR1065 (XIII) and the nitroxide derivative used for ESR studies (XIV).

be the reason for the weak positive NOE's observed. Therefore, spermine in the complex has relatively independent motion. Wemmer *et al.* argue that these results can be interpreted in terms of tight binding, with rapid diffusion between tight binding sites. This mechanism presumably involves the breaking of at least three strong amino-phosphate interactions and then rapid diffusion along the helix and the reformation of strong aminophosphate interactions. This is thought to be far less probable than the alternative of delocalised interactions with no discrete binding sites but rapid diffusion along the oligomer.

5.8.2 ESR Spectroscopy

Support for this model comes from ESR linewidth studies[33] of the spinlabelled polyamine derivative XIV. No increase was detected between the free cation and the DNA-cation systems. The geometry of the molecule would permit some rotational freedom of the side-chain even in the tightbinding limit. However, model studies[34] show that tight binding would severly restrict this motion and would result in considerably broadened lines. The absence of any broadening suggests a correlation time of less than about 10^{-9} s (comparable with NMR measurements[18]). With the correlation time of free spermine less than 0.3 ns and that of the DNA oligomer d(C-G-C-G-A-A-T-T-C-G-C-G) about 4 ns [35], the correlation time determined for XIV implies remarkable rotational and translational freedom.

5.8.3 Infra-Red Spectroscopy

Bertoluzza *et al.* [36,37,38] have studied the interaction of polyamines with phosphate, nucleic acids and DNA using Raman and infra-red spectroscopy. Complexes detected of phosphate hexahydrate salts of spermine and spermidine[36] are regarded as models of the molecular interactions between polyamines and nucleic acids. They appear, however, to be too simple to extend to the interactions between polyamines and DNA, in a similar manner to that of the complex between spermidine and adenosine monophosphate[12]. Evidence for the formation of medium strength hydrogen bonds between protonated polyamines and DNA phosphate groups has been found and it has been proposed that there is a direct interaction between ammonium ions and the DNA phosphate[38].

However, studies using spermine- and spermidine-DNA systems at concentrations of polyamine comparable to those in this study have detected no evidence for a direct interaction between the amino groups on the polyamines and the phosphate groups of DNA[39]. This suggests a "throughwater" (solvent separated) interaction which would support the theory of non-localised polyamine-DNA interactions.

5.8.4 Proton Linewidth Studies

The values of r^{o}/n^{o} for polyamines calculated in Chapter 4 and values of binding constants of the order of $10^{3}M^{-1}[40]$ provides evidence for the almost complete association of these compounds with DNA.

Whilst the values of linewidths for the neutral compounds IX and X and for the ruthenium-imidazole complex suggest an absence of interaction with DNA, the values of linewidth in the presence of DNA for the polyamines and polyaminothiols would appear to indicate a remarkable degree of freedom. As it is assumed that these polyamines are constrained to remain in close proximity to the DNA, from both the evidence given above and a consideration of the counterion condensation model, this freedom is interpreted as a rapid sliding along the DNA helix.

As mentioned above, this interpretation is considered more likely than that of Wemmer *et al.*[18] and should greatly facilitate the location of DNA damage sites when used as a model for the radioprotection of DNA. The absence of any appreciable linewidth increment upon addition of DNA tends to suggest that there is no complex formed with the amines lying along the major groove of DNA as this would be expected to significantly reduce the



Figure 5.12 Part of the 300 MHz ¹H spectrum of norspermidine in the presence (a) and absence (b) of DNA (0.12mmol DNA phosphate, 0.012mmol norspermidine).



Figure 5.13 Part of the 300 MHz ¹H spectrum of RW222 in the presence (a) and absence (b) of DNA (0.12mmol DNA phosphate, 0.012mmol RW222).



Figure 5.14 Part of the 300 MHz ¹H spectrum of WR1065 in the presence (a) and absence (b) of DNA (CH₂-N resonances, 0.12mmol DNA phosphate, 0.012mmol WR1065).

mobility of the cations and lead to a linebroadening effect in the NMR spectrum.

5.8.5 Model of DNA-Substrate Binding

Facilitated Transfer

The proposal of a rapid diffusion or sliding mechanism is supported by the model for protein binding to DNA[41,42]. Although applied to the location of its DNA target site, the operator O, by E. Coli lac repressor R[43,44,45,46,47,48,49] the model is sufficiently general to be applicable to the system of non-specific polyamine-DNA interactions[43].

Measured values of association constant k_a , for the repressor- operator interaction (Equation 5.2) were of the order of $10^2 - 10^3$ times greater than the maximum estimated value.

$$egin{array}{cccc} k_a & & & & & \ R+O &\rightleftharpoons & RO & & & \ & & & & \ & & & & \ & & & & \ & & & & \ & & & & \ & & & & \ & & & & \ & & & & \ & & & & \ & & & & \ & & & & \ & & & & \ & & & & \ & & & & \ & & & & \ & & & & \ & & & & \ & & & & \ & & & \ & & & & \ & & & \ & & & \ & & & \ & & & \ & & & \ & & & \ & & & \ & & & \ & & & \ & & & \ & & & \ & & & \ & & & \ & & & \ & & \ & & & \ & \ & & \ & \ & & \$$

A two-step process was proposed (Equation 5.3)[49] in which the first step is the diffusion controlled formation of a complex between R and a non-specific DNA site D, followed by a transfer process involving further non-specific RD complexes before the operator-repressor complex is formed.

$$k_{1} \qquad k_{2}$$

$$R + D + O \rightleftharpoons RD + O \rightleftharpoons RO + D \qquad (5.3)$$

$$k_{-1} \qquad k_{-2}$$

It was argued that since the overall rate must be speeded up by binding of R to D, such binding must increase the rate of target location and not slow it as would be expected if RD complexes acted as energy sinks for the repressor.

Application of the counterion condensation model[50,51,52] to this system gives a first step in Equation 5.3 that is a facilitated diffusion process as the counterions are found within a cylinder about the DNA in which the counterion concentration may approach molar concentration, even though bulk concentration may approach zero[52].

