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The Interaction of Methylene Blue with Nucleic Acids.

A Photophysical and Photochemical Study
THE INTERACTION OF METHYLENE BLUE WITH NUCLEIC ACIDS

A photophysical and photochemical study

by

Wilhelm J.M. van der Putten M.Sc.

A Thesis presented to the University of Dublin for the degree of

Doctor in Philosophy

Department of Chemistry, University of Dublin, Trinity College. June, 1987
DECLARATION

This thesis has not been submitted as an exercise for a degree at any other University. Except as where otherwise indicated, the work described herein has been carried out by the author alone.

Wilhelm J.M. van der Putten M.Sc.
The photophysical and photochemical properties of the dye 3,7 bis-(dimethylamino) phenazothionium chloride, methylene blue, with calf thymus DNA and the synthetic polynucleotides poly[d(A-T)] and poly[d(G-C)] have been investigated. The experimental techniques used were absorption and fluorescence spectroscopy, transient laser kinetic spectroscopy and molecular biology techniques.

A strong decrease in the fluorescence yield of the dye is observed when bound to CT-DNA and poly[d(G-C)]. This decrease is attributed to an electron-transfer from the guanine nucleotide to the excited singlet state of the dye. Laser flash spectroscopy on a picosecond time scale confirmed the electron-transfer hypothesis. Both CT-DNA and poly[d(G-C)] show a decrease in triplet yield. This decrease is concomittant with the decrease in fluorescence yield and a direct reaction of triplet state dye with these polymers is excluded. When methylene blue is bound to poly[d(A-T)] no change in either fluorescence yield nor triplet yield occurs.

Binding studies of the dye to the three polynucleotides showed a markable difference in the mechanism of binding. At low ionic strength the binding can be considered intercalative for all polymers. Increasing the ionic strength leads to a gradual decrease of dye binding to the polymer poly[d(G-C)]. The binding of the dye to poly[d(A-T)] consists of two different modes, one -presumably intercalation- which is extremely salt sensitive and one which is relatively invariant on addition of salt. Laser flash spectroscopy proved invaluable in the elucidation of these binding modes.

Methylene blue was found to cleave DNA directly, without the necessity of alkaline or nucleophilic additives. This cleavage was shown to occur both in the presence and absence of oxygen. Evidence for the involvement of singlet oxygen in the oxygen dependent mechanism was equivocal. The oxygen independent mechanism probably involves electron-transfer which had already been demonstrated in the picosecond flash spectroscopic measurements. Cleavage of the polymer backbone occurred always at a guanine nucleotide.

Computer simulation of energy transfer processes in DNA, from the nucleotides to intercalated dye molecules, indicate a possible transfer range of ca. 100 basepairs. Energy-transfer measurements between methylene blue and ruthenium tris-(1,10-phenanthroline) suggest that binding of dye molecules to DNA is not a static process, but that the molecules are mobile and able to diffuse along the polymer.
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love, the effort and labour put in the preparation of this thesis would
have been without meaning. I also sincerely apologize for the frequent
trips to the laboratory, when I really should have been home, cutting
the grass or washing the car.

I dedicate this thesis to the memory of my mother.
ABBREVIATIONS

A : Adenine
T : Thymine
C : Cytosine
G : Guanine
A-T, G-C : Nucleotide basepairs
poly[d(A-T)] : poly-deoxyadenylic-deoxythymidylic acid (double stranded)
poly[d(G-C)] : poly-deoxyguanylic-deoxycytidylic acid (double stranded)
CT-DNA : Calf thymus DNA
MB⁺ : Methylene blue
P/D : Polynucleotide phosphate to dye ratio
cccDNA : Covalently closed circular DNA
ocDNA : Open circular DNA
n : binding number
R : ratio $\frac{3 \text{MB}^2+}{3 \text{MB}^+}$
r : ratio of bound dye to concentration of binding sites
C_b : Bound dye
C_f : Free dye
C_t : Total dye concentration
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PREFACE AND AIMS OF WORK

It is worthwhile to give a brief summary of the origins and background of the research project from which the work presented in this thesis forms but a small part. The aims of the overall project were mainly centered on the use of short light pulses for the study of ultrafast processes in polymers and semiconductors. Polymers included biopolymers and synthetic conjugated polymers such as polydiacetylene. These polymers have ultra-fast excited state behaviour and possess large non-linear susceptibilities. The study of the interaction of very short light pulses with them could yield information on their non-linear photoproperties. These properties are important in areas such as high speed data transmission and image processing [Dennis, 1985].

The techniques developed in these studies were also to be applied to the biopolymer deoxyribonucleic acid (DNA). The study of the interaction of the polymer with short pulses of light could lead to a better understanding of the ultrafast opto-electronic properties of DNA. It is well known that DNA can be damaged by irradiation with light but the excited state deactivation processes involved in this are not very well understood. Singlet excited state energy-transfer along the polymer is proposed to play a role in the photoinduced damage of DNA [Nikogosyan et al., 1985] but the extent of such energy-transfer is currently the subject of some controversy [Anders, 1982; Rayner et al., 1980]. The direct measurement of relaxation processes in DNA is difficult due to the fast time-scale of deactivation. Small molecules that bind to DNA can however be used as probes in the investigation of photophysical properties of DNA and it was decided to utilise these in the determination of the extent of excited state energy-transfer in the
biopolymer.

A second overall aim of the project was the development of new methods to determine the sequence of the genetic code of DNA also using the interaction of DNA with light. Sequencing methods currently in use, i.e. the Sanger method [Sanger and Coulson, 1975] and the Maxam-Gilbert method [Maxam and Gilbert, 1977] can, at most, only sequence ca. 300 base-pairs/day. It has been proposed to sequence the genetic code of a typical human [Palca, 1986; Joyce, 1987]; as the human genome contains more than $3 \times 10^9$ basepairs [Saenger, 1984] it is obvious that novel methods to determine the basepair sequence of DNA are necessary. All nucleic acid sequencing presently in use starts by generating a series of fragments from a DNA polymer which are all terminated by the same target base. These fragments all have different lengths and can be separated by electrophoresis and analyzed. The Maxam-Gilbert method generates the fragments by cleaving the phosphodiester bond of the DNA at the particular base in a series of chemical reactions. To obtain different base-specific reactions, a different series of chemical manipulations has to be used and the total procedure is time consuming and inefficient.

A novel method to generate base-specific cleavage of the phosphodiester bond which would overcome the difficulties associated with the Maxam-Gilbert and Saenger methods involves the use of photosensitizing dyes which bind to the nucleic acid. When the dye binds to a particular base-pair of the polymer, then the effects of the photochemical damage induced by the dye can also be base or base-pair specific. This could lead to base-specific photochemistry and cleavage of the phosphodiester bond. Finally it was hoped that the interaction of DNA and DNA/dye complexes with ultrafast light-pulses could also lead to base-specific photochemistry. Evidence for this had already been presented in literature [Andreoni et al., 1980].
Summarizing, the main aims of the research program with relevance to nucleic acids were 1) the investigation of the possibility of energy-transfer in nucleic acids using dye molecules as probes and 2) the study of the interaction of photosensitizing dyes with DNA with as final aim the photosensitized generation of base-specific cleavage of the phosphodiester bond. The project obtained its main funding from the British Petroleum Venture Research Unit.

In this study it was decided to start with the dye Methylene Blue. Friedman and Brown [1978] already showed that this dye was able to initiate guanine specific damage to DNA via an as yet unspecified reaction mechanism. The investigation of the photosensitizing properties of this compound in the presence of DNA would hopefully lead to a better understanding of the general mechanism of photosensitized strand cleavage. Also, an overview of the literature indicated that the mode of binding of this compound to DNA was not very well known and it appeared important to assess its photosensitizing ability with respect to binding to the polynucleotide.
INTRODUCTION

The photophysical and photochemical interactions of the dye methylene blue with nucleic acids were investigated. The possibility of using methylene blue as a probe for the nucleic acid environment and its capability to mediate base specific photochemical cleavage of the sugar phosphate backbone of the polymer prompted our interest in the study of its interaction with DNA.

In the first two chapters of this thesis a brief introduction into the area is given. The first chapter discusses the structure of nucleic acids and the binding of small molecules (ligands) to it while the second chapter surveys the literature on the spectroscopic properties of methylene blue and of its binding to DNA, concluding with a discussion of the literature on the methylene blue photosensitized degradation of nucleic acids.

In chapter 3 the results of experimental investigations on the photophysical properties of complexes of methylene blue with natural and synthetic nucleic acids are presented. Chapter 4 describes the results of a study on the binding of the dye to the nucleic acids. In this chapter a novel method to measure association constants of ligands to nucleic acids is described, based on electrochemical measurements. Experimental results of an investigation into the mechanism of the methylene blue photosensitized degradation of nucleic acids are described in chapter 5.

In the first part of chapter 6 a computer simulation of fluorescence decay kinetics of dyes, sensitized by electronic energy transfer from
DNA, is presented. The model enables determination of electronic energy transfer rates in DNA from literature data. Actual results could not be obtained due to the absence of a suitable picosecond laser. In the second part of chapter 6 it is shown how energy transfer between different ligands, both bound to DNA, can be used to determine mobility of such compounds along the polymer. A description of materials and experimental methods is given in chapter 7 and finally, the results obtained this thesis are discussed in chapter 8.
CHAPTER 1

NUCLEIC ACIDS: THEIR STRUCTURE AND BINDING OF SMALL MOLECULES
1.1 DNA STRUCTURE

1.1.1 General considerations

Deoxyribonucleic acid (DNA) is the carrier of genetic information in living matter. A knowledge and understanding of the structure of this biopolymer will yield information on the genetic system and its regulatory mechanisms. Several excellent text books and review articles have recently been published on the structure and function of DNA [Jack, 1979; Zimmerman, 1982; Dickerson, 1982 & 1983; Kennard, 1984; Saenger, 1984; Shakked and Rabinovich, 1986]. In view of these excellent reviews only a brief summary of the main structural properties of the biopolymer is presented. Attention is focussed on those aspects of polynucleotide structure which are relevant to its interaction with small molecules (ligands).

The first and most important breakthrough in the study of DNA was the elucidation of the double helical structure by Crick and Watson from X-ray diffraction photographs of fibres of the polymer [Watson and Crick, 1953]. Figure 1.1 shows schematically this structure of DNA and of the individual subunits with associated nomenclature and numbering. DNA consists of two counterpropagating polymeric strands. The repeating unit in each polymer strand is a deoxyribose sugar unit (β-D-2-deoxy-ribofuranose in 2'-endo conformation), flanked by two phosphate groups attached to respectively the 3' and 5' carbon of the sugar. Attached to the 1' carbon of the sugar is a heterocyclic group, termed base. The nucleic acid bases are either purines: guanine (G) and adenine (A) or pyrimidines cytosine (C) and thymine (T). The two polymeric strands of DNA are coiled around a common axis in a right
Figure 1.1 Overview of the important characteristics of DNA. A: Sugar-phosphate backbone, showing numbering of sugar and position of bases. B: A-T and G-C Watson-Crick basepairs, showing numbering of atoms and hydrogen bonding. C: Schematic representation of double helix.
handed conformation and are linked via hydrogen bonds between the bases. In DNA the pairing of base pairs is such that G will only form hydrogen bonds with C and A with T. The structure is then the familiar double helix with the electronegative phosphate groups external to the main cylinder of the polymer and with the planar bases more or less perpendicular to the common long axis of the polymer. In the Watson-Crick configuration (termed B-DNA) the vertical distance between the bases is 3.4 Å, with an angle of 36° per residue. The polymer has a self-repeating substructure of 10 basepairs and repeats itself after 34 Å. The phosphate atoms are separated by 7 Å and are at a distance of 10 Å from the helical axis. This is the approximate conformation of the polymer at high humidity and in vivo.

DNA is not the rigid polymer as suggested by the Watson and Crick model, but can adopt a wide range of conformations. This was shown by accurate X-ray diffraction data of DNA-fibres [Arnott et al., 1974a,b,c; Mahendrasingham et al., 1983] and of single crystals of relatively large synthetic polynucleotides of known base sequence [Dickerson and Drew, 1981; Neidle and Berman, 1983 and Kennard, 1984]. Time dependent variations in the spatial structure of the polymer have been reported from high-field NMR experiments [Mitra et al., 1981; Patel et al., 1982; Borah et al., 1985]. The exact structural configuration of the biopolymer depends on solvent environment, ionic strength and on local variations in the base sequence and is also variable with time*.

Three main DNA structures can be identified: A-DNA, in solutions of low water content and low ionic strength; B-DNA the conformation at high humidity and in-vivo and Z-DNA, the conformation poly[d(G-C)] adopts at high ionic strengths. The B-DNA structure will be the

*X-ray crystallographic refinement procedures are not able to rigorously exclude all possible conformational permutations and this can explain some of the structural variations which have been observed.
conformation of the polynucleotides in this study under the experimental conditions used. This conformation and the factors determining its stability are described in the following section.

1.1.2 B-DNA conformation and stability

B-DNA is the general term for the DNA conformation of a right-handed helix with a 10_1 helical symmetry. As illustrated in figure 1.1, neighbouring bases will be positioned with their molecular planes more or less parallel to one another and with a considerable overlap of their π-electron systems of the basepairs. This overlap results in stacking and/or aggregation forces which will stabilize the helix. These forces, similar to the processes involved in the aggregation of dyes, are caused by hydrophobic interactions between nucleotide bases and the aqueous solvent. They are enhanced by permanent dipole-dipole and London dispersion interactions. The strength of the stacking forces has been estimated using quantum mechanical calculations and measured with differential scanning calorimetry. Basepair sequences involving only guanine and cytosine are substantially more stable than those involving only A-T base-pairs [Ornstein et al., 1978; Marky and Breslauer, 1982]. Additionally, G-C basepairs have an extra hydrogen bond compared to A-T basepairs. Both hydrogen bonding and stacking interactions infer that polynucleotides with a large G-C content possess a more stable helical structure than those with a large A-T content. This is observed experimentally [Marmur and Dothy, 1962].

The planes of neighbouring basepairs are found not to be parallel. Stacking interactions are maximised by a propellor twist of base-pairs which increases the overlap between one base and its neighbours up and down the chain [Wing et al., 1980] (see figure 1.2). Such a propellor twist can lead to steric interference between two adjacent purines on
Figure 1.2 A: "Stick and ball," model of DNA, after X-Ray crystallographic studies [Dickerson, 1983], illustrating local roll and twist of basepairs. Insert illustrates propellor twist in a basepair. B: Diagram illustrating local torsion angles in a single strand of the polymer.
opposite strands [Calladine, 1982]. This steric hindrance can be avoided by base-pair specific changes in the local helical and propeller twists and base roll angles. Propeller twists can assume a value of \(17^\circ\) in an A-T base-pair and \(11.5^\circ\) in a G-C base-pair [Dickerson and Drew, 1981] and the helical twist can vary between \(16^\circ\) and \(44^\circ\) [Dickerson, 1983].

The phosphate groups form the walls of two grooves in the helix, a minor and a major groove (Figure 1.1). The base of these grooves is formed by the nucleotide base-pairs, as seen edge on. This constitutes another base-specific variable in the DNA-structure. Different electrical and hydrogen bonding properties pertain depending on the groove and the base-pair sequence in it. This is illustrated in figure 1.3.

Water molecules bind to DNA via hydrogen bonds and the primary hydration shell of the polymer is estimated at 20 water molecules per nucleotide [Falk et al., 1963a,b]. These water molecules stabilize the secondary and tertiary structure of DNA [Perahia et al., 1977; Corongiu and Clementi, 1981]. An accurate determination of the position of the water molecules in a polynucleotide of the B-conformation was obtained from X-Ray crystallographic data obtained from a single crystal dodecamer [Kopka et al., 1982]. These studies suggest that in A-T rich regions the minor groove is filled with a regular spine of hydrogen bonded water molecules. This spine is not very prominent in G-C rich regions. The spine of hydration is of major importance to the stabilization of the B-DNA structure [Dickerson, 1983] and is also important for the binding of small ligands.

Single-crystal data suggests that the helical axis is not necessarily straight. In the crystal the axis traces a smooth bend with an equivalent radius of curvature of 112 Å. In comparison, the DNA cylinder radius is ca 20 Å. Although this bending could be caused by
Figure 1.3 Schematic diagram of pattern of hydrogen-bond donors and acceptors in the major groove of DNA [Felsenfeld, 1985].

- H-bond acceptor
- H-bond donor
- CH$_3$-group Thymine
crystal packing forces it does appear that DNA possesses more flexibility than was previously thought possible. Additional calculations of torsional and bending stiffness of DNA helices of different base-pair composition showed that such flexibility is strongly base-pair dependant. Poly[d(G)-d(C)] was found to be considerably stiffer than natural DNA or poly[d(A)-d(T)] [Hogan et al., 1983]. An interesting hypothesis regarding the flexibility of DNA has been given by Sobell et al. [1979, 1981]. He proposes the existence of regions of high flexibility with an alternating C_3,-endo-(3'-5')-C_2,-endo (etc.) pattern which was termed θ-DNA. These regions, which are in addition partially denatured, are formed by solvent bombardment and move as an acoustic wave along the polymer. Acoustic waves with a speed of 1800 m/s have been detected [Hakim et al., 1984]. This motion, termed "DNA-breathing", varies as a function of the nucleotide sequence and could be responsible for the intercalation of ligands. Whatever the exact mechanisms, it appears clear that local, base-pair dependent variations of structure play an important role in the mechanism of ligand binding and thus in the regulation of repression and transcription proteins [Wu and Crothers, 1984; Felsenfeld, 1985].

The B-DNA conformation discussed above appears to be valid for normal DNA and for the synthetic polynucleotide poly[d(G-C)]. However there is evidence that the synthetic polynucleotide poly[d(A-T)] does not conform to these rules. This is based on X-Ray crystallographic studies of the tetramer d(pATAT) and on molecular biological studies [Viswamitra et al., 1978; Klug et al., 1979]. The structure which this polynucleotide adopts has been termed "alternating" B-DNA and is unique in that the conformation of the sugars is now base-dependant ie. C_3,-endo for adenine and C_2,-endo for thymine, compared to C_2,-endo for all bases in "standard" B-DNA. Also changes in the P-O torsion angles are observed in this structure [Saenger, 1984]. Other authors have argued
the case for left-handed structures containing consecutive A-T basepairs [McLean et al., 1986].

1.1.3 Polyelectrolyte theories applied to DNA

DNA is a charged polyelectrolyte and is able to bind small cationic atoms and molecules (ligands) via electrostatic forces. It is important to assess these electrostatic effects as metal ions are used as part of buffer solutions. Metal ion binding is also important in the stabilization of the helix as is demonstrated by the observed increase in the denaturing temperature on increasing the concentration of salt [Marmur and Dothy, 1962; Ott et al., 1975]. The monovalent alkali metals Na\(^+\) and K\(^+\) and the divalent Mg\(^{2+}\) interact with the hydration shell around the polymer and bind solely to the DNA phosphate groups [Sissoef et al., 1976].