When applied to the example of polyamine sliding along the DNA, the non-specific DNA site D may be identified as the general DNA helix while O, the operator, corresponds to the damage site.

Polyamine Sliding Along the DNA Helix

The one-dimensional random walk process is assumed to increase the overall rate by increasing the target size to the DNA length over which the protein can slide before dissociating[41]. The lifetime of the complex is inversely proportional to ionic strength[45,46] which is in agreement with the finding that at increased concentrations of Na^+ and Mg^{2+} there is no detectable interaction between DNA and spermidine[53]. At sufficiently low ionic strength the rate of sliding is the rate limiting step. It has been shown that the rate of "hopping" (microscopic dissociation processes) has only a very marginal effect on the sliding result[45,46,47]. This is assumed to be because sliding provides a more efficient way of reaching nearby sites than dissociation-reassociation processes. This is especially valid for the polyamine-DNA systems in which association constants of the order of $10^3 M^{-1}[40]$ would appear to preclude disassociation-association processes in favour of the energetically more favourable sliding mechanism.

Approximate Sliding Rate Constants

An approximate sliding rate constant (or one-dimensional diffusion coefficient) has been calculated for the facilitated transfer of the repressor R on non-specific DNA and has a value of 9×10^{-10} cm²/s at 20°C[47,49].

Assuming a single, one-dimensional diffusion coefficient (D_1) for polyamines on DNA of $\geq 10^{-9}$ cm²/s (regarding the correlation time as a reorientational diffusion coefficient), and assuming that the rate of sliding is the rate limiting step, then the instantaneous random walk (or sliding) rate ($\Gamma_1=D_1/l^2$) corresponds to approximately 10⁶ 'jumps' between neighbouring phosphate groups per second (l is the interphosphate distance, i.e. 3.4×10^{-8} cm).

The length of such a random walk is given by $(D_1t/l^2)^{1/2}$, which gives a scanning of $\approx 10^3$ phosphates, or base pairs, per second (as the walk is random, and can therefore be in either direction). This approximate sliding length indicates that the proposed mechanism of polyamine-DNA interaction appears to be an effective method of drug delivery to DNA.

Molecular Interpretation of Polyamine Sliding

The sliding of repressor R, or a polyammonium cation, results in no net change in sodium ion displacement (Figure 5.15). The sodium cation displaced from in front of the sliding molecule is replaced by one 'binding' to the DNA behind the molecule as it slides along the helix. The molecule can thus be considered to be sliding over the DNA on an isopotential surface [54].



Figure 5.16: Schematic representation of the non-specific sliding interaction between polyamines and DNA phosphate groups (-)involving displacement of sodium ions (+).

As the sodium relaxation is rapid compared with the rate of sliding[55] the atmosphere is at equilibrium with respect to the sliding molecule and there is thus only a small thermodynamic barrier to sliding.

Direct interactions between radioprotectors and DNA have been suggested for aminothiols on the basis of geometrical configuration studies of the aminothiol molecules in isolation and during their approach to phosphates exposed on the DNA backbone[56,57].

The formation of an aminothiol-DNA complex by exact geometrical fitting of the amino and phosphate groups (of the aminothiol molecules and DNA) would presumably result in significant broadening of the proton NMR linewidths due to the lifetime of the distinct complex formed.

However, the sliding mechanism may be promoted through the attempted matching of amino and phosphate sites. The absence of a thermodynamic barrier to sliding and the uneven matching would tend to "pull" the aminothiol along the phosphate backbone.

5.9 Concluding Remarks

The results from Chapters 4 and 5 support the idea that the polyamines are constrained to remain close to the DNA polyion whilst existing as solvated species retaining a significant degree of independent motion, moving along the DNA backbone at approximately diffusion controlled rates. These results may have important consequences in the general area of drug delivery to DNA.

The territorial binding of certain transition metal complexes has also been demonstrated. The ability of these complexes to radioprotect DNA

from ionising radiation is reported below.

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Chapter 6

Studies of Irradiated DNA

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Systems

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6.1 Introduction

The previous chapters have shown that the territorial binding of certain polycations to DNA allows rapid sliding of the cations along the DNA helix, the cations retaining a significant degree of independent motion rather than being localised at specific binding sites. This result has applications in the design of potential radioprotecting agents and in the general area of drug delivery to DNA.

This chapter is intended as a brief review of work relating to the previous chapters. The ESR work described was carried out by Mr. A. Davies[1] and the plasmid assays by Mr. D. Elsy[2].

6.2 Polyamine Derivatives

Derivatives of polyamines have been investigated for anti-tumour activity for a number of years since studies indicated that polyamines were essential for cell growth[3]. Intracellular depletion of polyamines by chemical intervention in their biosynthesis activates a number of mechanisms aimed at conserving adequate intracellular polyamine concentrations, including increased uptake. This has been used to increase the uptake of spermidine derivatives following chemical treatment to deplete cells of polyamines [4,5]. Molecules exhibiting structural similarity to spermidine have been shown to utilize the transport carrier for spermidine[6,7] and structural analogues have been synthesised and investigated for potential anticancer activity following this scheme[8,9]. In addition to anti-tumour drugs, potential radioprotectors have been synthesised, based upon polyamines, to target the drug to DNA. The uptake characteristics of polyamines are thought to reside in the primary amine groups and previous derivatisation has concentrated upon the secondary amine[8,10,11,12]. Derivatised polyamines incorporating imidazole moieties, which have been shown to be effective electron scavengers[13], have been synthesised and work is in progress to determine their efficacy as radioprotection agents[14].

6.3 Aminothiol Compounds

The use of thiols in radioprotection was initiated with *in vivo* experiments using the sulphur-containing amino-acid cysteine (I)[15,16]. In 1959 the U.S. Army initiated an Anti-Radiation Drug Development Program at the Walter Reed Army Institute of Research. One of the drugs developed was WR2721, S-2(3-aminopropylamino)ethylphosphorothioic acid (II), the prototype aminothiol radioprotective drug, following clinical trials[17,18].

It was found that WR2721 differentially protected normal tissues with little or no protection of most experimental tumours[17,19,20]. The mechanism of this differential protection was thought to depend upon the facilitated uptake of WR2721 into most normal tissues[21] with minimal amounts of drug being absorbed by solid tumours by passive absorption[18,22].

Drug hydrophilicity has been demonstrated to be a major factor in this effect[23,24], dephosphorylation of WR2721, which reduces the hydrophilicity, allowing the resultant drug, WR1065 (III) to enter tumour cells more easily[23].

However, as the free, sulphydryl form of WR2721 (i.e. WR1065) is assumed to be the active metabolite of the drug[25] there is presumably a

I
$$H_3N^+CHCO_2^-$$

 $\Big|$
 CH_2SH

II
$$H_3N^+(CH_2)_3NH_2^+(CH_2)_3SPO_3H_2$$

III $H_3N^+(CH_2)_3NH_2^+(CH_2)_3SH$

IV $H_3N^+(CH_2)_2SH$

V $HO(CH_2)_2SH$

VI
$$-O_2CCH(CH_2)_2CONCHCONHCH_2CO_2$$

 $\begin{vmatrix} & & \\ & & \\ & & \\ & & NH_3^+ & CH_2SH \end{vmatrix}$

VII $H_3N^+(CH_2)_3NH(CH_3)^+(CH_2)_3NH_2^+(CH_2)_2SH$

VIII
$$H_3N^+(CH_2)_3NH(CH_3)^+(CH_2)_3NH_3^+$$

Figure 6.1: The protonated forms of cysteine (I), WR2721 (II), WR1065 (III), cysteamine (IV), 2-mercaptoethanol (V), glutathione (VI), RW222 (VII) and norspermidine (VIII).

correlation between activity and uptake time.

6.3.1 Protection of DNA by Thiols

Protection of DNA by thiols is thought to occur by hydroxyl radical scavenging and repair of DNA among other mechanisms[26]. The increased radioprotection shown by cationic thiols, e.g. WR1065 and cysteamine (IV) relative to the neutral 2-mercaptoethanol (V) and the anionic glutathione (VI) is thought to be due to the increased concentrations of drug in the proximity of the DNA[27,28,29].

Calculations based upon the counterion condensation model[30] have determined that monovalent cations are found within 20-30Åof the DNA cylinder, the concentration increasing by a factor of ten at approximately 10Åfrom the DNA[31]. Divalent cations show a greater increase in concentration near the DNA than monovalent cations. As, in bulk solution, glutathione, cysteamine and WR1065 react with hydroxyl radicals at essentially the same diffusion controlled rates [32,33], the increased protection afforded by cationic aminothiols is presumably due to the prevention of hydroxyl radicals impinging upon the DNA by the sheath of drug around the DNA cylinder. The proximity of the aminothiols is also beneficient in the repair of direct damage to the DNA, excluding oxygen from the region and also stabilising the helical structure thus allowing the repair mechanisms to be more effective.

6.3.2 Repair Mechanisms of Thiols

The main damage producing product of ionising radiation in aqueous systems is the hydroxyl radical[34]. The O-H bond energy is of the order of 50 kJmol⁻¹ stronger than most C-H bond energies[35], thus the hydroxyl radical reacts rapidly with most organic molecules to produce water and carbon-centred radicals. Addition to C-C double bonds can also occur. Reaction of OH radicals with DNA at the base sites is more common than reaction at sugar radicals, reaction at phosphate being insignificant[26].

The mechanism of protection of thiols (RSH) proceeds via donation of an hydrogen atom from the SH group, the S-H bond being substantially weaker than most C-H bonds[35]. The thiols thus destroy the damagecausing species (Equation 6.1), the thiyl radical formed not being sufficiently reactive to cause damage to the DNA[36], usually going on to form a disulphide (Equation 6.2).

$$RSH + OH^{\cdot} \longrightarrow RS^{\cdot} + H_2O$$
 (6.1)

$$RS^{\cdot} + RS^{\cdot} \longrightarrow RSSR$$
 (6.2)

However, whilst the RS^{\cdot} radical is not sufficiently reactive to cause damage directly to DNA, it has been shown that the thiyl radical of VI can react with O₂[37] and the resultant radical may be damaging to DNA[38].

The mechanism of thiol repair of DNA damage is complicated by the attack of OH radicals on both sugar and base components. In the sugar moiety (SH) damage is commonly H-atom abstraction. Thus donation of an H-atom from a thiol will result in restitution of the original molecule (Equations 6.3,6.4).

$$SH + OH^{\cdot} \longrightarrow S^{\cdot} + H_2O$$
 (6.3)

$$S^{\cdot} + RSH \longrightarrow SH + RS^{\cdot}$$
 (6.4)

Because damage to the sugar component usually results in strand breaks [39], most of the damage being decreased by thiols is presumably strand breaks.

Hydroxyl radical attack on the bases (B) however usually results in addition reactions and thus addition of an H-atom will not result in restitution of the original molecule [40,41,42] (Equations 6.5,6.6).

$$BH + OH^{\cdot} \longrightarrow BHOH^{\cdot}$$
 (6.5)

$$BHOH^{\cdot} + RSH \longrightarrow BHOH_2 + RS^{\cdot}$$
 (6.6)

Thiol repair is also in competition with molecular oxygen[43,44], TH representing intact DNA and T^{\cdot} a DNA radical (Equations 6.7,6.8).

$$T^{\cdot} + O_2 \longrightarrow TO_2^{\cdot}$$
 (6.7)

$$T^{\cdot} + RSH \longrightarrow TH + RS^{\cdot}$$
 (6.8)

$$TO_2 + RSH \longrightarrow TO_2H + RS^{-1}$$
 (6.9)

Whilst Equations 6.6 and 6.7 may lead to DNA damage, reaction of the oxygen adduct and thiol (Equation 6.9) has been found to prevent strand break formation in polyU[45]. This may be another means of DNA protection by H-atom donation.