The binding of charged ions and molecules to a polyelectrolyte such as DNA can be quantified by theories developed by Manning and others [Manning, 1969, 1978 and 1979; Record et al., 1976 and 1978; Anderson and Record, 1982]. These theories have been applied to the binding of repressor proteins to nucleic acids and pertain to the interaction of intercalating molecules with DNA [Record et al., 1977; deHaseth et al., 1977]. In the Manning theory, DNA is modelled as a thin one-dimensional polymer with a homogeneous charge distribution along its length. As such the theory is only approximate. Manning introduced a dimensionless charge density parameter \(\xi\):

\[
\xi = \frac{e^2}{k_B T b \varepsilon}
\]  \hspace{1cm} (1.1)

with \(e\) the electronic charge, \(\varepsilon\) the dielectric constant of the bulk solvent, \(k_B\) the Boltzmann constant, \(T\) the absolute temperature and \(b\)
the axial charge distance along the polyelectrolyte. For a given polymer, $\xi$ can be calculated and if $\xi > 1$ then, according to Manning, counterions will condense onto the polyelectrolyte by reducing the apparent value of $\xi$ to 1. This can only be done by increasing the axial charge distance $b$. In other words the actual value of the charge distance will, after condensation, be equal to the Bjerrum-length $e^2/\varepsilon k_b T$ [Glasstone, 1946]. The fraction of counterions condensed onto the polymer is then given by $\psi_c = 1 - \xi^{-1}$. The value of $b$ for B-DNA is 1.7 Å (2 phosphates every 3.4 Å). Substituting this in 1.1 yields a value of $\xi_{\text{DNA}}$ of 4.2. $\xi_{\text{DNA}}$ is larger than 1 and consequently a fraction of 0.76 (1 - 4.2$^{-1}$) phosphates will be "neutralized" by the condensed counterions. Additionally, a fraction $\psi_s$ of counterions will be bound by the screened polymer, subject to the standard Debye-Huckel approximation. Record et al. [1976] have calculated the latter contribution to be $(2 \xi)^{-1}$. This value is 0.12 for B-DNA. The total fraction of counterions bound to B-DNA is then $\psi_c + \psi_s = 0.88$. This amount of condensed counterions is constant and independent of the bulk ion concentration in the solution.

Stabilization of the helix by increasing the ionic strength of the solution can now be understood in terms of a lower charge density in denatured DNA. This has a $b$-value of ca. 4.3 Å [Record et al, 1978]. The fraction of neutralized phosphate ions $\psi_c'$, is then 0.39. This can be compared to the value of 0.76 for double helical DNA. Thus denaturing involves the release of counterions, which is an unfavourable process at higher ionic strengths.

The effect of ionic strength on the binding of small molecules to DNA can also be described within the framework of the Manning theory [Record et al., 1976; Wilson et al., 1979 & 1985a; Marcandalli et al. 1984]. These small molecules are often charged cations and electrostatic factors are thus involved in the binding process (see
also chapter 1.2). Simple competition between the salt ions and
cationic ligands will cause a number of these ligands to replace salt
ions in the condensation layer. Secondly, binding can change the
conformation of the polymer with a resultant decrease in the average
charge density leading to counterion release. The calculated dependance
of the binding constant $K_b$ on total (monovalent) salt concentration, is
summarized in equation 1.2 [Wilson et al, 1985a].

$$\log \frac{K_b}{\log [S^+]} = -2N(\psi - \psi^*) - m'\psi^*$$  \hspace{1cm} (1.2)

$[S^+]$ is the bulk salt concentration, $\psi$ and $\psi^*$ are the total
fractions of associated counterions per phosphate for respectively DNA
in the native and in the "intercalated" configuration. The value of $m'$
is the number of ion-pairs in the complex. This value of $m'$ is 1 in the
studies described in this work. Equation 1.2 has been confirmed
experimentally [Pasternack, 1986].

The polyelectrolyte theory, as described above, does not take
account of base, or base-pair sequence specificity in the binding
process. Calculation of electrostatic potential surfaces of molecules
has provided an insight in the mechanisms involved in this [Dean and
Wakelin, 1980a & 1980b; Weiner et al, 1982]. In addition, the
calculation of the electric field at the surface of the polymer can
possibly elucidate the mechanism and pathway of the ligand-polymer
association process as well as the binding of molecules with a
permanent dipole moment (ie. water) [Lavery et al, 1982]. The
calculations show that 1)local electrostatic potentials and fields are
very sensitive to the local geometry adopted by the nucleotides and any
conformational change in the geometry will markedly affect the shape
and intensity of the potential [Dean and Wakelin, 1980a]; 2) Water molecules bind very strongly to phosphates; 3) Electrostatic potential surfaces show base-pair specific variations of hydrogen binding and hydrophobic binding sites in the major and minor grooves of DNA, similar to those shown in figure 1.3. In vacuo calculations of the electrostatic potential showed that the electronegative charge on the carbonyl oxygen of the bases is as large as that on the fully ionized phosphate group [Dean and Wakelin, 1980a]. Further calculations which included counterion shielding indicate that the charge in the grooves of DNA is in effect larger than that on the phosphates [Lavery et al, 1982]. They reported that the largest potential was found for poly[dG]-poly[dC] in the major groove and for poly[dA]-poly[dT] in the minor groove. Their results are summarised in table 1.1. These results have to be treated with some caution as the dielectric shielding effect of water on the potentials and fields around the polymer is generally ignored or is not very well understood [MacDonaill, 1984]. However, in the future such calculations will be improved and can be expected to play an increasingly important role in the study of the binding of ligands to nucleic acids.
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<th>$(GC)_n$</th>
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<td><strong>Minor groove</strong></td>
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<tr>
<td>N3 (G)</td>
<td>- 4.21</td>
<td></td>
</tr>
<tr>
<td>07 (C)</td>
<td>- 4.08</td>
<td></td>
</tr>
<tr>
<td>N3 (A)</td>
<td></td>
<td>- 4.99</td>
</tr>
<tr>
<td>07 (T)</td>
<td></td>
<td>- 5.03</td>
</tr>
<tr>
<td><strong>Major groove</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N7 (G)</td>
<td>- 4.73</td>
<td></td>
</tr>
<tr>
<td>011(G)</td>
<td>- 4.64</td>
<td></td>
</tr>
<tr>
<td>N7 (A)</td>
<td></td>
<td>- 2.86</td>
</tr>
<tr>
<td>08 (T)</td>
<td></td>
<td>- 2.73</td>
</tr>
<tr>
<td><strong>Backbone phosphate</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anionic oxygens</td>
<td>- 2.95</td>
<td>- 2.34</td>
</tr>
</tbody>
</table>

**Table 1.1** Electrostatic surface potentials (Volts) calculated for synthetic polynucleotides poly[dG]-poly[dC] and poly[dA]-poly[dT]. Account is taken of screening by Na⁺. [from Lavery et al. 1982]. (For atom numbering see figure 1.1)
1.2 THE BINDING OF SMALL LIGANDS TO NUCLEIC ACIDS

The interaction of small molecules (ligands) with nucleic acids has been the subject of a large number of investigations. It is hoped that a better understanding of the processes involved in the binding of small ligands to nucleic acids can lead to an insight into, and manipulation of, the mechanism of protein/DNA recognition. Furthermore, the diagnosis of diseases associated with DNA, the development of novel chemotherapeutic agents and an understanding of the carcinogenic action of some compounds depends on a general knowledge of the binding mechanisms of small molecules to DNA. [reviews: Bloomfield et al., 1974; Berman and Young, 1981; Waring, 1981; Neidle and Waring, 1983; Dabrowiak, 1983; Dervan, 1986].

1.2.1 Modes of binding

The modes of binding of small ligands to DNA can most conveniently be divided in two separate categories, intercalating and non-intercalating (or externally binding) [Zimmer and Wahnert, 1986]. Intercalation is defined as a mode of binding where the ligand is inserted parallel and between the nucleotide base-pairs, whereas the term external binding will be applied to those ligands that bind physically or covalently to DNA, with minimal or no insertion of part of their structure between the nucleotides. This is a simplified division as numerous compounds can bind in both modes, sometimes simultaneously.
1) External Binding.

Several different types of molecules belong to this category. Firstly molecules which bind purely electrostatically to the electronegative DNA molecule, such as the salt ions eg. Na\(^+\), K\(^+\) and Mg\(^{2+}\). Lanthanide ions such as Eu\(^{3+}\) and Tb\(^{3+}\) which can be used as fluorescent probes for nucleotides bind in a similar manner [Eisinger and Lamola, 1971; Mushrush and Yonushot, 1983]. This mode of binding has already been discussed in 1.1.3. Other molecules which appear to bind predominantly via electrostatic interaction are the triphenylmethane dyes, such as methyl green and crystal violet [Krey et al., 1975; Zimmer and Wahnert, 1986]. (See figure 1.4 for structural formulas.)

In addition molecules can bind in the grooves of B-DNA. A number of antibiotics and anticancer agents, both naturally occurring and synthetically produced bind in this mode. The naturally occurring antibiotics Netropsin and Distamycin as well as the antitumor antibiotic CC-1065 and the stain Hoechst 33258 (used as a probe for chromosome structure) bind via a combination of hydrophobic and hydrogen bonding interactions in the minor groove of B-DNA [Hurley et al., 1984; Kopka et al., 1985a & 1985b, Latt and Brodie, 1974; Muller and Gautier, 1975; Latt et al., 1985]. This binding is selective for a consecutive sequence of dA-dT basepairs on the polymer. This is mainly due to the sequence of hydrogen donors and -acceptors as seen by the molecule (Figure 1.3). There is also crystallographic evidence that these molecules replace the hydration spine in the minor groove. A different naturally occurring antibiotic and antitumor agent which also binds in the minor groove is Mithramycin. This compound is unique in that it requires the presence of a stoichiometrically equivalent amount of Mg\(^{2+}\) to be active, as well as the presence of guanine bases [Waring, 1981]. Finally, a compound which is thought to bind via hydrogen
Figure 1.4 Structural formula of representative non-intercalating DNA-binding molecules.
bonding in the major groove of B-DNA is the polyamine spermine [Feuerstein et al., 1986].

A third category of non-intercalators bind covalently to DNA. The best known compounds in this class are those based on the potent antitumor compound cis-dichlorodiamine platinum (II) (cis-DDP or cis-platin) [Roberts and Pera, 1983; Saenger, 1984]. The platinum compounds are strongly electrophilic species which can react non-specifically with nucleophilic sites the nucleic acids.

The final category of externally binding molecules are cationic intercalating molecules which possess a strong aggregation constant. At low base-pair to ligand ratios some of such molecules will bind electrostatically to the outside of the polynucleotide, whereupon other such molecules can "stack" on top. This will be discussed in the following paragraph.

2) Intercalation

Intercalation is defined as a mode of binding in which the ligand is oriented parallel to and between nucleotide basepairs. Intercalation was initially proposed for the binding of acridine dyes to nucleic acids [Heilstein and Van Winkle, 1955; Lermann, 1961] but has been accepted as the predominant mode of binding for a wide range of compounds [Muller and Crothers, 1975; Muller et al., 1975; Lober, 1981; Neidle and Waring, 1983]. Figure 1.5 illustrates a number of drugs and dyes for which intercalation has been proposed.

Evidence for intercalative binding came initially from viscosity and X-ray fibre diffraction data [e.g. Lermann, 1961]. The intercalated dye will lengthen and partially unwind the helix and this increase in length leads to an increased viscosity and decreased sedimentation velocity [Waring, 1970]. Additional evidence for intercalation comes from induced dichroism in the visible absorption spectra of the dye.
Figure 1.5 Structural formula of representative molecules for which intercalation has been advocated as a major mode of binding to DNA.
caused by the asymmetry imposed upon the dye by its chiral binding site [Norden and Tjerneld, 1982].

The lengthening of the DNA helix by approx. 1 basepair (3.4 Å) will cause a distortion in the sugar-phosphate backbone. Model building studies have been used to propose structures for the intercalation site [Alden and Arnott, 1975] which have later been confirmed using X-ray diffraction on crystals of synthetic oligonucleotides with intercalated dyes [Sobell et al., 1981; Neidle and Bermann, 1983; Neidle and Sanderson, 1983]. The change in DNA conformation upon intercalation involves characteristic changes in the phosphate backbone angle \( \omega \) and the glycosidic bond angle \( \chi \) [Berman and Neidle, 1979], as well as a change in the sugar pucker from C\(_2\)-endo to C\(_3\)-endo at the 5'-end of the binding site [Saenger, 1984]. These structural changes make it impossible for a second ligand to intercalate next to an already intercalated one. This effect is often termed "neighbour exclusion" [Crothers, 1968]. Conformational changes at the 5'-end appear to be more facile for pyrimidine-(3'-5')-purine sequences as intercalation is preferred in these sites [Krugh and Reinhardt, 1975; Reinhardt and Krugh, 1978]. Theoretical calculations support these observations and suggest that in addition, basepair stacking and base-phosphate interactions are also important [Nuss et al., 1979; Ornstein et al., 1978 & 1979]. Others have also indicated that the selectivity for pyr-(3'-5')-pur sequences results from the ability to open the DNA helix for this particular sequence [Miller et al., 1980].

The structure of an intercalating ligand must conform to the steric requirements of the binding site. Hence intercalators will in general possess large aromatic ring systems. If they possess bulky side groups (e.g. Actinomycin) these will be outside the binding site ie. on the outside of the polymer. An intercalated dye will be stabilised in its binding site by hydrophobic and Van der Waals interactions between the
\[ \text{π-electronic systems of the bases and of the ligand. Additional stabilization of the intercalated ligand can come from electrostatic interactions between the anionic polymer and cationic intercalator as well as by hydrogen-bonding. The latter can either be H-bonding in the binding site, as has been proposed for some acridines [Georghiou, 1975; Gaugain et al., 1981] or external H-bonding as is the case for the drugs Daunomycin and Adriamycin [Neidle and Anderson, 1983]. The interaction between intercalated dye and the nucleotide bases serve to stabilise the polymer and an increase in the denaturing temperature will be observed for the complex. The increase in melting temperature can be more than 20 °C, depending on ionic strength [Gersch and Jordan, 1965; Kleinwachter et al., 1968; Kleinwachter and Balcarova, 1981; Tossi, 1987]. It has however to be born in mind that externally bound, non-intercalating ligands can also stabilize the DNA helix [Marky et al., 1983].}

The actual steps involved in the intercalation process are still not very clear. A two-step process is envisaged in which the first step involves an electrostatic binding of ligand to helix with a concomittant conformational change in the latter. This is then followed by the actual intercalation step [Li and Crothers, 1969; Wakelin and Waring, 1980]. This hypothesis is based on results obtained with "Joule-heating" temperature-jump relaxation studies of dye-DNA complexes. The sequence of events is given in scheme 1.3, with A = DNA, D = dye and I and II refer to the different binding modes. Huckel calculations appear to vindicate the two-step hypothesis [Pack et al., 1981].

\[ \begin{align*}
\text{A} + \text{D} & \xrightarrow{k_{12}} \text{[AD]}_I \xleftarrow{k_{21}} \text{A} + \text{D} \\
& \xrightarrow{k_{23}} \text{[AD]}_II \xleftarrow{k_{32}} \text{[AD]}_I
\end{align*} \] (1.3)

This hypothesis has however recently been challenged on experimental
grounds [Marcandalli et al., 1984]. These authors give strong arguments against the previous interpretation of the "Joule-heating" experiments and, using an iodine laser to heat the sample, could find no evidence for a two-step mechanism in a proflavine-DNA complex. The "DNA-breathing" hypothesis by Sobell et al. (see above) has been proposed as an alternative theory. It has been used to explain the intercalation of such bulky compounds as H$_2$TMPyP [Murphy, 1985]. The conclusion must be that this matter as yet remains resolved.

An area of particular interest to our work is the possible base-specificity of binding. This has been studied by numerous workers [Muller at al., 1975; Muller and Gautier, 1975; Muller and Crothers, 1975; Sharples and Brown, 1976; Baldini et al., 1981; Dougherty, 1982a; Newlin et al., 1984; Wilson et al., 1985; Dervan, 1986]. Apart from the pyr-(3'-5')-pur selectivity discussed above, some evidence has been presented that G-C (or C-G) specificity is associated with the polarizability of the molecule [Muller and Crothers, 1975]. Sharples and Brown [1976] associate G-C specificity of a ligand with its electron affinity. Outside (groove) binders appear to have specificity for A-T basepairs [Zimmert and Wahnert, 1986] although some exceptions to this rule have been found [Newlin et al., 1984]. Recently Wilson et al. [1985b] have studied hydroxyl derivatives of thiophene and anthracence which they claim intercalate between A-T basepairs. The reason for this specificity is thought to originate in a stabilization of the compound by the hydration spine in the minor groove next to the intercalation site (A-T basepairs). Conversely, G-C specificity is then caused by the inability of an intercalator to interact with this solvent lattice. This hypothesis is somewhat different from the reasons for G-C specificity given by Muller and Crothers.

Apart from full intercalation, with the ligand completely "buried" in the helix, part intercalation has also been proposed [Peacocke,
1973]. This modified intercalation model is more electrostatic in nature. The dye is more accessible to external solvent and hence ligand-ligand interactions can come into play. The cationic ligand molecules can bind electrostatically to the phosphate groups. Subsequently, other ligand molecules can aggregate onto the already bound ones. Thus extensive aggregation can be observed in the ligand absorption spectra. This "stacking" of ligands on the outside of the polymer is caused by a combination of electrostatic, hydrophobic and Van der Waals interactions. The compound for which these effects are most notable is the dye acridine orange [Stone and Bradley, 1961; Stone et al., 1970; Fredericq and Houssier, 1972; Vitagliano, 1983; Lober, 1981].
1.3 METHODS TO STUDY LIGAND BINDING

1.3.1 Experimental studies

Numerous methods have been used to study the binding of ligands to DNA and several critical reviews of the techniques used have been published [Dougherty, 1982b; Dougherty & Pigram, 1984; Saenger, 1984]. Examples of experimental methods which determine changes in the properties of the polymer upon ligand binding are helix unwinding studies [Waring, 1970], viscosity [Lerman, 1961], sedimentation [Waring, 1970] and thermal denaturation studies [Tossi, 1987]. Also NMR (both $^{31}$P and $^1$H) and X-Ray crystallography have been utilised [Patel, 1979; Neidle & Berman, 1983]. Absorption and fluorescence spectroscopy can be used to determine binding by measuring changes in the properties of the bound ligand. Thus, steady state, kinetic as well as polarized and unpolarized absorption and fluorescence spectroscopy as well as Raman spectroscopic measurements have been used in binding studies [Schipper et al., 1980; Fornasiero and Kurucsev, 1981; Lober, 1981; Chen and Morris, 1983; Bayley & Dale, 1985; Hard & Kearns, 1986]. Also electric field induced dichroism and the more traditional equilibrium dialysis and phase partitioning have found wide application [Hogan et al., 1979; Ridler & Jennings, 1980 & 1983; Ridler et al., 1986; Muller & Crothers, 1969; Waring et al., 1975]. Kinetic spectroscopy of the triplet state is another method to study ligand binding studies. The method has been used extensively in this work to study the binding of methylene blue to nucleic acids. Triplet state lifetimes of common dyes used as probes range from micro- to milliseconds, which is convenient for probing dynamical fluctuations in the polymer and
dissociation constants as these are of the same order of magnitude [Saviotti and Galley, 1974; Geacintov et al., 1976 & 1981; Wang et al., 1982; Hogan et al., 1982; Corin et al, 1985; Berkoff et al., 1986].

Most experimental methods are not able to distinguish between intercalative or external binding. Deductions regarding the exact mode of binding have to be made on the basis of more than one experimental method and very often by comparison with "known" intercalators or on the basis of molecular modelling.

1.3.2 Analysis of binding data

The aim of binding studies is to determine the strength of binding and the number and type of possible binding site(s). Several analytical procedures have been developed which yield these results from the acquired data. These have been extensively reviewed elsewhere [Freifelder, 1982; Van Holde, 1985; Orsi and Williams, 1985] and only a short overview will be given.

All methods depend on the determination of the amount of free and bound ligand. This is either determined directly, as in phase partitioning and in equilibrium dialysis, or indirectly. Indirect methods invariably rely on the measurement of a particular parameter of the ligand which changes upon binding. Then the relative change in that parameter can be related to the amount of dye bound. Examples of this are the changes in the extinction coefficients and fluorescence intensities of dyes when bound to DNA.

The most widely used method of analysis is that which has been derived by Schatchard [1949] for a single mode of binding with one unique association constant. Under these assumptions then:

\[
\frac{r}{c_f} = K (n-r) \quad \text{(I.4)}
\]
with $r$ the ratio of bound ligand to concentration of sites and $c_f$ the concentration of free ligand. $K$ is the association constant and $n$ is the binding number which is the maximum number of ligands that can be bound per site (i.e. $0 < n < 1$). The Scatchard plot of $r/c_f$ versus $r$ should then be linear with slope $-K$ and intercept $n$ for $r/c_f = 0$.

In practice linear plots are very rarely observed. Several reasons have been advanced for a curved Scatchard plot [Peacocke & Skerrett, 1955; Crothers, 1968; McGhee & Von Hippel, 1974]. These are 1) multiplicity of binding sites, each with a different $K$ and $n$, 2) neighbour exclusion and 3) cooperative binding of ligands. Unfortunately, these effects will give similar curvature in the Schatchard plots and it is then difficult to make deductions about the correct binding model. Another problem of the Schatchard model is related to its dependance on $c_f$. For strong binding measured at high $r$ ratios, $c_f$ will be very small and hence small experimental errors in its determination will make the value of the quotient $r/c_f$ unreliable.

A different type of analysis which is useful as a diagnostic test for the mode of binding is the simple binding-plot [Freifelder, 1982; Van Holde, 1985] which involves a plot of $r$ versus $c_f$. This plot can give information about cooperativity.
CHAPTER 2

THE PROPERTIES OF METHYLENE BLUE AND ITS INTERACTION WITH DNA
The compound with which the investigations of dye-nucleic acid complexes were started is Methylene Blue (3,7 Bis (dimethylamino) phenazothionium chloride) (figure 2.1).

Figure 2.1 Structural formula of methylene blue.

This choice was based on the observations by Friedman and Brown [1978] who showed that photosensitized damage to DNA yields cleavage of
phosphodiester bonds at guanine sites. The dye is used extensively as a biological stain, notably in the colouring of connective tissue [Bergeron and Singer, 1958; Padyakula, 1983]. It is also one of the well known photosensitizers and very often methylene blue is used as a standard by which other potential photosensitizers are compared (see section 2.3). The dye is easily reduced on photoirradiation and for this reason it has been extensively investigated with respect to its possible use in photogalvanic cells [Wildes et al., 1977]. Consequently, a considerable amount of work has been reported with respect to the photosensitizing and photoreductive properties of the dye and its triplet state. Surprisingly little is known about the behaviour of the excited singlet state of the dye. In the following sections the available literature on the singlet and the triplet state of methylene blue will be discussed. These are followed by a summary on the literature of the binding of methylene blue to nucleic acids. Finally the extensive literature on the methylene blue photosensitized degradation of DNA and its individual components is reported.

2.1 PHOTOPHYSICAL PROPERTIES

2.1.1. Singlet state

Figure 2.2 shows the ground state absorption spectrum of a dilute methylene blue solution in water. The spectrum is characterised by large absorption bands at 665 nm and at 290 nm, which are attributed to $S_1$ and $S_2 (\pi, \pi^*)$ transitions on the basis of their intensities. The
Figure 2.2 Absorption spectra of monomeric methylene blue (dotted line) and dimeric aggregates of the dye (solid line) [from Fornili et al., 1981].
molecule has $C_{2v}$ symmetry and thus the transition-dipole moment associated with excitation at these wavelengths is in the plane of the molecule and aligned along its long axis [Norden and Tjerneld, 1982]. A fairly prominent shoulder can be observed at ca. 605 nm. The dimer aggregate of the dye absorbs at a similar wavelength (see below), but as the shoulder is still observed at very low concentrations of the dye it appears that its coincidence with the dimer band is fortuitous and that it is probably a vibronic sublevel of the $S_1$ level. An analogous observation has been made for the similar dye thionine [Rabinowitch and Epstein, 1941]. In addition to these strong bands a weak absorption shoulder can be observed at 330 nm, the origin of which is ambiguous. There have been suggestions that this band may be due to a $(n, \pi^*)$ transition, in which case its associated transition dipole moment would be oriented perpendicular to the molecular plane [Norden, 1977]. It would then probably be a $S_2(n, \pi^*)$ excited state as its energy is higher than that of the $S_1(\pi, \pi^*)$ state. No evidence can be seen in the spectrum for a $S_1(n, \pi^*)$ transition which is probably obscured by the large 665 nm $S_1(\pi, \pi^*)$ transition. An alternative explanation for this band is given by Kern and Dorr [1961]. They give evidence for a transition dipole moment associated with the 330 nm absorption band, which is oriented in the plane of the molecule along the short axis of the molecule. The above assignments are however not secure as some evidence has been presented that methylene blue is not planar but instead has a dihedral angle of 153° [McDowell, 1974]. Most authors assume the molecule to be planar.

Very few studies have been reported on excited singlet state deactivation processes in methylene blue. Studies of comparable compounds such as acridines and substituted acridines indicate the complexity of such processes [Shapiro and Winn, 1980; Kasama et al., 1981a & b]. In these molecules excited state deactivation is
strongly dependent on the existence of possible hydrogen bonding with the solvent. A small energy gap between the $S_2(n,\pi^*)$ and the $S_1(\pi,\pi^*)$ level can provide an additional deactivation path for the excited state. Similar processes are expected to play a role in the case of methylene blue. Moreover, excited state deactivation is even more complex due to possible involvement of the sulfur 3d-orbitals whose energy levels are not far above those of the sulfur 3p-orbitals [McWeeny, 1979]. The multitude of possible deactivation pathways possibly explains the rapid radiative singlet decay which is observed for methylene blue. The fluorescence lifetime is measured as 350 ps [this work, chapter 4] which can be compared with that of the similar compound acridine orange (3,6 Bis(dimethylamino)acridine) which has a fluorescence lifetime of 1.8 ns [Kubota, 1973].

One of the aspects of methylene chemistry blue which has been studied extensively is the formation of dye aggregates, both in aqueous solutions and by aggregation onto polyanionic biopolymers [Bergeron and Singer, 1958]. Rabinowitch and Epstein [1941] discuss the effect of aggregation on the ground state spectrum of the dye. They showed that the maximum absorption of the dimer band is blue shifted by ca. 60 nm from the corresponding maximum in the monomer spectrum. Numerous authors have since measured the absorption spectrum of the dimer [Bergman and O'Konski, 1963; Braswell, 1968; Mukerdjee and Gosh, 1970; Fornili et al.,1981 & 1985] and figure 2.4 shows the absorption spectrum of the dimer as obtained by Fornili et al. [1981]. This dimer spectrum can be explained on theoretical grounds if it is assumed that the two dye molecules are oriented in a "sandwich" or "biplane" configuration [Kasha and McRae 1964]. These studies, and additional temperature-jump studies [Spencer and Sutter, 1979], showed that the formation of the dimer is driven by hydrophobic interactions. The equilibrium constant for dimer-aggregate formation has been determined
by Fornili et al. [1981] as $7.7 \times 10^3 \text{ dm}^3 \text{mol}^{-1}$ in pure deionized water at 20 °C.

2.1.2. Triplet state and radical intermediates

Transient intermediates in flash spectroscopic investigations of methylene blue were first reported by Parker [1959], Kato et al. [1964] and Matsumoto [1964]. In aqueous neutral solutions transient spectra are observed with maxima at 420 and 520 nm respectively. Varying the pH leads to a partial disappearance of the 420 nm band and the appearance of an absorption at 380 nm. These bands were assigned to respectively unprotonated (3\text{MB}^+) and protonated (3\text{MB}^{2+}) triplet state dye. Kato et al. determined a value of 6.7 for the pKₐ of the protonation of the triplet state. The other species which is observed at 420 nm and the 520 nm species are attributed to respectively a semi-reduced radical \text{MB}^- and a loosely combined pair of semi-reduced \text{MB}^- and semi-oxidized \text{MB}^{2+}. The assignments of these radical intermediates was later confirmed by pulse radiolysis measurements [Keene et al., 1965].

Danziger et al. [1967] reported laser flash photolysis data of methylene blue. Their observations are similar to those obtained with conventional flash photolysis. They reported the occurrence of transient maxima (in "plain" water) at 420 nm (triplet) and 520 nm (radical). They also observed a very fast (< 2 µs) transient at 550 nm, which was attributed to triplet state dye dimer. According to Danziger et al. the dimer can be excited as a separate entity and does not dissociate upon excitation. They confirm the assignment of Kato et al. of the 520 nm radical as an intra-dimeric charge transfer species ie. (\text{MB}^{2+} \rightarrow \text{MB}^-)

Triplet lifetimes of \text{MB}^+ depends strongly on the pH of the solution. This was inferred by Kato et al. [1964] and was demonstrated by
Bonneau et al. [1974,1975] who found a value of respectively 4 μs for the decay of $^3\text{MBH}^{2+}$ at pH = 4.5 and 85 μs for $^3\text{MB}^+$ at pH = 8.5. Similar values were later reported by Ohno et al. [1979]. The considerable discrepancies in triplet lifetimes which were earlier reported by Nilsson et al. [1972] could thus be attributed to ill defined pH-values.

The triplet-triplet absorption spectrum of $^3\text{MBH}^{2+}$ was presented by Wildes et al. [1977] and is shown in figure 2.3. The spectrum is characterised by a strong band at 370 nm and minor bands at longer wavelengths. Ohno et al. [1979] report the absorption spectrum for $^3\text{MB}^+$ and this is also shown in figure 2.4. These authors also show a dependence of the triplet decay rate on the buffer concentration (KH$_2$PO$_4$). This dependence could be attributed to the rate of protonation of the unprotonated triplet.

The quantum yield of triplet state formation (ie. the probability of population of the triplet state) of methylene blue has been reported as 0.58 [McVie et al., 1978]. The triplet state of the dye is responsible for the formation of $^1\text{O}_2$, a reactive oxidizing species, via a collision induced energy transfer [Frimer, 1983]. Bonneau et al. [1975] showed that the yield of $^1\text{O}_2$ from the triplet state can be as high as 1 at a pH of 9 with an overall yield of $^1\text{O}_2$ thus approximately 50 - 60%. This high yield is the reason for the widespread use of methylene blue as a generator of singlet oxygen. Methylene blue sensitized singlet oxygen reactions will be discussed more extensively in section 2.3.
Absorption spectra of MB⁺ in 0.01 N aqueous H₂SO₄ (---), 3MBH₂⁺ in 0.01 N aqueous H₂SO₄ (---).

**A**

S-S, --, and T-T, ---: absorption spectra of 2 µM methylene blue chloride buffered at pH 8.2 in 30% aq. ethanol by borate.

**B**

*Figure 2.3* Extinction coefficients of 3MBH₂⁺ (A) and 3MB⁺ (B). A: from Wildes et al., 1977; B: from Ohno et al., 1979.
Reduction of the dye will result in the formation of the colourless form of the dye, leucomethylene blue (MBH) according to scheme 2.1 [Khatchaturyan and Nikolaev, 1980]:

\[
\begin{align*}
\text{e}^- & \quad \rightarrow \quad \text{MB}^+ \\
\text{MB}^+ & \quad \rightarrow \quad \text{MB} \\
\text{MB} & \quad \rightarrow \quad \text{MBH}^+ \\
\text{MBH}^+ & \quad \rightarrow \quad \text{MBH}
\end{align*}
\]  

(2.1)

The reaction can be photoinitiated and this photoreduction of methylene blue has been the subject of extensive research. A wide range of electron donating substrates has been investigated such as nucleotides [Knowles, 1971], amines [Kayser and Young, 1976a,b], aromatic compounds [Kikuchi et al., 1977], Fe(II) [Wildes et al., 1977; Ohno and Lichtin, 1980; Kamat and Lichtin, 1982a] and ground state dye [Kamat and Lichtin, 1981a, 1982b].

A number of different pathways for the production of the reduced form of the dye (i.e. MB· or MBH+·) have been proposed. It has been suggested that photoreduction proceeds via the protonated form of the dye triplet. This was inferred from the fact that an increased phosphate buffer concentration accelerated photoreduction [Knowles and Gurnani, 1972]. Somer and Green [1973] propose that MB+ can be reduced without any additional electron donor. They showed that water is able to act as a reducing agent for the dye in an acidic solution and suggested a two-photon mechanism involving a long-lived dye dimer intermediate. The final products of these reactions are MBH and H₂O₂ and in a subsequent report polarographic evidence is given for the presence of H₂O₂ [Somer and Temizer, 1984]. Their proposed reaction mechanism is summarized in table 2.1.
### Table 2.1 Photoreduction of methylene blue by water as proposed by Somer and Green (1973). [S] is a long lived intermediate not further identified by the authors.

\[
\begin{align*}
\text{MB}^+ + \text{hv} & \rightarrow \text{1}_\text{MB}^{++} & (1) \\
\text{1}_\text{MB}^{++} & \rightarrow \text{3}_\text{MB}^{++} & (2) \\
\text{MB}^+ + 3\text{MB}^{+++} + \text{H}_2\text{O} & \rightarrow [\text{S}] & (3) \\
2[\text{S}] + \text{H}^+ & \rightarrow \text{MBH} + 3\text{MB}^+ + \text{H}_2\text{O}_2 & (4) \\
\text{backreaction: MBH} + \text{H}_2\text{O}_2 & \rightarrow \text{MB}^+ + 2\text{H}_2\text{O} + \text{H}^+ & (5)
\end{align*}
\]

A third mechanism for the formation of a reduced dye intermediate is reported by Kamat and Lichtin [1981]. They suggest that semireduced dye can be produced from the reaction of ground state dye with a solvated electron. The solvated electron in its turn is produced by photoejection from an excited triplet T\textsubscript{2} -state of the dye yielding the semi-oxidised dye radical MB\textsuperscript{2+}. This process occurs only in basic media. They rule out participation of dye dimer in the production of MB\textsuperscript{2+} and thus contradict Danziger et al. (see above). The radical intermediate MB\textsuperscript{2+} also has to be considered in any photosensitized oxidation. It is proposed as an oxidizing intermediate in the photosensitized oxidation of amino acids [Knowles and Gurnani, 1972].

Finally, a mechanism has been proposed which involves the excited singlet state of the dye [Lober and Kittler, 1978; Kittler et al., 1980]. This is based on quenching of dye fluorescence by nucleotides which could be correlated with the oxidation potentials of the nucleic acid bases according to scheme 2.2 (S is the reducing agent). However, no direct evidence was presented for the formation of either MB\textsuperscript{*} (or MBH\textsuperscript{+}).
Concluding this section it is clear that excited state deactivation processes in methylene blue are complicated and involve a variety of different intermediates. All of these can play a role in the photoinduced reactions of the dye. Table 2.2 summarizes the absorbing species which are likely to be observed in a flash photolysis experiment with methylene blue.

\[
\text{MB}^+ + h\nu \rightarrow \text{MB}^{+\ast} + S \rightarrow \text{MB}^* + S^{+\ast} \quad (2.2)
\]

Table 2.2 Predominant transient intermediates which have been observed in transient spectroscopy of methylene blue.
2.2 METHYLENE BLUE BINDING TO NUCLEIC ACIDS

Only a limited number of studies on the interaction of MB\(^+\) with DNA have been reported. Evidence for the intercalation of methylene blue in the DNA-helix comes from dichroism studies on dye-DNA complexes [Bradley et al., 1972; Schipper et al., 1980; Norden and Tjerneld, 1982; Hogan et al., 1982]. These studies used either flow orientation or electric field orientation of the DNA helix and reported that the main visible transition dipole moment of the bound dye is oriented more or less perpendicular to either the flow or electric field. The results by Norden and Tjerneld suggest that different intercalation modes are possible. This will be discussed at length in the section on dye binding. Preliminary results reported by Allison and Hahn [1977] show that methylene blue changes the superhelical density of a closed circular form of DNA. This is considered indicative for an intercalated form of binding.

Association or binding constants \(K_b\) of the dye to the polymer were reported by a number of authors and are given in table 2.3 with the binding number \(n\) (if reported).
<table>
<thead>
<tr>
<th>DNA-type</th>
<th>$K_b$ (M$^{-1}$)</th>
<th>n</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Calf thymus</td>
<td>1.6x10$^5$</td>
<td>0.19</td>
<td>[Tanaka et al., 1981]</td>
</tr>
<tr>
<td>(b) Calf thymus</td>
<td>7.0x10$^5$</td>
<td>0.13</td>
<td>&quot;</td>
</tr>
<tr>
<td>(c) Calf thymus</td>
<td>1.2x10$^4$</td>
<td>--</td>
<td>[Muller &amp; Crothers, 1975]</td>
</tr>
<tr>
<td>(d) Calf thymus</td>
<td>1.1x10$^6$ and 1x10$^5$</td>
<td>0.13</td>
<td>[Norden &amp; Tjerneld, 1982]</td>
</tr>
<tr>
<td>(e) Calf thymus</td>
<td>6.5x10$^4$</td>
<td>0.14</td>
<td>&quot;</td>
</tr>
<tr>
<td>(f) Calf thymus</td>
<td>6.0x10$^4$</td>
<td>--</td>
<td>[Hogan et al., 1982]</td>
</tr>
<tr>
<td>(f) Chicken DNA</td>
<td>5.0x10$^5$</td>
<td>--</td>
<td>[Hogan et al., 1983]</td>
</tr>
<tr>
<td>(f) poly[dG]-poly[dC]</td>
<td>2.0x10$^6$</td>
<td>--</td>
<td>&quot;</td>
</tr>
<tr>
<td>(f) poly[dA-dC]-poly[dT-dG]</td>
<td>1.0x10$^6$</td>
<td>--</td>
<td>&quot;</td>
</tr>
<tr>
<td>(f) poly[dA]-poly[dT]</td>
<td>1.0x10$^5$</td>
<td>--</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

Table 2.3 Binding constants for methylene blue to DNA. Experimental methods: (a) absorption; (b) calorimetry; (c) equilibrium dialysis; (d) dichroism 5mM NaCl, n for first binding mode; (e) dichroism .2 M NaCl, n see (d); (f) fluorescence quenching. Binding number n is defined as mole dye per mole phosphate of DNA.