6.4 Repair of DNA by Electron Transfer

The mechanism of aminothiol radioprotectors, H-atom donation, can effect restitution of the DNA molecule, generally after indirect damage effects, operating on sugar radicals at the proposed site of strand-breakage[46], thus restituting such damage.

The use of additives designed to repair the damage centres detected by ESR spectroscopy, under conditions of direct damage, has been studied in the frozen aqueous model system[47,48]. Those compounds used, e.g. nitroimidazoles, iodoacetamide, were found to lower the yield of either thymine or guanine by either electron acceptance or electron donation. The use of redox agents capable of both electron donation and electron acceptance has been investigated using transition metal complexes. The use of transition metal complexes in this manner depends upon the movement of the complexes between damage sites on the DNA molecule as opposed to the usual anti-tumour mechanism.

Repair of base damage by electron-transfer between the additive and the bases should decrease the yield of strand-breaks by reducing the incidence of those conditions necessary for strand breaks to occur[48].

6.5 Transition Metal Complexes

Transition metal complexes have had a wide range of applications as antitumour agents [49,50,51]. The usual mechanism of anti-tumour activity of these complexes involves disruption of the DNA helix via localised conformational changes [52,53,54].

A number of transition metal ions have been studied in DNA systems with respect to anti-tumour activity and the mechanism of action[51,55]. Chelates of ruthenium have been suggested as having oncostatic and virusidal activity since the 1960's[56,57]. The ready availability of the 2+ and 3+ oxidation states of ruthenium under physiological conditions and its well characterised coordination chemistry make ruthenium an ideal metal for study.

6.5.1 Ruthenium Anti-Tumour Agents

Ruthenium complexes have been found to be active against Ehrlich ascites tumour and leukemia L1210 cells[58], in a manner similar to that of cisplatin (cis-[Pt(NH₃)₂Cl₂]). The use of ⁹⁷Ru-containing radiopharmaceuticals as tumour imaging, location and diagnostic aids has been investigated[59,60] and the incorporation of ¹⁰³Ru and ¹⁰⁶Ru β -emitting radionuclides in radiotherapeutic drugs to provide a short range radiation dose at the tumour site has also been suggested[61]. A number of ruthenium-ammine complexes have shown anti-tumour activity[62,63] and ruthenium bipyridyl complexes have been found to sensitise DNA cleavage following photolysis[64,65].

These examples, and those of anti-tumour complexes in general, depend upon coordination of the metal centre to DNA, usually at the N7 site of guanine[66]. This site is available on the exterior of the DNA in the major groove and has been determined to be the most electron-rich, and consequently the most favourable, site for metal ion coordination[67].

The treatment of radiation damage depends not upon the disruption of DNA structure and inhibition of replication as for tumour cells but upon repair of the damage centres and prevention of cell death. Consequently, complexes used in this study were chosen such that no binding of the metal centre should occur.

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6.5.2 Ruthenium Solution Chemistry

Ruthenium occurs in aqueous solution predominantly as Ru^{II} and Ru^{III} . Complexes of Ru^{II} are octahedral and diamagnetic with a (t_{2g}^6) configuration. Ru^{III} compounds are octahedral, low-spin and have a (t_{2g}^5) configuration. Complexes of Ru^{II} and Ru^{III} with nitrogen bases are generally inert to substitution. The loss of ligands from $[\operatorname{Ru}^{II}(\operatorname{NH}_3)_5 L]$ (L=NH₃ or nitrogen heterocycle) is usually faster than in the corresponding Ru^{III} complexes but under physiological conditions the half-lives are still of the order of a day[68].

Electron exchange reactions between Ru^{II} and Ru^{III} have been extensively studied and have been shown to proceed via both inner- and outersphere mechanisms[69,70,71,72]. Redox reactions involving substitutionally inert complexes, e.g. the $[\operatorname{Ru}(\operatorname{NH}_3)_6]^{2+/3+}$ couple, usually participate in outer sphere reactions i.e. those in which the two reactants do not share a common atom or group, or, more generally, reactions in which the interactions of the relevant electronic orbitals of the two centres is weak. With low-spin (t_{2g}^6) and (t_{2g}^5) 4d electron configurations for the complexes there is no electronic spin-state change upon electron transfer. A single t_{2g} electron is transferred between a singlet and doublet state (Figure 6.2).

The ligands are firmly bound in both oxidation states and do not serve as facile channels for electron transfer. There is little inner coordination sphere reorganisation required because ruthenium-nitrogen bond lengths are essentially unchanged in passing from one oxidation state to the other[69,73]. Since the Frank-Condon barrier to electron transfer is small, redox reactions involving these systems are usually rapid, the rate of reaction being



Figure 6.2: Schematic energy level diagram showing electron transfer between the t_{2g} levels in the Ru(II/III) couple.

largely determined by outer-sphere, solvation, effects.

The use of macrocyclic compounds is expected to enhance electron transfer by an outer-sphere mechanism as the constraints of macrocyclic binding, either 4-coordinate or 6-coordinate, make the inner-sphere reorganisation minimal and the formation of an adduct between the metal complex and a site on the DNA is made unfavourable by the size of the macrocycle.

Outer-sphere reorganisation energies for macrocyclic complexes are expected to be smaller than for ruthenium ammines previously studied[69,70] as the increased complex size decreases the solvation strength.

6.6 ESR Spectroscopic Studies

6.6.1 Experimental Details

The metal complexes were studied for potential radioprotection using the technique of ESR spectroscopy. Under the conditions of direct damage, using frozen aqueous DNA at liquid nitrogen temperatures (77K), the damage centres, as detected by ESR, are the bases guanine, as the guanine radical cation $G^{,+}$ and thymine, as the thymine radical anion $T^{,-}$. Electron transfer, either from the anion or to the cation, would restitute the original DNA molecule. This is in competition with hydroxyl radical and oxygen addition, which would prevent restitution[42], and intramolecular H-atom abstraction which could lead to a possible β -elimination mechanism and strand breakage[45].

Electron transfer between the metal complex and the damaged base centre results in depletion of the detected base radical yields. Reduction of $G^{\cdot+}$ or $T^{\cdot-}$ centres should lead to a reduction in the number of strand breaks observed as these have been shown to lead to strand break formation[48].

As double strand breaks are assumed to occur when $G^{\cdot+}/T^{\cdot-}$ pairs are trapped between about 15 to 30Åin opposite strands, a reduction in either $G^{\cdot+}$ or $T^{\cdot-}$ yields should result in a greater percentage decrease in the yield of double strand breaks. A random 50% decrease in $T^{\cdot-}$, for example, would lead to the same percentage reduction in double strand breaks. However, as $G^{\cdot+}$ is essentially unchanged, and assuming $G^{\cdot+}$ and $T^{\cdot-}$ form strand breaks with comparable efficiency, a 50% reduction in $T^{\cdot-}$ reduces single strand breaks by only 25%. Previous results appear to support this[13].

The possible redox cycle between the bases and the transition metal

centre should lead to a greater percentage reduction in the yield of single strand breaks in addition to the reduction, and possible elimination, of double strand breaks.

Since double strand breaks lead to the separation of DNA fragments, due to diffusion, they are a major cause of cell death, preventing both repair and replication. The formation of single strand breaks, assuming they are separated by at least 30Å, should not result in any major disruption, allowing the cellular repair mechanisms to operate.

Analysis of the ESR spectra of DNA in the presence of these additives thus leads to an understanding of the mechanism of radioprotection of the additives.

6.6.2 Results and Discussion

6.6.3 Radiosensitisation of Tumour Cells

The ruthenium-imidazole complexes (IX, X, XI) are similar to those studied previously for radiosensitising ability in Chinese hamster ovary (CHO) cells[75]

The complex IX has been found to show a higher sensitising enhancement ratio (SER) than those complexes incorporating imidazole ligands with large aliphatic or aromatic groups, and than the free imidazole ligands themselves. The SER is defined as the ratio of X-ray doses between the hypoxic control and drug at 1% cell survival. Thus, enhanced sensitisation results in a lower radiation dose needed to produce the same percentage cell death.

The improved radiosensitising ability of these complexes may depend



XI L =

$$\begin{array}{c}
H \\
N \\
N \\
H \\
N \\
CH_{3}
\end{array}$$

$$\begin{array}{c}
H \\
N \\
CH_{2}CH_{2}CH_{2}OH \\
H \\
CH_{3}
\end{array}$$

Figure 6.3: Structures of the ruthenium-imidazole complexes RuCl₂(DMSO)₂(4-nitroimidazole)₂ (IX), RuCl₂(DMSO)₂(2-Me-5-nitroimidazole)₂ (X) and RuCl₂(DMSO)₂(metronidazole)₂ (XI), (S=sulphur bonded DMSO).

upon the increased reduction potential of the imidazole ligand (binding of a nitroimidazole ligand to the metal centre results in an increased reduction potential of the ligand, i.e. an increased electron affinity). The electron affinity of ligands is often correlated with radiosensitising ability[76].

Radiosensitising ability in tumour cells is maximised by the use of electron-affinic ligands, which appear to mimic molecular oxygen which is known to interact with radiation damage in DNA, thus leading to effective treatment[77]. Many tumour cells are low in oxygen content (hypoxic) and are consequently resistant to radiation treatment[78].

Attachment of these radiosensitising ligands to metals that are expected to remain close to the DNA, either through binding or electrostatic attraction, increases the local concentration of radiosensitiser at the target and thus decreases the required concentration and any deleterious sideeffects[79].

6.6.4 Radioprotection of DNA

Ruthenium–Imidazole Complexes

The use of these complexes in the treatment of radiation damage to DNA, i.e. radioprotection rather than radiosensitisation, is expected to proceed via the same mechanism. A similarly low dose is available and there is no evidence of any binding to the DNA under experimental conditions (see above). Results show a significant decrease in the thymine radical yields after irradiation in the presence of the complexes IX to XI[1]. The reduction in T^{-} was greater than the reduction measured in the presence of the free ligand alone[13]. The concentration of G^{++} was unaffected. The radical anions of the imidazole ligands were observed and it is assumed that the metal centre increases the electron affinity of the ligands and the proximity to the DNA without participating in the reaction directly.

Inclusion of metronidazole has been found to reduce significantly the number of both single and double strand breaks and it is presumed that the ruthenium-imidazole complexes have a similar effect.

Macrocyclic Complexes

Irradiation of DNA samples in the presence of the thiamacrocyclic ruthenium complexes also resulted in reduced radical yields. The inclusion of XII resulted in a decreased radical yield of $G^{\cdot+}$ and a signal attributable to Ru³⁺ was observed. Irradiation in the presence of XIII gave reduced radical yields of $T^{\cdot-}$ and the signal due to Ru³⁺ was seen to decrease. The radical yield of $T^{\cdot-}$ was reduced to a greater extent than that of $G^{\cdot+}$. These two results are presumed to be due to electron transfer between the metal centre and the damage sites. Since, in both cases the yield of the complementary base damage site, i.e. $T^{\cdot-}$ in the case of XII and $G^{\cdot+}$ for XIII, was essentially unchanged, it is assumed that there is no redox cycling between the Ru²⁺/Ru³⁺ states.

The inclusion of XIV however, resulted in decreased radical yields of both $T^{\cdot-}$ and $G^{\cdot+}$. The yield of $T^{\cdot-}$ was significantly reduced whilst the yield of $G^{\cdot+}$ was reduced to a lesser degree. This appears to indicate that a redox cycling effect is in operation, although the intensity of the signal due to Ru³⁺ was not significantly reduced.

These results tend to suggest that electron transfer between T^{-} and Ru^{3+} is more favourable, as may be expected. However, the fact that



Figure 6.4: Structures of the macrocycles in the ruthenium complexes $[Ru(9S3)]^{2+}$ (XII), $[Ru(16S4)Cl_2]^+$ (XIII) and trans- $[Ru(cyclam)Cl_2]^+$ (XIV).

reduced yields of $G^{\cdot+}$ were observed does apparently indicate that electron transfer between Ru^{2+} and the $G^{\cdot+}$ radical occurs.