There is considerable variety in the value of the association constants $K_b$. The value of the binding number n is however more consistent. This appears to be a general feature of the Scatchard equation used to determine $K_b$ and n and has been observed before [Tossi, 1987]. Tanaka et al. [1981] determined the thermodynamic binding parameters $\Delta G, \Delta H, \Delta S$ as -33 and -5 kJ.mol$^{-1}$ and 95 J.K$^{-1}$.mol$^{-1}$ respectively. The increase in entropy when methylene blue binds to DNA can be compared with a $\Delta S$ of 12 J.K$^{-1}$.mol$^{-1}$ found for the intercalating acridine compound proflavine.

Base-pair specific binding of methylene blue to DNA was determined by
Muller and Crothers [1975]. They found a clear binding preference for G-C basepairs which they attributed to a polar interaction between the dye chromophore and the G-C basepairs. This was confirmed later by Sharples and Brown [1976]. Muller and Crothers could not detect any cooperativity in the binding of the dye to the polymer. In cooperative binding the association constant is a function of the amount of dye bound which can lead to dye stacking on the outside of the polymer. This is well established for acridine orange, an intercalator with a similar structure to methylene blue.

Hogan and coworkers have pioneered the use of laser flash photolysis with methylene blue as a probe for physical properties of DNA [Hogan et al., 1982; Wang et al., 1982; Hogan et al., 1983; Berkoff et al., 1986]. They have determined molecular movements and flexibility of the helix utilising absorption anisotropy of the triplet state of the dye assuming intercalative binding. They used an excitation wavelength of 604 nm in these experiments which complicates interpretation of their experimental results as will be discussed later in this thesis.

Summarizing, comparatively few studies of the binding of methylene blue to DNA have been made. This is surprising in view of the extensive literature on the photolysis of DNA sensitized by this dye. Studies reported so far indicate that methylene blue binds by intercalation with a specificity for G-C basepairs. No cooperativity could be observed in the binding. There is some indication of a salt dependence of the binding.
In this project an attempt was made to determine the mechanisms via which methylene blue acts as photosensitizer for nucleic acids. Methylene blue is an efficient sensitizer of singlet oxygen \(^1\text{O}_2\) but is also an efficient radical generator and it was hoped to clarify the role of the different reaction pathways (i.e. Type I -direct; or Type II \(-\text{O}_2\) mediated) as well as the nature of the reactive substrate. This would give information with regard to the mechanism of the previously reported base-specific modification of DNA photosensitized by MB\(^+\).

It has to be realised that an extremely large number of chemicals can act as photosensitizers, ranging from food-colorants and drugs to paints and textile-dyes. Santamaria and Prino (1972) report 417 compounds as such and the list can -15 years later- only be regarded as a conservative estimate. The determination of possible mechanisms of photosensitized damage to nucleic acids is thus pertinent. Several excellent reviews on the general topic of photosensitized damage of DNA have appeared recently [Cadet et al., 1983, 1985, 1986]. In the overview presented here emphasis will be on the methylene blue sensitized reactions of DNA and its constituents.

### 2.3.1 Initial studies and studies on isolated nucleotides

One of the first studies to report MB\(^+\) photosensitized damage to DNA was reported by Simon and Vunakis [1962]. They reported a polymer degradation which was preceded by partial denaturing of the double
helix. Separate photolysis experiments with nucleotides showed preferential destruction of dGMP as inferred by loss of its UV absorption at 260 nm. The nucleotides dUMP, dCMP and dTMP were, in contrast, completely unreactive. It was shown that this photolysis of dGMP proceeded with an associated uptake of $O_2$ from the solvent. The authors discussed the possibility of a correlation between guanine destruction and DNA strand breaks. Such a correlation is not straightforward as was shown by Vunakis et al. [1966] who reported (using a $^{14}C$ labeled TRIS buffer) that the radioactive label became incorporated in the DNA strand and -in a separate experiment- in photoproducts formed on irradiation of MB$^+/dGMP$ complexes.

Simon et al. found that the (deoxy-)ribose group of the nucleotides does not affect the photooxidation and it was concluded that the main target of the reaction is the purine base. They subsequently showed (in DNA) that guanine residues were preferentially destroyed by the action of MB$^+$ and light [1965] and that $O_2$ was necessary. No mention was made of the possible reaction mechanisms which destroyed the bases and caused strand degradation. Bellin and Grossman [1964] studied the dye-sensitized photochemical degradation of nucleic acids by a variety of dyes. A substantial decrease in melting temperature was observed after irradiation of the complex indicating degradation of the polymer. The extent of degradation was correlated to the G-C content of the different native DNA used. Furthermore dye binding to the polymer was seen to significantly increase the extent of damage. The degradation of monomeric guanine compounds by photosensitization was examined by Sussenbach and Berends in a number of papers [1963, 1964, 1965]. Reaction products could only be observed in aerated solutions and were mainly $CO_2$, guanidine and a peroxide product which subsequently was photosensitized to parabanic acid.

Waskell et al. [1966] found similar breakdown products on the
photolysis of methylene blue/guanosine mixtures in an aerated pH=9.2 bicarbonate buffer. The main reaction products they could identify were guanidine, free urea, ribosyl-urea and free ribose. Free guanine could not be found. Similar products were found on the irradiation of guanosine in the presence of acridine orange. It was found that the rate of formation of free ribose is proportional to the destruction of guanosine whereas the destruction of ribose proceeds via different pathways. With reference to the degradation of nucleic acid strands as observed by Simon et al. (see above), the authors speculated that scission of the glycosidic bond takes place with an unstable intermediate of the nucleoside. Subsequent reactions lead to an oxidative modification of the ribose moiety which cause strand break formation.

The involvement of $^{1}O_2$ in the destruction of nucleic acid components was convincingly demonstrated by Hallet et al. [1970] and by Rosenthal and Pitts [1971]. Singlet oxygen was generated in the former case via the peroxide/hypochlorite reaction and in the latter case via a microwave discharge. Guanine and associated compounds such as guanosine and deoxyguanosine were by far the most susceptible to damage by $^{1}O_2$. Rosenthal and Pitts again showed the importance of buffer and pH. Using respectively a phosphate (pH=6.8), a Tris (pH=8.5) and a carbonate buffer (pH=10.5) they showed that destruction proceeded at a low rate in the first and at the highest rate in the last buffer solution.

Methylene blue is a well known sensitizer of $^{1}O_2$ with a high quantum yield of singlet oxygen production [Bonneau et al.,1975]. Thus, Nilsson et al. [1972] report evidence for the MB$^+$ mediated oxidation of amino acids by $^{1}O_2$. However, type I reactions were shown to be involved in the MB$^+$ sensitized degradation of nucleotides as well as amino acids [Knowles et al., 1971, 1972].

In a careful study Kornhauser et al. [1973] compared the reaction
products of methylene blue photosensitized, radioactively labeled guanosine with that of guanosine subjected to \(^1\text{O}_2\) generated by microwave discharge. Comparison of radiograms of TLC plates with the products from the different reactions showed peaks with identical \(R_f\) values, indicating similar compounds, but also peaks which indicated the involvement of non-singlet oxygen reactions in the dye sensitized photolysis of guanosine. Similar conclusions were also reached by Saito et al. [1975], Singh and Vadasz [1977] and by Cadet et al. [1978, 1983, 1986]. Singh and Vadasz found that approximately 60% of the inactivation of E.Coli ribosomes was due to \(^1\text{O}_2\) and 40% due to other mechanisms. This is based on a study of the effect of addition of specific \(^1\text{O}_2\) quenchers such as NaN\(_3\) and 2,5 dimethylfuran. Cadet et al. [1983, 1986] carefully analysed and examined the reaction products of methylene blue photosensitized reactions with 3',5'-di-O-acetyl yl-2'-deoxyguanosine, a water soluble guanine derivative and reported similar percentages. The authors were able to identify a wide range of different reaction products attributable either to type I or to type II reactions.

Figure 2.4 [from Cadet et al. 1986] shows possible intermediate products postulated in the photosensitized degradation of the guanosine derivative. The initial products of the reaction with \(^1\text{O}_2\) are formed by either cycloaddition across the 4,5 bond to form a dioxetane or the formation of a 4,8-endoperoxide and both react further. The authors postulate that the type I photoproducts are formed by an initial hydrogen or electron abstraction from the purine ring to the excited triplet-state of the sensitizer with subsequent further reaction with oxygen.

This summary shows that a considerable amount of work has been done on the determination of the methylene blue photosensitized damage to isolated nucleotide bases. It appears that 1) guanine and its
Figure 2.4 Reaction products formed from the methylene blue photosensitized photolysis of the water soluble guanine derivative 3'5'-di-O-acetyl-2'deoxyguanosine. Illustrated are products identified after both anaerobic (Type I) and singlet oxygen (Type II) reactions [Cadet, 1986].
derivatives are mainly susceptible to damage and 2) that this damage is both by type I and type II mechanisms.

2.3.2 Polymer sensitization studies

One of the first studies on the effect of photosensitization on the guanine moiety in-situ in the polymer was reported by Piette et al. [1981]. Polymer-dye complexes were submitted to low light fluences which did not significantly degrade the DNA (as observed by UV absorbance) The photosensitizer used was proflavine. After removal of the dye the polynucleotide was subjected to various hydrolytic and enzymatic treatments, where the reaction products were subsequently analysed via chromatographic and spectrometric techniques. They could show that the guanine residues were altered to two different photoproducts and that these were still incorporated in the polymer. Involvement of $^{1}O_2$ was inferred by the decrease of the photolysis in the presence of NaN$_3$. It was suggested that one of the photo products might be a 4,8-endoperoxide, but no evidence could be given. In striking contrast, Roberts [1981] did not find any damaged guanine (or adenine) residues of the DNA of HeLa cells photolyzed in the presence of proflavine. Instead cytosine and thymine residues were reported to be destroyed. Also no effect of $^{1}O_2$ quenchers or D$_2$O could be observed. This would suggest a type I mechanism for this reaction.

Methylene blue photosensitized damage in native and in synthetic polynucleotides was determined by Feldberg et al. [1983] using a DNA-binding protein which was specific for damaged lesions in the polymers. They could only detect damage in polynucleotides containing guanine after photosensitization with methylene blue. The effects of the addition of NaN$_3$ or D$_2$O suggested a type II mechanism. Interestingly, only limited damage in poly [d(G-$^5$C)] was found at a high salt
concentration when this polynucleotide is in the Z-configuration. They speculated that accessibility of the $^{1}$O$_2$ is limited under these conditions and that especially steric hindrance at the purine C-4 position would constrain reactivity. Their conclusions have to be regarded as somewhat tentative as we have shown (see later) that the binding of MB$^+$ to DNA is salt dependent and also that photosensitized damage to the polymer is dependent on the amount of dye bound. Indeed, Kawanishi et al. [1986] have demonstrated that methylene blue is more effective in damaging double stranded DNA than single stranded indicating the importance of intercalative binding to the photosensitization. This is consistent with the earlier observations of Bellin and Grossweiner (see above).

Rose bengal was used as photosensitizer in a study by Nieuwint et al. [1985] who reported strand breaks in covalently closed circular (ccc) DNA with the dye dissolved in the solution. Removal of oxygen strongly prevented cleavage whereas the use of D$_2$O as solvent increased the yield of strand breaks. Both factors seem to indicate the involvement of $^{1}$O$_2$ in the formation of the breaks. However the authors compared this method of generating singlet oxygen with a method in which the reactive oxygen species is generated from the slow decomposition of a water soluble endoperoxide (NDPO$_2$: endoperoxide from disodium 3,3'-(1,4-naphthylidene) dipropionate) and did not find any overt strand breaks in the latter case but alkali labile sites instead. The reason for the apparent discrepancy between the results obtained by the two different methods is not known and the authors suggested that a possible intermediate formed by a back reaction between $^{1}$O$_2$ and sensitizer might be involved. Direct phosphodiester strand breaks were not seen in plasmid DNA pBR322 when it was subjected to $^{1}$O$_2$ generated via the method of Wang and Midden [1983] [Hildebrand et al. 1986], although alkaline sensitive sites were observed. Ciulla et al. [1986]
have reported an increase in strand breaks on removal of oxygen in contrast to Nieuwint et al. in a similar experiment with dissolved rose bengal. They also reported the quantum yield for single strand break formation in an aerated solution to be $1.7 \times 10^{-8}$ thus indicating the inefficiency of the cleavage process. Praseuth et al. [1986] on the other hand conclude that the cleavage of cccDNA by water soluble porphyrins is predominantly by $^1O_2$.

Recently, it has become possible to measure the time resolved infrared luminescence of $^1O_2$ enabling direct determination of reaction rates. Thus, Lee and Rogers [1987] reported the rates of reaction of $^1O_2$ with DNA and dGMP as respectively $5.1 \times 10^5$ and $5.3 \times 10^5$ M$^{-1}$s$^{-1}$. This is very low and it is not surprising that other workers [Bensasson (private communication)] measured no effect of addition of DNA on the lifetime of $^1O_2$.

Concluding the overview of the literature on the dye sensitized damage of nucleic acids and its components, several observations can be made. Firstly, the exact mechanism of the methylene blue sensitized damage is reasonably well established for the isolated nucleotide and indicates an approximately 40:60 ratio between type I and type II reactions whereby the latter involves addition reactions and the former hydrogen or electron transfer reactions. Secondly, the observations made on polynucleotides are not clear cut and even contradictory and do not give a good indication of the importance, if any, of $^1O_2$ or other oxygen radicals in the reaction. Thirdly, this large number of contradictory and conflicting reports indicate the difficulty of the precise determination of the exact reaction mechanism of the strand cleavage (see also Cadet et al. 1986).

The experiments which are described in chapter 5, concentrate on the possible elucidation of the reaction mechanism of the sensitized backbone cleavage and its efficiency. Sequencing has been carried out
to enable the detection of base-specific cleavage. It was already known that MB\(^+\) cleaves DNA specific at G-residues in aerated solutions after treatment with piperidine (from the work of Friedman and Brown, 1978) and the effect of different treatments on the base-specificity was to be investigated. It does appear that singlet oxygen modification of the polymer is guanine specific and it is conceivable that type I -radical- mechanisms might be specific for other nucleotides.
CHAPTER 3

PHOTOPHYSICAL PROPERTIES OF METHYLENE BLUE
AND METHYLENE BLUE/DNA COMPLEXES
INTRODUCTION

Photosensitized reactions involve electronically excited states of the photosensitizer. In such reactions, a distinction can be made between two different reaction pathways. The first (termed Type I) involves a direct reaction of the (singlet or triplet) excited state of the sensitizer with the substrate. The second (Type II) reaction involves the generation of a reactive oxygen species: singlet oxygen ($^1O_2$) from a collision between the triplet state of the sensitizer and the triplet (ground-) state of oxygen. It has already been shown that the binding to DNA of a dye can change its excited state properties i.e. those responsible for photosensitized reactions [Geacintov et al., 1981]. The study of the mechanism of the methylene blue photosensitized damage to DNA involves thus the study of its singlet and triplet state properties when the dye is bound to DNA.

Methylene blue is a very efficient sensitizer of singlet oxygen ($^1O_2$) with a reported quantum yield of production which may exceed 0.55 [Bonneau et al., 1975] and thus the destruction of nucleic acids can occur via a Type II mechanism. It might be expected that singlet oxygen is less important in the photodegradation of MB$^+$-DNA complexes as the triplet yield of the dye is reported to be quenched on binding [Hogan et al., 1983]. In addition, the close proximity of sensitizer and substrate in the intercalated dye-DNA complex could facilitate direct radical reaction between dye sensitizer and polymer. It is thus of interest to know how the yield and reactivity of the triplet state (i.e. the excited state responsible for $^1O_2$ formation) is modified on binding and to determine whether any transient photochemical products (e.g. formed by redox reaction of the dye and nucleic acid bases) can be
observed. Furthermore, studies of the photophysical properties of the complex can give information on the binding of the dye to the polynucleotide.

Conventional absorption and fluorescence spectroscopy was used to study the ground state of free dye and dye/DNA complexes. These results are presented in section 3.1. Laser flash photolysis results on transient intermediates of both free dye and dye/DNA complexes are reported in section 3.2. These results were obtained with an excimer laser as excitation source. Additional experiments, to ascertain the effects of variations in ionic strength and in pH were performed with a dye laser as excitation source and are reported in section 3.3. Preliminary results of picosecond laser flash photolysis experiments, carried out in the University of Manchester, are reported in section 3.4. Each section is concluded with a discussion of the results presented.
3.1 ABSORPTION AND FLUORESCENCE SPECTROSCOPY

Experimental results on the absorption and fluorescence properties of methylene blue and methylene blue/nucleic acid complexes are reported in this section.

3.1.1 Methylene blue dimer absorption spectrum.

The absorption spectrum of monomeric methylene blue has already been discussed in chapter 2 and will not be further discussed here. Methylene blue is well known to form dimer aggregates at the concentrations required for the laser flash photolysis experiments (section 3.2). The extent of dimerization is expected to be ionic strength dependent, therefore it was decided to calculate the monomer-dimer equilibrium constant at the ionic strength at which the laser flash photolysis experiments (see later) were carried out. In the literature, only the equilibrium constant for aggregation in deionised water has been reported. Furthermore, this would enable the determination of extinction coefficients of the dimer in the ultraviolet i.e. at the wavelength of the excimer laser.

The dimer absorption spectrum was determined using the method of West and Pearce [1965]. In this method the contributions of monomeric and dimeric absorbances to that of a measured solution are determined in an iterative manner, assuming initially that the monomer spectrum does not overlap with the dimer spectrum and that the dimer spectrum is symmetrical around its maximum. The molar concentration of the monomer $C_m$ at a total dye concentration $C_t$ can then be calculated from a knowledge of the monomer extinction coefficient and the pathlength of
the cell. The dimer concentration $C_d$ at the total dye concentration $C_t$ is calculated from equation (3.1):

$$C_d = \frac{(C_t - C_m)}{2}$$ (3.1)

The value of $C_d$, with the value of the cell pathlength yields the extinction coefficients of the dimer.

This procedure was carried out for methylene blue in the 50 mM phosphate buffer (pH = 6.9) for a series of dye concentrations. The dye concentrations were determined spectrophotometrically. Table 3.1 lists respectively the total dye concentration $C_t$, the cell thickness $l$, the observed optical density and the calculated monomeric density at the monomeric maximum and the dimer association constant $K_d$, calculated from $K_d = \frac{C_d}{C_m^2}$.

<table>
<thead>
<tr>
<th>$C_t$ (M)</th>
<th>$l$ (mm)</th>
<th>$OD_m$ (x 10^-5)</th>
<th>$OD_{calc}$ (665 nm)</th>
<th>$K_d$ (M^-1) (x 10^-4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.1</td>
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<td>.347</td>
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<td>2.4</td>
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<td>.337</td>
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<tr>
<td>0.071</td>
<td>10.00</td>
<td>.131</td>
<td>.130</td>
<td>1.22</td>
</tr>
</tbody>
</table>

average $K_d = 1.21 \pm 0.07 \times 10^4$ (M^-1)

Table 3.1 Association constants of dimer formation for methylene blue in a pH = 6.9, 50 mM phosphate buffer at T = 22 ± 1°C.

The values of $K_d$ obtained for the different dye concentrations do not show any systematic deviation with dye concentration. This rules out any contribution of higher oligomers at the high dye concentrations.