The possible reversible electron transfer reaction exhibited by XIV may reflect the increased stability of ruthenium-ammine complexes, although the possible loss of a chloride ligand during the reaction may be involved in the process. Incorporation of the encapsulated complex $[Co(diAMsar)]Cl_3$, or the ruthenium analogue, should help to clarify the mechanism with regard to this question.

6.7 Plasmid DNA Strand Break Assays

6.7.1 Strand Breaks

The experimental details for the strand break assay can be found elsewhere [80]. Plasmid DNA can be extracted in a superhelically coiled form. The introduction of a 'nick' (a single strand break) allows the superhelical twists to unwind to give a 'relaxed', open-circular form of the plasmid. if the strands are broken at a coincident site (a double strand break) the plasmid is obtained in a linear form. These forms can be easily separated and measured by gel electrophoresis.

6.7.2 Results

The strand break assay results from experiments conducted at room temperature (293–298K) are outlined for both single strand breaks and double strand breaks (Figure 6.5). The results indicate a correlation between the charge on the additive and the protection conferred. This is consistent



Figure 6.5: The effect of WR1065 (III), cysteamine (IV), 2-mercaptoethanol (V), RW222 (VII) and norspermidine (VIII) on strand breaks induced by γ -irradiation of plasmid DNA at 293–298K. The percentage form of plasmid DNA indicates single and double strand breaks.

with the idea expressed previously that the increased proximity of a drug to DNA increases the protection[27,28,29]. Indeed, double strand breaks are only observed at higher doses for V and VIII.

The predominant mechanism under the experimental conditions is indirect damage. The increased protection offered by the aminothiols is therefore due largely to interception of the damage causing hydroxyl radical.

Results from experiments conducted at liquid nitrogen temperature (77K) would be expected to show damage at higher doses of radiation than those conducted at room temperature. As radicals generated in the ice-phase are localised away from the DNA, only those radicals generated within the DNA phase itself are capable of causing damage. Experiments at this temperature are currently in progress.

6.8 Continuing Work

There is a large amount of research possible following on from the work mentioned above. The use of polyamines and encapsulated metal complexes can be combined in the use of pendant arm macrocycles[81,82] and further work is necessary on those classes already synthesised in order to investigate further the mechanisms of electron transfer and radioprotection.

The polyamine-imidazole class of compounds recently synthesised[14] requires further investigation having shown some initial activity.

Throughout this work the subject of study has been the sodium salt of DNA, and has been studied *in vitro* exclusively. The next step, once the efficacy of a particular class of drug has been established, is to extend the scope of experiment to encompass cellular targets. These would include cell membrane permeability studies and a study of their interaction with cellular DNA.

The majority of cellular DNA is bound to histones and to other positively charged compounds[83], thus reducing the ability of the polyammonium cations and other positively charged additives to associate with the DNA. However, those sites involved in replication are not bound to histones and are more likely to be susceptible to radiation damage[84]. This is the region to which the cationic drugs are most likely to bind. The work described here may thus be appropriate in describing the interaction between these drugs and the region of DNA most susceptible to radiation damage.

Once the work has been extended to cellular systems, the question of the potential mutagenesis of the complexes becomes important. The more stable a complex is, the longer it will remain active in the radioprotective sense. Thus, the need for further encapsulating ligands seems to be indicated for the continuance of these mechanisms.

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Appendix A

Sodium Relaxation Theory

Both the longitudinal and transverse relaxation rates produced by a quadrupolar interaction are simple exponential decays if:

- 1 the nucleus has a spin $I \leq 1$
- 2 the electric field gradients fluctuate much more rapidly than the Lamor frequency of the nucleus, the "extreme narrowing" condition [1].

If neither of these conditions is satisfied, as is the case for sodium at fields of 200 MHz and above in solutions of aqueous DNA [2], then the longitudinal and transverse relaxations produced by a quadrupolar interaction are the sums of I exponentials, if I is an integer, or (I+1/2) exponentials, if I is a half-integer. For the case of sodium (I=3/2) the relaxation decays as the sum of two exponentials.

The transverse relaxation is given by:

$$M_T(t) = M_T(o) \Big((0.6 \exp(-a_1 t) + 0.4 \exp(-a_2 t)) \Big)$$
 (A.1)

where:

$$M_T(o) =$$
 equilibrium magnetisation

$$a_1 = p_F R_F + p_B / T_{2f}$$
 (A.2)

$$a_2 = p_F R_F + p_B / T_{2s}$$
 (A.3)

Here p_F and p_B are the mole fractions of nuclei in the free and bound states respectively and the fast and slow components of the relaxation are given by:

$$\frac{1}{T_{2f}} = \frac{\pi^2}{5} \chi^2 \tau_c \left(1 + \frac{1}{(1 + \omega^2 \tau_c^2)} \right)$$
(A.4)

$$\frac{1}{T_{2s}} = \frac{\pi^2}{5} \chi^2 \tau_c \left(\frac{1}{(1+4\omega^2 \tau_c^2)} + \frac{1}{(1+\omega^2 \tau_c^2)} \right)$$
(A.5)

where:

$$\chi$$
 = quadrupole coupling constant
 τ_c = correlation time for bound cations
 ω = resonance frequency (rad s⁻¹)

If $\omega \tau_c$ is sufficiently small (< 1.5) then equation (A.1) is approximately exponential with the relaxation rate:

$$R_2 = T_2^{-1} = p_F R_F + p_B \left(0.6(T_{2f}^{-1}) + 0.4(T_{2s}^{-1}) \right)$$
(A.6)

If $\omega \tau_c \ll 1$ then:

$$R_2 = T_2^{-1} + p_F R_F + p_B \frac{2\pi^2}{5} \chi^2 \tau_c \tag{A.7}$$

The high resolution spectrum is obtained by taking the Fourier Transform of equation(A.1). The result is a superposition of two Lorentzian components, if second-order dynamic frequency shifts can be neglected [3]. When equation(A.7) applies the spectrum is observed to be a single Lorentzian.