The value of the association constant obtained here is somewhat higher than that reported by Fornilí et al. [1981] (i.e. 7.7x10^3 M^-1). This
value was obtained in deionized water and the higher value found here is presumably attributable to the higher ionic strength of the solution (100 mM) where ion-pairing ensues.

The concentration of monomeric dye molecules $C_m$ in solution can be calculated from the total dye concentration $C_t$ by solving equation 3.1 (with $K_d = C_d/(C_m^2)$). This then yields:

$$C_m = \frac{1 + 8 \times K_d \times C_t}{4 \times K_d}$$

(3.2)

The absorption spectra of the dye monomer and dimer are shown in figure 3.1. The visible dimer absorption spectrum is characterised by a large absorption band at 610.5 nm and a smaller band with a maximum at 680 nm. The spectrum obtained here is in good agreement with those reported previously [Fornili et al., 1981]. The single absorption band in the monomer is observed to split into two bands in the dimer. This is in agreement with theoretical predictions for a dimer with the two molecules of the complex oriented parallel to one another in a "sandwich" or "biplane" configuration [Kasha and McRae, 1964]. The interaction of excited and ground state wavefunctions which gives rise to this splitting apply apparently only to the lowest singlet $S_1$ state as no splitting is observed at 290 nm in the higher energy $S_2$ state of the monomer. The extinction coefficient of $101,490 \text{ M}^{-1}\text{cm}^{-1}$ determined here for the dimer band at 610.5 nm is somewhat lower than the value of $132,000 \text{ M}^{-1}\text{cm}^{-1}$ reported by Bergman and O'Konski [1963].

3.1.2 Absorption in the presence of polynucleotides.

The spectral changes associated with the binding of a low concentration of dye to CT-DNA in a 50mM phosphate buffer (pH = 6.9), are a red shift in the long wavelength transition from 665 nm to 673 nm.
Figure 3.1 Experimentally determined monomer (dotted line) and dimer (solid line) spectrum for methylene blue in a 50 mM phosphate buffer ($\mu = 100$ mM).
and a hypochromicity of 22% at the highest P/D ratio* (Figure 3.2). Similar effects are observed for the polynucleotides poly[d(G-C)] and poly[d(A-T)]. The former polymer yields a red shift of 9.5 nm with a hypochromicity of 26%, whereas the latter exhibits a red shift of 6 nm with a hypochromicity of 23%. Under the conditions employed (High P/D ratio, pH = 6.9, μ = 50 mM phosphate, T = 22 °C) all dye/polymer spectra clearly show the existence of an isosbestic point. This is in contrast to the absorption spectra of MB+/CT-DNA complexes in a 100 mM Tris/HCl buffer, reported by Tanaka et al. [1981]. The presence of an isosbestic point is generally thought to indicate the presence of only two species in solution ie. free and bound dye. Binding parameters derived from these spectra will be discussed in chapter 4.

Addition of polynucleotides to a relatively high concentration of dye (> 2x10⁻⁵ M) results in a decrease of the 610.5 nm dimer absorption band. This is observed both in CT-DNA and poly[d(G-C)] and to a somewhat lesser extent in poly[d(A-T)]. Figure 3.3 shows the experimental observations for CT-DNA. The results obtained for the two synthetic polynucleotides are similar. The decrease of the absorption band associated with the dimer could be due to binding of monomeric dye to the polymers. Alternatively, the dye could be bound as a dimer aggregate with a different structure from that in aqueous solvents. This will discussed further in chapter 3.3. The apparent deaggregation is indicative of an absence of external stacking on the outside of the polymer associated with electrostatic binding, such as is observed for the dye acridine orange [Vitagliano, 1983]. The absence of cooperative binding was already inferred from results obtained with equilibrium dialysis [Muller and Crothers, 1975].

* P/D ratio : ratio of DNA phosphate concentration ν dye concentration. Used to express polymer concentration relative to that of dye.
Figure 3.2 Absorption spectra of 2.4x10^{-6} M methylene blue in 50 mM phosphate buffer (pH ~ 6.9) with CT-DNA (A), poly[d(G-C)] (B) and poly[d(A-T)] (C). P/D ratios are (A): 130.9, 101.2, 78.2, 60.4, 46.7, 36.1, 27.9, 21.5, 16.6, 12.9, 9.9. (B): 50.1, 38.7, 29.9, 23.1, 17.9, 13.8, 10.7, 8.2. (C): 57.8, 44.7, 34.5, 26.7, 20.6, 15.9, 12.3, 9.5. In all cases a clear isosbestic point is observed.
Figure 3.3 Absorption spectrum of $6.2 \times 10^{-5}$ M methylene blue in a 50 mM phosphate buffer (pH = 6.9) on addition of DNA. Note decrease of absorption band associated with dimer aggregate at 600 nm. Similar spectra are observed under identical conditions with poly[d(A-T)] and poly[d(G-C)].
3.1.3 Fluorescence in the presence of polynucleotides.

The fluorescence emission of methylene blue is quenched on the addition of polynucleotides. The quenching is dependent on the polynucleotide and the ionic strength of the solution (Figure 3.4). The fluorescence quenching observed here confirms the observations reported by Tomita [1968] and Hogan et al. [1983]. Fluorescence quenching can be used to determine binding constants of the dye to the polymer [LePecq and Paoletti, 1967]. The ionic strength dependence of binding will be discussed in greater detail in chapter 4.

The quenching of the fluorescence on addition of the polymer can arise due to an electronic interaction between the excited state of the dye and the polymer. This can be elucidated by the determination of the quantum yield of fluorescence. Figure 3.5 shows the corrected fluorescence spectra of MB⁺ with CT-DNA, poly[d(G-C)] and poly[d(A-T)] in a 50 mM phosphate buffer. Excitation was at 600 nm and the spectra were corrected using reference compounds as described in chapter 8. The spectra are broad and featureless with a maximum emission wavelength at 684 nm for the free dye which shifts on addition of CT-DNA and poly[d(A-T)] to 691.5 nm and 693.4 nm respectively. No shift in the wavelength of maximum emission was observed on addition of poly[d(G-C)].

The fluorescence quantum yield of methylene blue in ethanol has been reported as 0.04 (± 50%) [Olmsted, 1979]. From the integrated fluorescence emission of an ethanolic dye solution, the fluorescence yield of the dye in an aqueous solution was determined as 0.016 (± 50%). The fluorescence quantum yields for the dye/polynucleotide complexes can then be determined using this value and are given in table 3.2. The relative accuracy of the data is ± 5%. The absolute value of the quantum yields is only accurate to within ± 50%, which
Figure 3.4 Quenching of fluorescence emission of methylene blue on addition of CT-DNA. Dye concentration and ionic strength 1: $2.4 \times 10^{-6}$ M and 0 mM; 2: $1.2 \times 10^{-6}$ M and 100 mM; 3: $1.9 \times 10^{-6}$ M and 200 mM.

Phosphate buffer, pH = 6.9. Excitation at 665 nm, emission at 690 nm.
Figure 3.5 Corrected fluorescence spectra of methylene blue with CT-DNA (A), poly[d(G-C)] (B) and poly[d(G-C)]. Dye concentrations and P/D: (A) $4.6 \times 10^{-6}$ M; 65, 35.5, 19.3, 10.5, 5.8, 3.1, 1.7, 0. (B) $1.2 \times 10^{-6}$ M; 56, 29.3, 15.4, 8, 4.2, 0. (C) $1.2 \times 10^{-6}$ M; 63.5, 33.3, 17.4, 9.1, 4.8, 0. Buffer 50 mM phosphate, pH = 6.9. Excitation wavelength 600 nm.
is the reported accuracy for the literature value of the fluorescence quantum yield of MB⁺ in ethanol.

<table>
<thead>
<tr>
<th>CT-DNA</th>
<th>poly[d(G-C)]</th>
<th>poly[d(A-T)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>P/D</td>
<td>〈Φ_f〉 (x10⁴)</td>
<td>P/D</td>
</tr>
<tr>
<td>0.0</td>
<td>16</td>
<td>0.0</td>
</tr>
<tr>
<td>1.7</td>
<td>14.9</td>
<td>4.2</td>
</tr>
<tr>
<td>3.1</td>
<td>14.1</td>
<td>8.0</td>
</tr>
<tr>
<td>5.8</td>
<td>13.3</td>
<td>15.4</td>
</tr>
<tr>
<td>10.5</td>
<td>11.7</td>
<td>29.3</td>
</tr>
<tr>
<td>19.3</td>
<td>10.4</td>
<td>56.0</td>
</tr>
<tr>
<td>35.5</td>
<td>7.7</td>
<td></td>
</tr>
<tr>
<td>65.0</td>
<td>5.6</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2 Fluorescence quantum yields for complexes of methylene blue with CT-DNA, poly[d(G-C)] and poly[d(A-T)] in a 50 mM phosphate buffer, pH = 6.9. Dye concentration: 4.7 x 10⁻⁵ M (CT-DNA); 1.2 x 10⁻⁶ M (poly[d(A-T)]); 1.2 x 10⁻⁶ M (poly[d(G-C)]).

The yield of fluorescence emission is reduced by approximately the same amount on addition of the polymers CT-DNA and poly[d(G-C)]. The fluorescence quantum yield of methylene blue is unchanged on addition of the polynucleotide poly[d(A-T)], although the dye does exhibit substantial changes in its absorption spectrum on addition of this polymer. These observations suggest that the quenching of the emission is strongly basepair dependent with G-C basepairs quenching fluorescence and not with A-T basepairs.

3.1.4 Discussion

The results from these steady state absorption and fluorescence experiments support, in general, the findings of other workers [Tomita, 1968; Tanaka et al., 1981; Hogan et al., 1983]. Compared to free dye, a red shift and associated hypochromism is observed in the absorption
spectrum of all three dye/polymer complexes, compared to that of the free dye. A red shift is observed in the absorption spectrum of most molecules that bind to DNA and a number of hypotheses have been reported to account for this red shift.

- It has been suggested that the shift reflects the transition for the molecule from an aqueous to an organic -less polar- environment. This is based on a similarity between the maximum wavelengths of absorption and fluorescence of the dye proflavine in ethanolic solutions and in complex with DNA [Lober, 1981] compared to those in water. However, similar observations could not be made for methylene blue in our experiments. In fact the absorption spectrum is blue shifted in EtOH with a $\lambda_m$ of 650 nm in EtOH, compared to $\lambda_m$ of 665 nm in H$_2$O. Similarly, the maximum of the fluorescence emission is blue shifted from 690 nm in H$_2$O to 682 in EtOH. In DNA the opposite is seen (see above).

- An alternative hypothesis for the red-shift is given by Philpott [1970]. His theory envisages coupling of the ground and excited state wavefunctions of the dye with those of the polymer and relates the observed red-shift to an interaction energy $U_{dp}$ which expresses the strength of the coupling between the dye and the polymer transition dipole. Direct evidence for such a coupling of the dye and polymer wavefunctions comes from observations by Nastasi et al., [1974], who reported a dimer-like exciton splitting in the absorption spectrum of the dye quinacrine when the dye is intercalated in the double helix.

The relation between the observed red-shift and the interaction energy is given in equation (3.3). (This is equation 2.29 in [Philpott, 1970]) In its derivation nearest neighbour interaction between the dye and the monomer units of the polynucleotide have been assumed only.

\[
E_p - E_o = \frac{(2 \times U_{dp}^2)}{(E_{DNA} - E_p)} \]  

(3.3)
In this equation \( E_o \) is the centre absorption wavelength of the unbound dye, \( E_p \) is that of the bound dye, and \( E_{DNA} \) is the centre wavelength of the polymer absorption band (here taken as 260 nm).

Table 3.3 shows values of \( U_{dp} \) calculated for methylene blue and for the intercalating dyes proflavine, acridine orange and ethidium bromide. The values of \( U_{dp} \) are compared with values of \( \Delta G \) for binding (calculated from \(-RT \times \ln(K_b)\)).

<table>
<thead>
<tr>
<th>compound</th>
<th>( E_p - E_o )</th>
<th>( E_{DNA} - E_p )</th>
<th>( U_{dp} )</th>
<th>( \Delta G )</th>
<th>(ref)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB</td>
<td>245</td>
<td>23424</td>
<td>1694</td>
<td>2289</td>
<td>(I)</td>
</tr>
<tr>
<td>PF</td>
<td>831</td>
<td>15071</td>
<td>2502</td>
<td>2842</td>
<td>(II)</td>
</tr>
<tr>
<td>AO</td>
<td>682</td>
<td>17227</td>
<td>2424</td>
<td>2842</td>
<td>(II)</td>
</tr>
<tr>
<td>EB</td>
<td>1497</td>
<td>16717</td>
<td>3537</td>
<td>3026</td>
<td>(III)</td>
</tr>
</tbody>
</table>

Table 3.3 Comparison of values of the calculated interaction energy \( U_{dp} \) [Philpott, 1970] of intercalators with the \( G \) values for binding from literature. MB: methylene blue; PF: proflavine; AO: acridine orange; EB: ethidium bromide. All energy values in cm\(^{-1}\). Other symbols see text. References: (I) Hogan et al., 1982; (II) Tanaka et al., 1981; (III) LePecq and Paoletti, 1967.

The data show that there is a reasonable correlation between the \( \Delta G \) value of the binding process and the calculated value of \( U_{dp} \). The theory developed by Philpott thus appears to give an explanation for the observed red-shift in the absorption spectrum of the dye. The theory predicts a total lowering of the dye excited state energy on binding and does not take account of the vibronic substructure of the electronic energy levels. Thus, it could also explain the red-shift in the maximum of the fluorescence spectrum as is observed for methylene blue bound to CT-DNA and poly[d(A-T)]. The absence of a red-shift in the fluorescence of the MB\(^+\)/poly[d(G-C)] complex is less clear and will be discussed below.
The absorption spectra of the concentrated dye solutions with polynucleotides failed to give evidence for a cooperative stacking of methylene blue dimers onto the outside of the polynucleotides. The possible binding of dye dimers to polynucleotides will be discussed in more detail in section 3.3.

The quenching of the fluorescence of methylene blue bound to polynucleotides appears to be related to the G-C content of the particular polymer. Decreased fluorescence emission can be due to an enhanced radiation-less decay to the ground state, to increased intersystem crossing to the triplet state, or to chemical reactions. In chapter 3 it is shown that the decrease in fluorescence yield of the dye/polynucleotide complex is similar to the decrease in triplet yield of the complex. This appears to rule out an increased rate of intersystem crossing. Photosensitized electron transfer has been proposed as a mechanism of quenching [Lober and Kittler, 1978; Kittler et al., 1980] and is schematically illustrated in scheme 3.4:

\[ \text{MB}^+ + \text{G} \rightarrow \text{MB}^{+*} + \text{G} \rightarrow \text{MB}^- + \text{G}^+. \]  (3.4)

The possibility of quenching is governed by the oxidation and reduction potentials of respectively the nucleic acid bases and the excited state of the dye. Table 3.4 shows the oxidation potentials of the nucleic acid bases compared with the reduction potential of methylene blue in the ground state and in the excited singlet state. The latter is calculated from equation (3.5):

\[ E^* = E_o + (N \ h \ c \ \psi)/F \]  (3.5)
E*: excited state reduction potential; E°: ground state reduction potential; N: Avogadro's number; h: Planck's constant; c: velocity of light; \( \nu \): energy of the 0-0 transition; F: Faraday's constant. The value of E° was measured in a cyclic voltammetry experiment (see chapter 4).

<table>
<thead>
<tr>
<th>Oxidation potential</th>
<th>Reduction potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guanine E(_{ox}) = 1.53 V</td>
<td>Methylene blue E° = 0.05 V</td>
</tr>
<tr>
<td>Adenine = 1.63 V</td>
<td>E* = 1.89 V</td>
</tr>
<tr>
<td>Thymine = 1.73 V</td>
<td></td>
</tr>
<tr>
<td>Cytosine = 1.88 V</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.4 Oxidation potential of nucleic acid bases and reduction potential of methylene blue ground and excited singlet state. All potentials vs. standard hydrogen electrode.

Guanine has the lowest oxidation potential of the four bases and is thus most easily oxidized by the excited state of the dye. Kittler et al. correlate the fluorescence quenching of a substantial number of intercalating dyes with the above theory. However, no direct experimental evidence has ever been given for the electron transfer. In section 3.4 data is reported which shows direct evidence for electron transfer in MB\(^+\)/polynucleotide complexes.

Although the quenching of the dye fluorescence can be explained on the basis of electron-transfer from guanine, it is somewhat surprising that the quantum yield of fluorescence of MB\(^+\) bound to poly[d(G-C)] is not lower than that in CT-DNA (table 3.2). Most of the dye can be considered bound (chapter 4) which rules out emission from free dye. It is possible that only a certain number of dye molecules is in a favourable position to transfer its electrons and will have their emission quenched. The remainder would not interact as strongly with
the nucleic acid bases and hence fluoresce. This weak interaction implies a less strong coupling between the dye and nucleotide wavefunctions according to the theory of Philpott and consequently the dye emission would not be red-shifted.
3.2 LASER FLASH SPECTROSCOPY OF METHYLENE BLUE WITH NUCLEIC ACIDS

308 nm EXCITATION

3.2.1 Transient spectra

3.2.1.1 Free dye

The transient absorption spectrum of a degassed, aqueous MB+ solution (concentration 1.2 \(10^{-5}\) M) in the pH = 6.9, 50 mM phosphate buffer is shown in figure 3.6. Excitation was by a 20 mJ, 308 nm laser pulse of ca. 15 ns duration. The spectra were determined ca. 50 ns after excitation. The prompt transient absorption region is characterised by bands at 380, 420, 520 and 710 nm and an absorption region > 740 nm. All but the 520 nm band have been previously assigned by other workers as due to protonated triplet \(3^{MBH^{2+}}\) (380 and 710 nm) or unprotonated triplet \(3^{MB^{+}}\) (420 and > 740 nm) (see also chapter 2.1).

The pKₐ of the protonation-deprotonation equilibrium has been determined as 6.9 [Kato et al., 1964] or 7.1 [Ohno et al., 1979] and therefore at a pH of 6.9 both species will be observed.

The species whose absorption can be observed at 520 nm has been assigned as a semi-oxidized radical \(MB^{2+}\). It has a long lifetime compared to the triplet and is easily observed in a transient spectrum recorded 100 \(\mu s\) after the exciting laser flash (Figure 3.7). Its precise origin is somewhat disputed and has been attributed to either photoinduced electron ejection from a highly excited \(T_{2}\) state of the unprotonated dye [Kamat and Lichtin, 1981b] or to intramolecular charge transfer in \(MB^{+}\)-dimers [Danziger et al., 1967]. (In the latter case...
Figure 3.6 Prompt transient absorption spectrum of a 1.2x10^{-5} M solution of methylene blue in 50 mM phosphate buffer. Excitation by a 20 mJ, 308 nm, 15 ns laser pulse. Spectrum ca. 50 ns after excitation.
Figure 3.7 Transient absorption spectra of three different methylene blue concentrations in 50 mM phosphate buffer, taken at 100 μs after exciting laser pulse. Laser as in figure 3.6. Arrow denotes transient absorption band of semi-oxidized radical.
also MB\(^+\) will be observed). Alternatively, it can be produced by ground state quenching of protonated dye triplet [Kamat and Lichtin, 1981a] (see also chapter 2). This semi-oxidised species could conceivably be involved in the oxidation of nucleic acid bases and in a number of experiments the origin of this radical species was investigated. Possible involvement of dye dimers in the formation of this radical has been proposed [Danziger et al., 1967]. Several experiments performed here seem to confirm this.