The broad component, whose integrated intensity is 60% of the total, has a linewidth $\Delta \nu_{\frac{1}{2}} = R_{2f}^{Na}/\pi$ (Hz). The narrow component, comprising 40% of the signal, has a linewidth $\Delta \nu_{\frac{1}{2}} = R_{2s}^{Na}/\pi$ (Hz). Small magnitudes of quadrupolar coupling constants have been evaluated for ${}^{23}Na$ in polyelectrolyte systems [4,5]. This has suggested that the relaxation in these systems occurs as a two-step process [6,7].

This two-step relaxation is described as a fast anisotropic reorientation superimposed on a more extensive slow motion.

The fast component of the relaxation is explained as an averaging of the quadrupolar interaction, by a fast, slightly anisotropic motion, to a small non-zero value. This is attributed to rapid molecular motions within the first hydration shell of the sodium ion or fast diffusion of the sodium around the polyelectrolyte. The second component is the averaging to zero of the residual quadrupolar interaction by a much slower motion. This could be due to internal motion within the polyelectrolyte molecule or translational diffusion of the sodium ions away from the influence of the polyelectrolyte.
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Appendix B

Publication

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CHEMICAL PHYSICS LETTERS

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MOTION OF POLYAMMONIUM IONS ON AQUEOUS SOLUTIONS OF DNA

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Magnetic resonance techniques have been used to establish or confirm that (i) various polyamines are present almost completely as polyammonium cations at pH 7, (ii) that these have the very high affinity for DNA expected between a polycation and polyanion, and (iii) that this is a loose electrostatic interaction which does not significantly hinder motion (rotational and translational) of the cations close to DNA, and hence that migration of the polyammonium ions along the DNA must be extremely rapid.

1. Introduction

Several polyamines, especially spermine, spermidine, putrescine and cadaverine (fig. 1) are abundant in nature; but their precise rôles remain relatively poorly understood, despite the fact that polyamines are necessary for the normal growth of cells. A key property is that in solutions buffered to a pH of ≈ 7 (the normal body pH) they are effectively fully protonated. Hence we are really considering polyammonium cations (PAC) rather than polyamines. As expected from polyelectrolyte theory [1], PACs have a very high affinity for the polyanion. DNA, and it is probably this affinity that makes them important in nature. Our interest centres on the effective rate at which PACs migrate along DNA strands. In the course of this study we have used (i) ¹H NMR of C-H protons adjacent to nitrogen (α -protons) to obtain approximate protonation constants, (ii) ²³Na resonance to assess the extent to which PACs displace Na⁺ from the close vicinity of DNA [2], (iii) ¹H resonance linewidths to gauge the freedom of motion of PACs associated with DNA, and (iv) ESR spectroscopy of a spin-labelled PAC (fig. 1) to extend the time range for (iii).

I $H_{3}^{+}(CH_{2})_{3}^{-}H_{2}^{+}(CH_{2})_{4}^{+}H_{2}^{-}(CH_{2})_{3}^{+}H_{3}^{+}$ II $H_{3}^{+}(CH_{2})_{3}^{-}H_{2}^{-}(CH_{2})_{4}^{-}H_{3}^{+}$ III $H_{3}^{+}(CH_{2})_{4}^{-}H_{3}^{+}$ IV $H_{3}^{+}(CH_{2})_{3}^{-}H_{3}^{+}(CH_{3})^{-}(CH_{2})_{3}^{-}H_{3}^{+}$ V $H_{3}^{+}(CH_{2})_{3}^{-}H_{3}^{+}(CH_{3})^{-}(CH_{2})_{3}^{-}H_{3}^{-}$

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Fig. 1. The protonated forms of the naturally occurring polyamines, spermine (I), spermidine (II), and putrescine (III); the NMR probe molecule N^4 -methylnorspermidine (IV), and the nitroxide derivative used for ESR studies (V).

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2. Protonation constants

Our results show that the proton chemical shifts change markedly when nitrogen is protonated (fig. 2), and hence are ideal for measuring the extent of protonation. However, for spermidine, the shifts for the protons of the four methylene groups differ by only 0.12 ppm [3], and hence only an overall value for protonation can be obtained directly. This difficulty does not apply for the N-methylated derivative. (IV). This problem has been overcome for spermidine using two-dimensional ${}^{1}\text{H}{-}{}^{13}\text{C}$ correlated NMR spectroscopy, and accurate pKa values for the three different protons have been derived [4]. Their results show that protonation starts at a pH of



Fig. 2. Proton chemical shifts as a function of pH for the PAC (IV) (a) for the methyl protons, (b) for the inner CH₂ protons and (c) for the outer CH₂ protons.

H; NCH2	CH_2	NH ⁺ -CH ₂ -CH ₂ -NH ₃ ⁺]			
t	t	~	t	t	
L c	b	CH3	ь	c	Ţ
		(a)			

 \approx 12. and is effectively complete at pH 7. Site preferences are almost negligible for the first proton but double protonation is slightly favoured at the terminal nitrogens. Ease of addition of a second proton is barely affected by the first (\approx 0.2 pK units) but the third is added with greater difficulty (\approx 1.2 pK units).

Our results for (the N-methylated derivative (IV)) compare well with these conclusions. The shift experienced by the methyl protons (a) of (IV) is entirely due to protonation on the central nitrogen (fig. 2). Curve a gives an apparent pK value of 8.0. Shift data for the CH₂ protons adjacent to the central nitrogen (b) give the same pK value, but the extra inflection at higher pK shows that these protons are also sensitive to protonation of the outer nitrogens. The outer CH₂ protons are mainly sensitive to terminal protonation and give an apparent pK value of 10.3 for both. Again, a smaller shift occurs when the central nitrogen is protonated.