- The long-lived transient spectrum shows appreciable amounts of transient absorption at 430 nm. This can be associated with MB\(^+\).
- It was found that the intensity of transient absorption at 520 nm was independent of whether the solution was degassed or not and thus it is unlikely that the species is formed from the triplet state of the dye. Knowles and Gurnani [1972] reported that the 520 nm species has a grow-in of its transient absorption, concomitant with a decay of the triplet absorption at 420 nm. In no experiment has it been able to confirm these observations. Instead, it was found here that the grow-in of the transient absorption at 520 nm is instantaneous with the exciting laser pulse.
- The position of a large depletion band around 600 nm corresponds approximately to the wavelength of maximum absorption of the dye dimer. This would make it seem likely that this depletion is attributable to the dimer of MB\(^+\).
- In a separate experiment a correlation was found between observation of the 520 nm transient absorption band and the presence of the 600 nm depletion band. This experiment was performed by comparing transient spectra of identical dye concentration in aqueous and ethanolic solutions. UV/VIS absorption indicated that dimerization was not observable in a 1:2 EtOH/H\(_2\)O solution as indicated by absence of the 600 nm band and in this solution the long-lived 520 nm species was
- Finally, strong evidence for the involvement of the dye dimer in the production of the $\text{MB}^{2+}$ is obtained from a plot of the prompt transient optical density at 520 nm against the percentage of dye dimer in solution. This experiment was performed by varying the concentration of dye. The concentration of dimer was calculated from equation 3.2. This is shown in figure 3.8. Although the data has some scatter (the transient optical densities were $< 4 \times 10^{-3}$) it does appear that there is a correlation between the amount of dimer in solution and the amount of $\text{MB}^{2+}$ produced.

In view of the above evidence it appears likely that $\text{MB}^{2+}$ is produced from a dimeric dye species. The mechanism of this production is however not very clear. Figure 3.9 shows a log-log plot of transient optical densities measured respectively at 520 nm and 420 nm vs. incident laser intensity. The plots yield a slope of 0.8 for production of the triplet as measured at 420 nm. This is close to a slope of 1 as expected for a monophotonic process. In contrast, the intensity dependence of the production of $\text{MB}^{2+}$ follows a slope of 1.6 which indicates, in part, a bi-photonic component in its mechanism of production. It is conceivable that excitation from an excited dimer triplet state might be responsible for the formation of $\text{MB}^{2+}$. (The excited dimer singlet state has a lifetime of ca. 5 ps and is not very likely to be involved. See section 3.4).

3.2.1.2. $\text{MB}^{+}$/nucleic acid complexes

The transient absorption spectrum of $^3\text{MB}^{+}$ is affected by addition of CT-DNA (Figure 3.10). The spectra are similar to that of the free dye, although the transient optical density decreases with increasing DNA-content. The bands at 380, 420 and 710 are clearly visible and no new
Figure 3.8 Graph of prompt transient absorption at 520 nm. versus percentage of methylene blue dimer in solution. The latter was calculated from equation 3.2.
Figure 3.9 Log-log plot of intensity of exciting laser pulse versus concentration of triplet (as estimated by the transient optical density at 420 nm) and yield of \( \text{Mb}^{2+} \) (as measured by prompt optical density at 520 nm). N.B. Buffer is 50 mM pH 8.9 phosphate. Alkaline buffer increases yield of \( \text{Mb}^{2+} \).
Figure 3.10 Prompt transient absorption spectrum of $1.1 \times 10^{-5}$ M methylene blue with different amounts of CT-DNA added. Excitation and buffer as in figure 3.6.
bands appear in the spectrum. The band at 520 nm is much less prominent in the dye/DNA complex. The red shift of the ground state absorption spectrum is reflected in a similar red shift in the depletion region. In figure 3.11 the prompt transient absorption spectrum of $^{3}\text{MB}^{+}$ on addition of poly[d(A-T)] and poly[d(G-C)] is compared with that of free $^{3}\text{MB}^{+}$. From these spectra it is clear that the triplet is strongly quenched in poly[d(G-C)] in comparison with poly[d(A-T)]. The transient absorption spectrum of $\text{MB}^{+}$-poly[d(G-C)], although weak, shows features similar to those of the free dye with no obvious strong new bands and no new long-lived species. This suggests that if any new chemical species are produced (as suggested by the reactions proposed in the explanation of the fluorescence quenching) they are very short lived. In particular there is no evidence for significant amounts of $\text{MB}^{*}$ ($\lambda_{\text{max}} = 430$ nm) having a lifetime much greater than 50 ns (see scheme 3.4).

The transient absorption spectrum observed for methylene blue following 308 nm excitation in pH=6.9 buffer consists of those of $^{3}\text{MB}^{+}$ and of $^{3}\text{MBH}^{2+}$, accompanied by smaller amounts of $\text{MB}^{2+*}$. In the presence of poly[d(A-T)] the transient absorption below 400 nm is decreased, so that the band at 420 nm is now dominant, and increased absorption is also noted at about 530 nm and above 730 nm. Whereas some of these features are found in a combination of $^{3}\text{MB}^{+}$ (band at 420 nm) and $\text{MB}^{2+*}$, the spectrum is strikingly similar to that reported for $^{3}\text{MB}^{+}$ in alkaline media (Figure 3.12). A possible explanation for this is that the protonation of the triplet state of the dye is substantially lower when bound to the polynucleotide, compared to free dye.

The two radical species that are observed in the transient spectrum of free methylene blue recorded after decay of the triplet, are $\text{MB}^{*}$ (at 430 nm) and $\text{MB}^{2+*}$ (520 nm) (figure 3.7). The intensities of both these absorption bands decreases on addition of polynucleotides. The decrease in the transient absorption of $\text{MB}^{*}$ explains the
Figure 3.11 Prompt transient absorption spectrum of 1.1x10^{-5} M methylene blue with poly[d(G-C)] (A) and poly[d(A-T)] (B). Spectra of free dye (P/D = 0) shown for comparison (dotted). Excitation and buffer as in figure 3.6.
Figure 3.12 Comparison of transient absorption spectra of 1.1x10^{-5} M methylene blue with poly[d(A-T)] (P/D = 24) in a 50 mM, pH = 6.9 buffer and a 50 mM, pH = 8.9 buffer. Note similarity in region 350 - 500 nm. Excitation as in figure 3.6.
decrease in the formation of leuco-methylene blue (MBH) when the dye is bound to DNA (see chapter 5). MB* has been shown to be an intermediate in the formation of MBH (scheme 2.2). The decrease in the transient absorption of both MB* and MB$^{2+}$ can be explained on the basis of both being produced from the dye dimer state. Intercalative binding mode will bind monomeric dye molecules and will thus reduce the amount of dimer in solution.

3.2.2 Triplet yield determination

The absolute yield of triplet state formation was determined using the comparative method as described by Bensasson et al. [1978]. In this the triplet yield of the unknown is compared with that of a standard according to equation (3.6):

$$\phi^X = \phi^A \times \frac{OD^X \times \varepsilon^A}{\varepsilon^X \times OD^A} \quad (3.6)$$

In this equation $\phi^A$, $\varepsilon^A$ and $OD^A$ are respectively the triplet yield, the triplet extinction coefficient (at wavelength of observation $\lambda$) and the experimentally observed transient optical density (at $\lambda$) of the compound A, which is used as a standard. The properties of the unknown compound X are denoted similarly. The standard which was used in the triplet yield determinations was methylene blue in an aqueous buffer solution; the triplet yield of the dye has been determined as 0.58 [McVie et al., 1978].

In order to apply equation (3.6) it is necessary to know the values of the extinction coefficients of the triplet-triplet transitions. These were determined from transient optical density measurements in
which complete conversion of the dye to the triplet state is assumed. This assumption is based on the invariance of the transient optical density with laser intensity. The necessary high laser intensity could not be achieved with the Lambda Physik excimer laser. Instead, a Q-switched Ruby laser was used which emits pulses of ca 20 ns duration, and up to 1.5 Joule per pulse at a wavelength of 694 nm. The intensity of this laser was sufficient to achieve the necessary invariance of the transient optical density as is shown in figure 3.13. Assuming complete conversion of the dye to its triplet state, the T-T extinction coefficients can be determined from the transient optical density (ΔOD) from:

$$\text{OD} = [\text{DYE}_{\text{total}}] \times (\varepsilon^T - \varepsilon^S)$$  \hspace{1cm} (3.7)

In this equation $\varepsilon^T$ and $\varepsilon^S$ are the extinction coefficients of the triplet and singlet dye, respectively. The T-T extinction coefficients were determined for methylene blue in the 50 mM, pH=6.9, phosphate buffer without polymer as well as in complex with CT-DNA and the synthetic polynucleotides poly[d(A-T)] and poly[d(G-C)]. The resulting extinction coefficients are shown in figure 3.14.

The triplet yields could now be determined using equation 3.6. The transient optical densities were obtained using the excimer laser as excitation source in the same 50 mM, pH=6.9 buffer. The yields were determined at three wavelengths (380, 420 and 480 nm) and subsequently averaged. This was done to maximise the accuracy of the determination.

The triplet yields are given in table 3.5 and are determined relative to a triplet yield of 0.58 for methylene blue in water [McVie et al., 1978].
Figure 3.13 Saturation of transient optical density, measured at 420 nm, for a 2.2 x 10^{-5} M methylene blue concentration as a function of incident laser energy (Ruby laser, 15 ns pulse, 694 nm). 100% is equivalent to 1 Joule per pulse.
Figure 3.14 Triplet-triplet extinction coefficients determined for methylene blue, free and in complex with polynucleotides. Buffer 50 mM phosphate, pH = 6.9.
Table 3.5 Quantum yields of triplet formation for complexes of MB+ with CT-DNA, poly[d(A-T)] and poly[d(G-C)]. [MB+] in DNA complex: 1.0x10^-5 M; [MB+] in complex with poly[d(A-T)] and poly[d(G-C)]: 1.1x10^-5 M. Buffer 50 mM, pH=6.9 phosphate buffer. Standard deviation ± 20 %.

The quantum yields of triplet formation relative to that of free dye are shown in figure 3.15. They are compared with the relative quantum yields of fluorescence which had been determined previously (see table 3.2). The reduction in triplet yield which is observed when the dye binds to either CT-DNA or poly[d(G-C)] follows the same trend as the reduction in the fluorescence quantum yield. A small amount of additional quenching of the triplet state by the G-C containing polymers is also evident.

3.2.3 Kinetics

3.2.3.1 Triplet

Representative traces of triplet decay curves measured at 420 nm are shown in figure 3.16 for free dye and for dye in complexed to CT-DNA. A long lived transient absorption can be seen which is most likely attributable to semi-reduced dye radical MB•. In general, lifetimes of
Relative Fluorescence Quantum Yields and Triplet Yields

Figure 3.15 Graphical comparison of fluorescence yields (Table 3.2) and triplet yields (Table 3.5).
Figure 3.16 Representative decay traces of methylene blue triplet, measured at 420 nm in deoxygenated 50 mM phosphate buffer with CT-DNA. Dye concentration $1.2 \times 10^{-5}$ M. (A): P/D = 0, $t = 6.8 \mu s$; (B) P/D = 27.0, $t = 8.0 \mu s$. Decays are single-exponential. Note different transient absorbances for the two solutions.
radical species (MBH+, MB or MB2+) were found to be larger than 100 us (see below), and thus did not interfere in the determination of the lifetimes of the triplet state. Additionally, the magnitude of all long-lived transient absorptions decreased on addition of polynucleotides. This is in agreement with the absence of any new transient absorption bands in the dye/polymer complexes (see section 3.2.1.2). Table 3.6 shows triplet decay times obtained for a series of dye/polynucleotide ratios in both degassed and aerated solutions. The triplet decays were measured at 420 nm (triplet decay times at other wavelengths are similar). It can be seen that addition of the polymer increases the lifetime of the triplet excited state. Also given in table 3.6 are rate constants for oxygen quenching of the triplet as determined from the degassed and aerated triplet lifetimes with equation 3.8

\[ k_{\text{air}} = k_{\text{degas}} + [O_2] \times k_q \]  \hspace{1cm} (3.8)

\( k_{\text{air}} \) and \( k_{\text{degas}} \) are respectively the decay rates for the triplet in aerated and degassed solutions, \( [O_2] \) is the oxygen concentration, assumed to be \( 2.65 \times 10^{-4} \) M [Murov, 1973] and \( k_q \) is the oxygen quenching rate constant (M\(^{-1}\)s\(^{-1}\)). From the yield of oxygen quenching it is possible to estimate a yield of production of singlet oxygen, \( ^1O_2 \) (see below).
<table>
<thead>
<tr>
<th></th>
<th>Triplet lifetime (µs)</th>
<th>( k_q ) ((M^{-1}s^{-1}))</th>
<th>( ^{1}O_2)-yield (estimated)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>degassed</td>
<td>aerated</td>
<td></td>
</tr>
<tr>
<td>Free dye</td>
<td>6.2</td>
<td>3.0</td>
<td>( 6.5 \times 10^8 )</td>
</tr>
<tr>
<td>CT-DNA (P/D=29)</td>
<td>14.0</td>
<td>11.4</td>
<td>( 6.0 \times 10^7 )</td>
</tr>
<tr>
<td>poly[d(G-C)] (P/D=26)</td>
<td>13.2</td>
<td>10.8</td>
<td>( 6.4 \times 10^7 )</td>
</tr>
<tr>
<td>poly[d(A-T)] (P/D=42)</td>
<td>7.4 &amp; 22.8</td>
<td>10.8</td>
<td>( 3.7 \times 10^7 )</td>
</tr>
</tbody>
</table>

Table 3.6 Triplet lifetimes, rate-constants of oxygen quenching and estimated yields of \(^1 O_2\) formation for complexes of MB\(^+\) with CT-DNA, poly[d(G-C)] and poly[d(A-T)]. Dye concentration \(1.0 \times 10^{-5}\) M. The yields of \(^1 O_2\) formation are calculated using equation 3.9. \( k \) and \(^1 O_2\)-yield for poly[d(A-T)] are calculated taking account of different amount of long- and short lived species. Double exponential decays obtained using "stripping" method.

The decay of \(^3 MB^+\) bound to CT-DNA or poly[d(G-C)] appears to follow first order kinetics, both in degassed and in aerated solutions and increases with polymer concentration. It may be noted that the signals for CT-DNA and poly[d(G-C)] are rather weak and multi-exponential decays might be obscured.

The decay of \(^3 MB^+\) to poly[d(A-T)] is more complicated and can be analysed as consisting of two consecutive first order decays in a solution purged with argon (Figure 3.17). The double exponential decay is independent of incident laser intensity (not shown). With each of the polymers the decay follows approximately first order kinetics on admission of oxygen.

It is recognised that the methylene blue triplet state is able to

* These experiments were performed when the BBC microcomputer for signal averaging and smoothing was not yet available. See also chapter 3.3.
Figure 3.17 Decay of transient optical density at 420 nm for an deoxygenated (●) and an aerated (■) solution of a complex of methylene blue with poly[d(A-T)]. P/D = 42. Dye concentration is 50 mM phosphate. Excitation as in figure 3.6. Decay times are given in table 3.6.
efficiently produce singlet oxygen by energy transfer to ground state $O_2$. Assuming that each triplet quenched yields a $^1O_2$ molecule, the yield from each of the dye/polynucleotide complexes can be calculated from measurements of the decay of the triplet state in argon purged and aerated solutions according to equation 3.9 [Bonneau et al., 1975]:

$$\Phi_{^1O_2} = \left(1 - \frac{\tau_{\text{air}}}{\tau_{\text{degas}}}\right) \times \frac{\Phi}{T}$$  \hspace{1cm} (3.9)

The yields so calculated are shown in table 3.6. It may be observed that the $^1O_2$ yield is predicted to be much lower for MB bound to the polynucleotides than for free dye and that it is particularly low for poly[d(G-C)]. Implicit in the calculation is the assumption that the reaction of ground state oxygen ($^3O_2$) is identical for free dye and bound dye.

### 3.2.3.2 Radical species

Figure 3.18 shows a typical trace of the decay of MB$^{2+*}$, measured at 520 nm after excitation with a 15 ns, 20 mJ, 308 nm laser pulse. The decay is first order and is a function of the stoichiometric dye concentration as is shown in figure 3.19. The quenching rate constant for this quenching is determined from this plot as $2.7 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$. The mechanism of this quenching is not clear.

The semi-oxidised radical MB$^{2+*}$ has been proposed as an active intermediate in the oxidation of amino acids [Knowles and Gurnani, 1972]. It is conceivable that it could also play such a role in the observed oxidation of nucleic acids (chapter 5). A decrease in the lifetime of the semi-oxidised species could indeed be observed on the addition of CT-DNA (Figure 3.20). The decays are all first order and the quenching rate constant obtained from this is $5.3 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ (expressed in mol DNA-phosphate groups). The species could thus
Figure 3.18 Representative decay traces of methylene blue semi-oxidized radical $\text{MB}^{2+}$, measured at 520 nm in deoxygenated 50 mM phosphate buffer with CT-DNA. Dye concentration $1.2 \times 10^{-5}$ M. (A) : P/D = 0, t = 203 $\mu$s; (B) : P/D = 27, t = 168 $\mu$s.
Figure 3.19 Quenching of MB$^{2+}$ by ground state dye MB$^+$. Quenching rate constant as determined from plot is 2.7x10$^7$ M$^{-1}$s$^{-1}$. 
Figure 3.20 Quenching of MB$^{2+}$ by CT-DNA. Plot shows observed decay times for semi-oxidized radical as a function of CT-DNA phosphate concentration. Quenching rate constant as determined from slope is $5.3\times10^6 \text{ M}^{-1}\text{s}^{-1}$ (per Mole phosphate).
possibly be involved in the oxidation of the polymer. It is not expected to be a major pathway for the photosensitized oxidation of DNA as the amount of MB\textsuperscript{2+} produced on excitation decreases rapidly on increasing DNA concentration. In fact, hardly any MB\textsuperscript{2+} could be detected in the MB\textsuperscript{+}/poly[d(G-C)] complex. It is not likely that its reaction with poly[d(G-C)] is so fast that it would not be detected in view of the relatively slow rate constant for its reaction with CT-DNA.

3.2.4 Discussion

The transient triplet-ground state difference spectra obtained for the free dye agree well with those reported previously in literature. Our experimental data appears to indicate that dye dimer is involved in the production of MB\textsuperscript{2+}, although the precise mechanism could not be determined.

The absolute triplet-triplet absorption spectrum for the free dye agrees reasonably well with a spectrum which is a mixture of published values of protonated and unprotonated dye T-T spectra [Wildes et al., 1977; Ohno et al., 1979]. No new transient absorption bands could be observed after addition of polynucleotides. The triplet-triplet spectra of the dye/polymer indicates that there is some interaction between the triplet state of the dye and the polymer. The absorption band around 520 nm has been reported for 3\textsuperscript{MB+} in the free dye [figure 2.4, Ohno et al., 1979] is absent in the polymer complexes figure 3.14). The magnitudes of the extinction coefficients of the triplet-triplet transitions are however fairly small. Relatively small experimental errors will thus have a significant effect on the final values of the triplet-triplet extinction coefficients.

The transient difference spectrum of MB\textsuperscript{+}/poly[d(A-T)] resembles that
of the dye in alkaline solution which is somewhat surprising. It might be expected that an equilibrium of the type:

\[
(\text{MB}^+ + \text{H}^+) \rightleftharpoons (\text{MBH}^{2+})
\]

should be displaced in favour of the divalent species when the dye is bound to the negatively charged polymer - the opposite to what is observed here. A possible explanation is that when bound to the polymer, the protonation of the dye is substantially slower than in aqueous solution. This was confirmed in flash photolysis experiments with a dye laser excitation source (section 3.3).

The triplet yield when methylene blue is bound to CT-DNA or poly[d(G-C)] is much less than that when it is bound to poly[d(A-T)] consistent with the quenching of the excited singlet state of the dye by guanine. In both cases the decays of the triplet state appear to follow first order kinetics, although multi-exponential decays can be obscured by the low signal/noise ratio due to the low triplet yield with these polynucleotides. The decay of the triplet state of the dye bound to poly[d(A-T)] is puzzling, with a clear double-exponential decay in an argon purged solution, which reverts back to an (approximate) single-exponential decay in aerated solutions. Similar double-exponential decays have also been reported by Berkoff et al. [1986]. A possible explanation could be that the dye involves species in two sites having different accessibility to the protonating species (either \(H_3O^+\) or \(H_2PO_4^-\)) but similar accessibility to oxygen molecules. This however appears unlikely as the short component of the triplet decay in the argon purged solution is faster than the (approximate mono-exponential) triplet lifetime in an aerated solution. Another explanation could be possible involvement of bound dimeric dye species to this polynucleotide. \([\text{MB}^+]_2\) absorbs substantially at 308 nm (Figure
3.1) and could be excited by the laser flash. Berkoff et al. [1986] also observed two-exponential decays after excitation at 604 nm i.e. the maximum of the dimer absorption spectrum. This matter was resolved using a dye-laser as excitation source (section 3.3).

Finally, it is observed that the estimated yield of $^{1}O_2$ is predicted to be much lower for MB$^+$ bound to the polynucleotides than for free dye and that it is particularly low for poly[d(G-C)]. As it is known that photosensitization causes damage preferentially at guanine residues [Friedman and Brown, 1978; This work, chapter 5], it is probable that it is either due to a direct singlet state reaction with guanine or to singlet oxygen formed either in the bulk solution or at A-T rich sites.
In the previous section the effects of nucleic acids on the singlet and triplet state properties of the dye methylene blue were reported. These experiments were carried out at a relatively high ionic strength (100 mM) and it became clear from other studies, notably the investigation of topoisomerase unwinding (see chapter 4), that the binding of methylene blue to nucleic acids is very sensitive to changes in ionic strength. The first objective of the experiments reported in this section was to determine the effect of ionic strength on the triplet properties of the dye in complex with polynucleotides. A second objective was to establish the effect of pH on the triplet parameters of the dye/polymer complex. In the previous section we proposed (without giving any supporting evidence) that protonation of the triplet state of MB⁺ is much slower when the dye is bound to a polynucleotide. Measurement of triplet state properties of dye/polymer complexes at different pH could confirm this. Also more extensive measurements of the oxygen quenching of the triplet states are reported as a function of solvent environment. The determination of these rates is important in assessing possible roles for reactive oxygen species such as ¹⁰₂ in photosensitized reactions which damage DNA. The oxygen quenching rates also yield information on the accessibility of the dye to oxygen. This provides information on the nature of the dye binding site and the binding mode.
Finally, the experiments presented in section 3.2 were performed with a XeCl excimer laser (308 nm) as the excitation source. During the course of the project a dye laser became available as excitation source for the laser flash apparatus and it was decided to compare the results of excitation at 650-660 nm (monomer absorption maximum) with those of excitation at 600 nm (dimer absorption maximum). Thus, selective excitation of monomeric and dimeric dye species is possible and can be compared with excitation in the violet where both are excited simultaneously. This enables a study of possible binding of dimeric dye species to the polynucleotides.

3.3.1. 650 nm excitation

3.3.1.1 Transient absorption

Figures 3.21-3.23 show transient absorption spectra obtained in argon saturated solutions for conditions of different ionic strength and pH, for respectively free MB⁺, poly[d(A-T)]/MB⁺ and poly[d(G-C)]/MB⁺. Figure 3.21 shows the transient spectra for free MB⁺. Bleaching can be observed with a maximum around 660-670 nm which is close to that expected for the dye monomer. The transient absorption maxima at 380 nm and 420 nm are similar to those observed upon excitation at 308 nm and can be attributed to absorption from respectively the protonated and unprotonated triplet state of the dye (see section 3.2). Small changes are noticeable between the spectra obtained at pH = 5.3 in the 6 mM and the 200 mM buffer which are consistent with a larger amount of \(^3\)MBH\(^{2+}\) being formed in the latter case. The transient spectra of the dye complex with poly[d(A-T)] are
Figure 3.21 Prompt transient absorption spectra of MB⁺ (2.2 \times 10^{-5} M) in a pH=5.3 and a pH=8.3 phosphate buffer. Ionic strengths 6 and 200 mM. Excitation wavelength 650 nm.
Figure 3.22 As Figure 3.21. MB⁺-poly[d(A-T)]; P/D=30.6.
Figure 3.23 As Figure 3.21. MB+ -poly[d(G-C)]; P/D=28.7. Buffer pH=8.3, ionic strengths 60 and 200 mM.
shown in figure 3.22. In the pH = 5.3 buffer the effects of protonation on the triplet state of the dye are very noticeable. At the lowest ionic strength very little dye is protonated when it is bound to the polynucleotide, whereas at an ionic strength of 200 mM the transient absorption bands associated with $^3\text{MBH}^{2+}$ (380 and 710-720 nm) are clearly observable. The transient bleaching of the complex exhibits a blue shift at high ionic strength. The transient spectra of dye with poly[d(G-C)] are shown in figure 3.23. Only the spectra obtained at a pH of 8.3 are shown, as at the other pH values the measured absorption values are extremely low, especially for the low ionic strength solutions. (The dye laser intensity is approximately a factor 10 smaller than the excimer laser output, resulting in extremely small transient optical densities).

The extent of protonation of the dye bound to poly[d(A-T)] was studied by determining the ratios of prompt transient absorption at 380 nm to that at 420 nm as a function of ionic strength and comparing them to those obtained for free dye. The results are given in figure 3.24 for the three pH values used. From the plots it may be seen that protonation is substantially inhibited when the dye is bound to poly[d(A-T)]. The slow protonation of the triplet state of the dye when bound to poly[d(A-T)] at an ionic strength of 6 mM (pH=5.3) is clearly demonstrated by the slow risetime of the transient absorption of $^3\text{MBH}^{2+}$ (monitored at 380 nm) compared to that of $^3\text{MB}^+$ (monitored at 420 nm) (Figure 3.25). At higher ionic strengths the risetimes at 380 and 420 nm are indistinguishable (not shown). The ratios in the MB$^+/\text{poly[d(A-T)]}$ complexes at low ionic strength are different from those at high ionic strength. At low ionic strength protonation of the dye is almost completely prevented. This suggests different modes of binding at low and high ionic strength. This conclusion is substantiated by results obtained from thermal denaturation studies.
Figure 3.24 Ratio of prompt transient absorbance of $^{3}\text{MBH}^{2+}$ (measured at 380 nm) and $^{3}\text{MB}^{+}$ (measured at 420 nm) as a function of the ionic strength of the solution. A: MB+ in buffer ($2.2\times10^{-5}$ M); B: MB+–poly[d(A-T)] (dye=$2.2\times10^{-5}$ M, P/D=30.6). a) pH=8.3, b) pH=6.9, c) pH=5.3.
Figure 3.25 Risetime of transient absorbance of MB⁺ (2.2 10⁻⁵ M) in a pH = 5.3, μ = 6 mM phosphate buffer. A: a) measured at 380 nm ("MBH⁺"); b) measured at 420 nm ("MB⁺"). B: measured at 380 nm, increased time-resolution compared to Fig. A-a Excitation wavelength 650 nm.
3.3.1.2 Kinetics

Tables 3.7-3.9 give the rates of triplet decay of the dye with and without polynucleotides measured as a function of pH and ionic strength in an argon saturated solution after excitation with a 10 ns, 650 nm laser pulse. Triplet decays measured at both 420 and 650 nm are similar. The decays are mono-exponential in all cases, in contrast with the results obtained with the excimer laser (section 3.2).

Oxygen quenching rate-constants ($k_Q$) were calculated from the measurement of the triplet decay rates in respectively argon saturated, aerated and oxygenated solutions using equation 3.8. The oxygen quenching rates so determined for free MB$^+$, MB$^+$/poly[d(A-T)] and MB$^+$/poly[d(G-C)] are shown in Tables 3.10-3.12 and figures 3.26-3.28 show the dependence of the oxygen quenching rates $k_Q$ on ionic strength and pH. No quenching rates could be determined at pH=5.3 in MB$^+$/poly[d(G-C)] due to the extremely low transient optical densities and fast triplet decays which are observed in this buffer. Figure 3.29 shows two representative plots of individual decay rates as a function of oxygen concentration. Other plots are similar.

It can be seen from figure 3.26 that the oxygen quenching rates of the triplet state of the free dye are strongly dependent on the ionic strength of the buffer solution. Also, the highest quenching rates are observed in alkaline solution. A similar dependence of the oxygen quenching of triplet states on the pH of the buffer is observed for the xanthene photosensitizer Rose Bengal [Lee and Rogers, 1987]. The dependence of $k_Q$ on ionic strength is less in the pH=8.3 buffer, than in either neutral or in acid buffer.

The quenching rates for the triplet state of MB$^+$ are similar for the two polynucleotides at low ionic strength, with the values for
<table>
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<th>6.9</th>
<th>8.3</th>
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<tr>
<td>200</td>
<td>2.6</td>
<td>1.6</td>
<td>4.8</td>
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</tbody>
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Table 3.7. Triplet decay rates of \(\text{MB}^+\) in buffer (in [s\(^{-1}\)]) as a function of ionic strength and pH. (All values ± 10%) 

<table>
<thead>
<tr>
<th>(a_1) (mM)</th>
<th>5.3</th>
<th>6.9</th>
<th>8.3</th>
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Table 3.8. Triplet decay rates of \(\text{MB}^+\)/poly[d(A-T)] (in [s\(^{-1}\)]) as a function of ionic strength and pH. (All values ± 10%) 

<table>
<thead>
<tr>
<th>(a_1) (mM)</th>
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<th>8.3</th>
</tr>
</thead>
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Table 3.9. Triplet decay rates of \(\text{MB}^+\)/poly[d(G-C)] (in [s\(^{-1}\)]) as a function of ionic strength and pH. (All values ± 10%) n.m.: not measurable.

N.B. Table 3.7 - 3.9. Rates determined in deoxygenated solutions. Excitation with dye laser: wavelength 650 nm.
<table>
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<tr>
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**Table 3.10.** Oxygen quenching rate-constants of MB$^+$ in buffer. (in [M·s$^{-1}$]) as a function of ionic strength and pH. (All values ± 10%) n.m.: not measured.

<table>
<thead>
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<th>pH 6.9</th>
<th>8.3</th>
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<tr>
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<td>1.8</td>
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**Table 3.11.** Oxygen quenching rate-constants of MB$^+$/poly[d(A-T)]. (in [M·s$^{-1}$]) as a function of ionic strength and pH. (All values ± 10%)

<table>
<thead>
<tr>
<th>$A_1$ (mM)</th>
<th>5.3</th>
<th>pH 6.9</th>
<th>8.3</th>
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<tr>
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<td>1.9 $10^8$</td>
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<td>n.m.</td>
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<tr>
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<td>n.m.</td>
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</table>

**Table 3.12.** Oxygen quenching rate-constants of MB$^+$/poly[d(G-C)]. (in [M·s$^{-1}$]) as a function of ionic strength and pH. (All values ± 10%) n.m.: not measurable.
Figure 3.26 Oxygen quenching rates for MB⁺ as a function of ionic strength calculated using equation 3.8. a) pH = 5.3; b) pH = 6.9; c) pH = 8.3 Note different scale for pH=8.3.
Figure 3.27 As Figure 3.26. MB⁺-poly[d(A-T)]; P/D=30.6.
Figure 3.26 As Figure 3.26. $\text{MB}^+\text{poly[d(G-C)]}; P/D=28.7.$

a: pH = 6.9; b: pH = 8.3
Figure 3.29 Representative plots of triplet decay-rates as a function of dissolved oxygen concentration. Dye $2.2 \times 10^{-5}$ M. P/D for complex with poly[d(G-C)] = 28.7. Oxygen concentrations from Murov [1973].
poly[d(G-C)] somewhat lower than those for poly[d(A-T)]. At higher ionic strengths the \( k_q \) for MB\(^+\)/poly[d(G-C)] increases rapidly, compared to the values for MB\(^+\)/poly[d(A-T)] which do not change much. The oxygen quenching rates for MB\(^+\)/poly[d(A-T)] are substantially lower at all ionic strengths and pH-values than those for the free dye. The quenching rates for MB\(^+\)/poly[d(G-C)] are very much lower at low ionic strength but increase rapidly on increasing ionic strength and attain values similar to those for free MB\(^+\) at ionic strengths larger than 100 mM.
3.3.2. 600 nm excitation

3.3.2.1 transient spectra

The methylene blue dimer has its maximum absorption spectrum at 600 nm and selective excitation of the dimer might be achieved with this wavelength. The results obtained here also pertain to the picosecond flash photolysis results reported in section 3.4. Figure 3.30 shows prompt transient absorption spectra of an argon saturated $4.3 \times 10^{-5}$ M dye solution in a pH=6.9 phosphate buffer (ionic strength 100 mM) after excitation at 580 nm and 650 nm. The spectra show very similar transient absorption in the main triplet regions, as indicated by the two bands at 380 and 420 nm and the wavelength region beyond 700 nm. The ground state depletion region in the two spectra is however different. The spectrum obtained after excitation at 580 nm shows a large bleaching centered around 600 nm, with a small depletion band visible at 650 nm. In stark contrast, the spectrum obtained after excitation with a 660 nm pulse shows a maximum bleaching band in the wavelength region around 650-660 nm where ground state absorption is maximal and a much smaller depletion at 600 nm and is comparable to the spectrum obtained with excimer laser excitation (Fig. 3.6). (N.B. Due to the large ground state absorption of methylene blue at 660 nm the monochromator slits had to be opened wide. This precluded a more accurate measurement of the transient spectrum around 660 nm due to scattered laser light.)

Figure 3.31 shows the prompt transient absorption spectra of a MB$^+$/DNA complex ($[\text{MB}^+] = 2.2 \times 10^{-5}$ M; P/D=89) in pH=6.9 phosphate buffers of different ionic strength, obtained after excitation with a 600 nm
Figure 3.30 Prompt transient absorption spectrum of MB$^+$ (4.2 $10^{-5}$ M) in a pH-6.9, 100 mM, phosphate buffer. Excitation wavelength 600 nm and 650 nm.
Figure 3.31 Prompt transient absorption spectrum of MB⁺ (2.2×10⁻⁵ M) with CT-DNA (P/D-89) in a pH-6.9 phosphate buffer. Ionic strength of the solutions: 20 mM (a), 200 mM (b), 1.2 M (c). Excitation wavelength 600 nm.
laser pulse. At low values of the ionic strength the observed spectra are similar to those reported previously (Fig. 3.10) with the maximum of the depletion region situated around 660 nm. At high ionic strengths, where most of the dye will be displaced from the polymer (see chapter 4), the spectrum is similar to that obtained for free dye after excitation at 600 nm (Fig. 3.30). The effect of addition of salt on the transient absorption of the dye triplet is similar to that observed with 650 nm excitation (see section 3.3.1) i.e. an increase in the absorption of the band associated with $3\text{MBH}^{2+}$ (at 380 nm), compared to the band at 420 nm ($3\text{MB}^+$).

3.3.2.2 Kinetics

Excitation of dye/polymer complexes at a wavelength of 650 nm yields transient decay kinetics which are mono-exponential. However, a multi-exponential decay can be observed after excitation of a $2.2\times10^{-5}$ M dye solution (without polymer) at 580 nm. The decay of the transient depletion, measured at 620 nm, is mainly mono-exponential, with a small initial fast decay. This fast decay is only visible after averaging and smoothing the data and is too small to be detectable in the decay of the triplet absorption (measured at 420 nm) which appears thus to be single-exponential. In contrast, the transient decays measured after excitation at 650 nm are, however, mono-exponential. Representative decays of these transients of $\text{MB}^+$ in buffer are shown in figure 3.32.

Figure 3.33 shows the decays of the triplet state of a $\text{MB}^+/\text{poly[d(A-T)]}$ sample (P/D=30.5), measured at 420 nm in an argon saturated 10 mM, pH=6.9 phosphate buffer on excitation at respectively 600 and 650 nm. Excitation at 600 nm clearly leads to a double exponential decay with lifetimes of respectively 4.7 and 19.1 $\mu$s, in good agreement with the results obtained after excitation at 308 nm (section 3.2). The
Figure 3.32 Decay of transient absorbance of MB$^+$ \( (2.2 \times 10^{-5} \text{ M}) \) in an argon saturated, pH=6.9, 100 mM phosphate buffer. A: measured at 620 nm, excitation at 600 nm; B: measured at 600 nm, excitation at 650 nm.
Figure 3.33 Decay of transient absorbance of MB+-poly[d(A-T)] (dye=2.2 $10^{-5}$ M, P/D=30.6) in an argon saturated, pH=6.9, 100 mM phosphate buffer, measured at 420 nm after excitation with 600 nm (A) and 650 nm (B). See also figure 3.33A.
Figure 3.33A Decay analysis of trace of MB⁺/poly[d(A-T)], excited by 595 nm and measured at 420 nm (also shown in figure 3.33) with computer program ABSDUB. Double exponential decay yields the best fit.
analysis of the data was performed using Marquardt least-square analysis (program ABSDUB). In contrast to this, the decay after excitation at 650 nm is mono-exponential with a decay of 18.3 μs. In an aerated solution the decays mono-exponential curve fitting yields best results for both excitation wavelengths (Figure 3.34).