The value of 10.3 agrees well with that of 10.4 given for the terminal amino groups in spermidine [4]. However, the value of 8.0 is ≈ 1 pK unit less than that for the central nitrogen of spermidine (II) (≈ 9) [4]. This is presumably largely a function of the greater proximity of one of the two outer positive charges for (IV) relative to (II). Also, the methyl group may slightly reduce the basicity of this nitrogen. Our results confirm that the outer NH₂ groups are effectively more basic than the central nitrogen.

It seems that proton resonance shift studies are more suitable than 13 C or 15 N studies [5-8], mainly because distance effects from the more remote protonation sites are insignificant. The key result is that the central nitrogen is the last to be protonated but that this is effectively "complete" at pH 7.

3. Binding constants

Polyelectrolyte theory requires that there be strong binding between DNA in its uncomplexed form and PACs. One of the most convincing measurements of this effect is the change in the linewidth for the ²³Na resonance line of aqueous DNA [2.9]. When sodium ions are close to DNA the strong, asymmetric electric field from the DNA (which has an effectively linear structure over small distances) causes a Volume 165, number 2.3

marked line-broadening via quadrupole alignment. The "near" and "far" Na⁺ ions are in rapid equilibrium so the measured broadening is a time average. When PACs are added, they displace Na⁺ from the vicinity of the DNA. thus causing a sharpening of the ²³Na resonance. This can be shown as a simple equilibrium (1) although intermediate stages may be significant (f and b indicate free and bound ions).

$$(PAC)_{f}^{n+} + nNa_{b}^{+} = (PAC)_{b}^{n+} + nNa_{f}^{+}$$
 (1)

(Once one positive unit in a PAC ion is close enough to DNA to displace one Na⁺, there will be a high probability that the other will follow, because of the pseudo "chelate" effect which accounts for the high binding constants.) Much work has been directed towards discovering what happens at high degrees of loading DNA, and at high concentrations of sodium ions [9]. Obviously, an excess of Na⁺ will tend to reverse equilibrium (1), and differences between different polyamines will become marked when the loading is high. Our concern is with the motion of PACs which are close to DNA, and hence we have removed and excess of sodium ions, and have kept the concentration of bound PACs as low as possible. Under these conditions binding constants are in the region of 10³, and the assumption of almost complete binding is justified.

Our results for PAC IV are shown in fig. 3. The trend is effectively linear, and the line extrapolates to unity, i.e. to complete loading of the PAC onto DNA. (The unit N/P used here is the ratio of the number of positive charges to the number of negative charges, i.e. the number of N atoms to the number of P atoms.) This result accords with effectively complete binding [9], and is similar to that found by Burton et al. [2] for spermidine.

These studies establish strong binding, but give no information about preferential binding sites, if any, nor about the degree specificity of binding.

4. Freedom of motion of bound PACs

It is commonly assumed that spermine forms a bridge across the minor grove of B-DNA, the two ammonium groups on each and being close to phosphate groups of either strand [11]. More recently, X-ray analyses of tRNA containing two spermine



Fig. 3. Trend in the width increment, Δ , for the ²³Na⁺ resonance of calf-thymus Na-DNA on adding the PAC (IV). Concentration is expressed as N/P (see text).

units and of a double-helical B-DNA dodecamer with a spermine counter ion have not supported this specific structure, the former having a spermine ion lying along a major grove of the anticodon stem [12], and the latter having it bridging the major groove [13]. Nevertheless, all these results can still be used to infer "tight" DNA binding.

Here we define "tight" binding as involving PACs attached in some way to the DNA anions such that they are stationary at some site on the DNA during the period of interaction. On the other hand, "loose" binding is defined in terms of almost free diffusion of the PACs along the DNA strands, in such a way that they are close to the DNA for long periods. Thus, binding constants are high, but they remain loosely bound.

In a recent study, Wemmer et al. [14] showed by NMR spectroscopy that, in the presence of a small, double stranded DNA oligomer, d(C-G-C-G-A-A-T-T-C-G-C-G) spermine behaves as if it were quite free to rotate, with no restrictions caused by tight binding. Our results for calf thymus DNA are fully in accord with this finding. On the time-scale of the NMR experiment, the PACs are effectively free (fig. 4). In view of the high binding constants, we interpret this result to mean that these cations have almost complete rotational and translational freedom

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but that they are constrained to remain close to the DNA. This must mean that their rates of movement along DNA must be extremely high.

This result is supported by ESR linewidth studies of the spin-labelled PAC(V). There was no detectable linewidth increment for the DNA-cation system (width of the $|0\rangle$ feature in the absence of DNA=1.67±0.02 G, and in the presence of DNA=1.65±0.02 G for 10⁻⁵ M solutions). In this case, there must be some rotational freedom even in the tight-binding limit because of the non-rigid side chain. However, model studies show that tight binding would severely restrict this motion and should result in considerable broadening. Absence of any significant broadening suggests a correlation time less than $\approx 10^{-9}$ s, which again implies remarkable freedom.

Wemmer et al. argue that their NMR results can still be interpreted in terms of tight binding, as is usually envisaged for PACs, provided there is very rapid jumping from site to site [14]. We think that this alternative is far less probable than the loose binding concept with rapid diffusion close to the



Fig. 4. Part of the 300 MHz ¹H NMR spectrum of (IV) in the presence (a) and absence (b) of DNA (0.15 M in phosphate units). Apart from a very small general broadening from viscosity changes there is no significant broadening from binding to the DNA.

DNA. Tight binding would be maximised by hydrogen-bonding between the phosphate anions and the ammonium protons. If, for spermine, say, three out of four ammonium groups were bonded strongly, it would be necessary for at least three H-bonds to break in a short time period in order to give freedom for diffusion. This "chelate effect" is expected to give a long enough lifetime for such tight complexes to give marked NMR and ESR line-broadening, which is not observed.

We conclude that these PACs are constrained to remain close to the DNA polyanions, that they are not precisely bound, but exist as solvated species having freedom to move at approximately diffusioncontrolled rates along the DNA strands.

This result may be of importance in the general area of drug delivery to DNA [15].

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