Further transient decay kinetics were measured at 580 nm and 600 nm with excitation at respectively 600 and 650 nm. The aim was to investigate the possible role of dye dimer in the excitation wavelength dependent decay characteristics. Special emphasis was given to the grow in of the ground state depletion at these wavelengths as the dye dimer has its maximum absorption there. It was hoped that this could yield information on the dimer association or dissociation after absorption of a photon. Figure 3.35A shows grow-in of the transient depletion for a 2.2x10^{-5} M dye solution in 10 mM buffer after excitation at 600 and 650 nm. Within the accuracy of these experiments it can be seen that the grow-in of the depletion region is instantaneous after absorption of a photon for both excitation wavelengths. Thus absorption of either 600 or 650 nm light has no noticeable effect on the dye dimer. A similar experiment performed on a MB^{+}/poly[d(A-T)] (P/D=30.5) solution under identical conditions yielded the results shown in figure 3.35B. Again no noticeable difference is observable for the transients obtained with the two excitation wavelengths. The conclusion from this must be that the multi-exponential decays are not attributable to dye dimer dissociation upon excitation. These results corroborate those of Danziger et al. [1967]. Thus irradiation of the dye dimer with a laser pulse excites the species as a single entity and does not dissociate the dye aggregate.
Figure 3.34 As Figure 3.33. Solutions are aerated.
Figure 3.35 A: Grow-in of transient absorbance of MB\(^+\) \((2.2 \times 10^{-5} \text{ M})\) in an argon saturated, pH=6.9, 100 mM phosphate buffer, measured at 580 nm with 600 nm excitation (a) and at 600 nm with 650 nm excitation (b). B: As A; MB\(^+\)-poly[d(A-T)] \((\text{dye } 2.2 \times 10^{-5} \text{ M}, \text{ P/D}=30.6)\).
3.3.3 Discussion

The observations obtained with dye laser excitation pertain to our own results reported previously on transient spectroscopy with an excimer laser as excitation source (section 3.2) and to MB+/DNA triplet studies reported by Berkoff et al. [1986]. In section 3.2 it was reported that the prompt transient spectrum of the triplet state of MB+ in complex with poly[d(A-T)] is very similar to that of the dye in an alkaline buffer. It was not clear whether this effect was attributable to a possible alkaline environment of the polynucleotide or to a reduced accessibility of the dye to the protonating species. The observations reported here clearly show that the dye is not very accessible to the protonating species when bound to the polynucleotide. Both the transient absorption spectra (Fig. 3.21-3.23) and the figures illustrating the ratio of transient absorption of $^{3}{\text{MBH}}^{2+}$ and $^{3}{\text{MB}}^{+}$ (figure 3.24) indicate that the dye is not protonated as readily when bound to poly[d(A-T)]. The slow rate of protonation of the triplet state of the dye when bound to poly[d(A-T)], compared to that of free dye, is illustrated in figure 3.25. The ratio of the transient optical densities of the protonated and unprotonated triplet species of the free dye increases with increasing ionic strength. This suggests that the predominant protonating species is $\text{H}_2\text{PO}_4^-$ from the buffer (and not $\text{H}_3\text{O}^+$). Limited access of $\text{H}_2\text{PO}_4^-$ to the electronegative polynucleotide poly[d(A-T)] can then possibly explain the slow protonation of the dye when bound to this polymer.

The binding of the dye to poly[d(A-T)] at an ionic strength of 20 mM
or less is most likely intercalative. Evidence for this comes from thermal denaturation studies performed for this polymer (chapter 4). At higher ionic strengths other modes of binding will have to be considered. The binding of the dye to poly[d(A-T)] is not predominantly electrostatic to the electronegative phosphate groups of the biopolymers as the ratio of $^{3}\text{MBH}^{2+}/^{3}\text{MB}^{+}$ for the $\text{MB}^{+}$/poly[d(A-T)] complex is remarkably invariant to changing the ionic strength of the solution from 20 mM to 200 mM. One possible mode could be hydrogen-bonding in one of the grooves of the polynucleotide. The apparent selectivity of this binding for poly[d(A-T)] might reflect the specific sequence of hydrogen-bond donors and acceptors in this polymer (see figure 1.3). Similar evidence for a strong binding mode at high ionic strength also comes from our studies on the oxygen quenching rates (see below).

The decay kinetics of the triplet state of the dye were single exponential after excitation at 650 nm. Changes in ionic strength, pH and on whether the dye was free or not, did not change the first order nature of the decay. This is in contrast with the observations reported in section 3.2 with excitation at 308 nm and with the observations of Berkoff et al. [1986] who excited the dye at 604 nm. Single exponential decay of the triplet state of a number of different dye-DNA complexes have been reported previously. Examples of first order kinetics are measurements of the triplet decay of the intercalators acridine orange and proflavine [Geacintov et al., 1981], benzopyrene [Geacintov et al., 1976] and furocoumarines [Beaumont et al., 1980] in complex with DNA. The binding of a dye to DNA is an equilibrium process and first order decay of the triplet state of ligands intercalated in DNA has been attributed to a rapid equilibration process between free and bound dye [Geacintov et al., 1981]. A similar process could be involved in the binding of methylene blue.
The decay rates of the triplet state of MB⁺ are substantially decreased on binding to both nucleotides. A possible explanation is that the helical environment of both poly[d(A-T)] and poly[d(G-C)] limits access of adventitious quenchers to the dye. More likely is a decrease of non-radiative decay processes due to the rigid polymer environment. Increasing the ionic strength will in general decrease the binding of intercalated dye molecules [Gilbert and Claverie, 1968; Record et al., 1976]. In agreement with this it is observed that the decay rates of the MB⁺ triplet in complex with polynucleotides increase with the ionic strength of the solution as the binding equilibrium shifts towards non-intercalated or free dye.

Measurement of the oxygen quenching rates of the dye triplet in the two polynucleotides as a function of ionic strength and pH clearly illustrates the decreased accessibility of the dye to molecular oxygen. Interestingly, the dependence of the $k_q$ on ionic strength illustrates the qualitatively different modes of the binding of methylene blue to poly[d(G-C)] and poly[d(A-T)]. At the lowest ionic strength, values of $k_q$ are similar for both polynucleotides and are in reasonable agreement with the values reported by Berkoff et al. [1986]. Increasing the ionic strength has very little -or no- effect on the values of $k_q$ determined for poly[d(A-T)] but sharply increases those for poly[d(G-C)] at all pH's measured.

Berkoff et al. [1986] report oxygen quenching rates which are of the same order of magnitude as those determined in this study. These authors use the values of the oxygen quenching rates to draw conclusions about the flexibility of the polynucleotides poly[d(A-T)] and poly[d(G-C)]. They concluded on the basis of this hypothesis that the interior of the polymer poly[d(A-T)] is much more accessible to oxygen than that of poly[d(G-C)]. The conclusion from Berkoff et al. is that poly[d(A-T)] is more flexible than poly[d(G-C)]. The results
presented in this work indicate however that such an interpretation of the oxygen quenching data is not without problems due to the dependence of the binding of the dye on the ionic strength. It appears more likely that the rate of oxygen quenching reflects the mode of binding of the dye and not the accessibility of oxygen to the interior of the polynucleotide.

The tentative conclusion which is drawn from these observations is that at a low ionic strength, binding of the dye to both polynucleotides is very similar, and most probable intercalative in nature [Our results chapter 4; Norden and Tjerneld, 1982; Wang et al., 1982]. In separate experiments (chapter 4) it has been shown from simple Scatchard analysis that binding of the dye to poly[d(G-C)] yields only a single binding constant (i.e. only one mode of binding is possible). Increasing the ionic strength leads in this case to a shift in the binding equilibrium towards free dye. Dye which is bound is still intercalated. The decrease in association constant thus leads to an increase in quenching efficiency and at the highest ionic strength (200 mM) the oxygen quenching rates of the triplet of MB⁺ with poly[d(G-C)] approach those of free dye.

Increasing the ionic strength of MB⁺-poly[d(A-T)] solutions from 6 mM to ca 20 mM results in a sharp increase in the protonation rate as observed from the ratio of concentrations of $[^3]MBH^{2+}$ and $[^3]MB^+$. This change occurs at a similar ionic strength where Norden and Tjerneld [1982] observe a change in the circular dichroism spectrum of the dye bound to calf thymus DNA. They attributed this to a rotation of the dye in its intercalated pocket. We have however shown on the basis of topoisomerase unwinding experiments [chapter 4] that intercalation at the higher ionic strengths is not very likely and an alternative explanation needs to be found. A possible hypothesis which accounts for the observations reported here and for those reported by Norden and
Tjerneld is where an increase in ionic strength causes the dye to change from an intercalated position to one where the dye is bound in either the major or minor groove of the polymer. Electrostatic attraction of the dye to the nitrogen N-3 of adenine and/or oxygen O-2 of thymine in the minor groove of poly[d(A-T)] has been suggested as a possible mode of binding [Saenger, 1984]. However, increasing the ionic strength of the solution to 200 mM has only a marginal effect on both oxygen quenching and protonation and this appears to rule out electrostatic binding to any large extent. In the case of proflavine, an intercalator with a structure not dissimilar from MB⁺, hydrogen bonding of dye to polynucleotide has been proposed [Georghiou, 1975]. It is possible that H-binding occurs in the interaction of MB⁺ with poly[d(A-T)] but in the absence of any experimental evidence this remains purely speculative. Concluding, the binding of methylene blue to poly[d(A-T)] is most likely intercalative at ionic strengths below 20 mM. At higher ionic strengths the mode of binding changes to one which is probably not intercalative nor electrostatic but still offers considerable protection from oxygen and protonating species. Binding of the dye to poly[d(G-C)] is intercalative. Increasing the ionic strength will decrease the association constant of the dye to this polynucleotide and thus increase the amount of free dye in solution. This accounts for the increase in the rate-constant of oxygen quenching.

B. Dimer binding

Irradiation of free dye and dye-polymer complexes with 600 nm light allows for selective excitation of the dye dimer, if present. Our results with relative high dye concentrations (4.3x10⁻⁵ M; Figure 3.30)
show that it is indeed possible to selectively excite the dimer band as shown by the transient depletion which corresponds to the ground state absorption band of the dimer. No such dimer depletion is observed in a complex of MB\(^+\) with CT-DNA at low ionic strength (Figure 3.31). This is consistent with monomeric binding of the dye (intercalation). Increasing the ionic strength will a) release dye from the polymer and b) decrease the electrostatic repulsion between the cationic dye molecules. Both will result in an increase in the concentration of dimer in solution which can be observed as an increase in the transient depletion region around 600 nm.

The kinetics of the transient absorption decays of both free dye and dye-poly[d(A-T)] complexes observed here after 600 nm excitation in an argon saturated solution are double exponential, in contrast to the decays of the identical solutions after 650 nm excitation. (Note. These experiments could not be carried out with poly[d(G-C)] due to the very low triplet yield of MB\(^+\) with this polynucleotide). Double exponential decays have been reported previously for MB\(^+\)-polynucleotide samples (section 3.2) and in the light of the observations of the transient absorption spectra, the fast component of the decay is attributed to that of dimeric dye species. It is not clear whether this decay is attributable to the dimer triplet or to another dimer excited state such as an intra-dimeric charge transfer species. The lifetime of the dimer triplet has been previously reported as < 2 \(\mu\)sec [Danziger et al., 1967]. If we assume that the observed decay is that of the dimer triplet then the observed decrease in decay rate could be due to protection from extraneous quenchers. This is analogous to what is observed for the decay of the triplet state of the monomer. In an aerated solution the fast component of the decay cannot be observed. This is similar to the observations of Danziger et al. [1967] who could not detect any dimeric triplet species in an aerated solution.
The reason for this is not quite clear. It is unlikely to be due to a rapid quenching of dimer triplet by oxygen although Nilson et al. [1972] report a somewhat higher oxygen quenching rate-constant for the dimer of methylene blue compared to monomer.

Another possible cause for the double exponential decays might be dissociation of the dimer. Experiments at the highest time resolution of the detection system demonstrate that the kinetics observed for the grow-in of the dye depletion region are similar, whether excited by 600 nm or by 650 nm. This confirms the conclusion of Danziger et al. that the dye dimer is excited as a separate species which remains so during its excited state. No grow-in of dye monomer absorption could be observed which corresponds to dissociation of the dimer. Possible dissociation of the dimer can thus also not explain the difference observed for aerated and degassed solutions.

A third hypothesis to explain the double exponential decay is to attribute the fast and slow decay rates to those of the triplet state of free and bound dye respectively. However, an aerated solution is then still expected to exhibit a double exponential decay upon excitation. This is not what is observed here and thus this hypothesis is rejected.

The above arguments lead us to believe that dimeric methylene blue can bind to poly[d(A-T)], to calf thymus DNA and possibly to poly[d(G-C)]. This dimeric species is the origin of the double exponential decay which we and others [Berkoff et al., 1986] have observed in laser flash photolysis experiments. Absence of an increased aggregation band in the UV/VIS absorption spectra of MB⁺/polynucleotide complexes is explained by an absence of cooperativity in the binding process.
3.4 PICOSECOND TRANSIENT ABSORPTION SPECTROSCOPY
OF METHYLENE BLUE/NUCLEIC ACID COMPLEXES

3.4.1 Introduction

In section 3.2 it was shown that the quantum yield of fluorescence of methylene blue bound to DNA is strongly quenched when the dye is bound near G-C base-pairs. Secondly, it was shown that the combination of dye and visible light is able to induce strand breaks at guanine nucleotides in the presence and absence of oxygen (chapter 5). Both the base-pair specific fluorescence quenching and the formation of single strand breaks can be explained on the basis of electron transfer from the guanine nucleotide to the excited state of the dye [Lober and Kittler, 1978, Kittler et al., 1980]. Electron transfer was proposed on the basis of calculations of the reduction potential of the dye in its excited singlet state and the oxidation potentials of the nucleotides (Table 3.4). Selective oxidation of guanine (and to a lesser extent adenine) by the excited state of the dye methylene blue is predicted but no evidence for such excited state electron transfer in dye-nucleic acid complexes has ever been presented. No evidence could be found for any transient species resulting from a redox reaction in the experiments carried out with nanosecond time resolution (Sections 3.2 and 3.3).

It can be expected that electron transfer between the intercalated electron acceptor (excited singlet state of the dye) and the donor nucleotide will be extremely fast due to the close distance between the two. In this section picosecond flash photolysis experiments on
methylene blue/DNA complexes are reported. The results indicate a very fast base-specific electron transfer between guanine and dye and to a lesser extent between adenine and dye. These results are explained on the basis of standard electron transfer theory and appear to confirm the existence of an inverted "Marcus" region. This is the first time that such results have been obtained in a dye/DNA system.

The experiments were performed with the assistance of Dr. Godfrey Beddard in his laboratory in the University of Manchester. His help and hospitality is gratefully acknowledged.

3.4.2 Picosecond laser system

Time-resolved transient spectra and decay measurements were performed using amplified pulses from a synchronously pumped, mode locked Rhodamine 6G dye laser excited by a mode locked argon ion laser (Coherent Innova 18/468). The 100 ps argon ion pulses produces 1.5 ps (FWHM) dye laser pulses, which are amplified in a four stage amplifier chain. The dye amplifier chain is pumped by the output of a Q-switched, frequency-doubled Nd:Yag laser (Spectron Laser Ltd.). Final output from the laser system is 750 µJ in a pulse of 1.5 ps (FWHM) at 580 nm. The pulse-width of the dye laser pulse is measured with a scanning autocorrelator, using second harmonic generation detection. A schematic diagram of the picosecond spectrometer is given in figure 3.36.

The amplified dye laser pulses are split into a "probe" and "pump" beam. The former is, after travelling through a variable delay, focussed into a 2 cm cell filled with a 1:1 H₂O/D₂O mixture to generate a picosecond continuum. Pump beam and probe continuum pulses are focussed to a diameter of ca 1 mm into the sample cell, passing colinearly through it. Subsequently, they fall on the entrance slit of
Schematic of the picosecond laser spectrometer. amp1 – amp4, dye laser amplifiers pumped by 532 nm Nd: YAG laser; s.f., spatial filter; sat. abs, Malachite green saturable absorbers; f, filters; c.g., continuum generation cell; Ar⁺ ion, argon ion laser; r.f., mode-locker r.f. source; sync., synchronization circuit between the mode-locked and Nd: YAG lasers; spectr, 1/2 metre spectrograph; oma, optical multichannel analyser; comp, microcomputer

Figure 3.36 Schematic of picosecond spectrometer as used in University of Manchester (from Irvine et al., 1985).
a spectrometer (Spex 1870). The detector was either an optical multichannel analyser (EG&G 1245,1216) or a pair of photodiodes. Part of the continuum beam was used as reference signal which was passed to a discriminator to eliminate low-energy pulses. The same laser system was also used to measure time resolved fluorescence emission with a Hadland Photonics Imacon III streak camera as detection system, attached to the spectrometer. Both transient absorption and emission systems were under computer control and extensive data averaging was employed. Extensive discussion on the construction of the spectrometer can be found in Irvine et al. [1985] and Irvine [1986].

3.4.3 Results

Fluorescence emission was measured with the streak camera. Samples were buffered in the standard 50 mM phosphate buffer. All samples contained 2.2x10⁻⁵ M dye. Representative decay traces are shown in figure 3.37A-C and an overview of the decay kinetics obtained from the fluorescence emission is given in table 3.13. The fluorescence lifetime measured for the free dye is ca. 400 ps., is single-exponential and in good agreement with that reported earlier [i.e. 365 ps; Ohno and Lichtin, 1980b]. In contrast, all lifetimes measured for dye/polynucleotide complexes are double-exponential. These initial measurements were performed without any "magic-angle" polarizer in the optical system of the detector and the decay kinetics are complicated by kinetics due to rotational reorientation. It was not possible to perform additional measurements due to failure of the argon-ion laser. The emission measurements show that the fluorescence decay of methylene blue in complex with polynucleotides with G-C basepairs contains a component which is considerably shorter than either the MB⁺/poly[d(A-T)] or the free dye emission.
Figure 3.37
Figure 3.37 Picosecond fluorescence decay traces, obtained with laser system from Fig. 3.36, with Hadland Photonics Imacon III streak camera. Dye concentration $2.2 \times 10^{-5}$ M. Buffer 50 mM phosphate, pH = 6.9. A: free dye; B: MB$^+$/poly[d(A-T)], P/D = 34; C: MB$^+$/poly[d(G-C)], P/D = 40. Lifetime values in table 3.13.
Table 3.13 Excited state lifetimes measured either via fluorescence emission or via transient absorption. Phosphate/dye ratio and dye concentration (in $10^{-5}$ M) are given in brackets. Buffer concentration 50 mM phosphate in the fluorescence experiment and 10 mM in transient absorption. Values with * are used in determination of rate constants.

<table>
<thead>
<tr>
<th>complex</th>
<th>fluorescence (λ &gt; 600 nm)</th>
<th>transient absorption 430 nm</th>
<th>transient absorption 670 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB$^+$</td>
<td>409</td>
<td>[P/D=0; 2.2]</td>
<td>313</td>
</tr>
<tr>
<td>MB$^+$/polyGC</td>
<td>53 &amp; 432</td>
<td>[P/D=40; 2.2]</td>
<td>3.8 &amp; 148.6*</td>
</tr>
<tr>
<td>MB$^+$/polyAT</td>
<td>261 &amp; 780</td>
<td>[P/D=34; 2.2]</td>
<td>332.8</td>
</tr>
</tbody>
</table>

Transient absorption decays were measured in the 10 mM phosphate buffer at 430 nm, where semi-reduced dye (MB$^+$) absorbs, as well as in the ground state depletion region at 670 nm. Figure 3.38 shows typical profiles of the decay of transient absorption measured at 430 nm for complexes of the dye with the synthetic polynucleotides poly[d(G-C)] and poly[d(A-T)]. Figure 3.39 shows similar traces for the decay of the transient depletion, measured at 670 nm. It was difficult to determine any long-lived decay kinetics from these data and subsequent more transients were collected at longer time-delays. These are shown in the figures 3.40A-C. In the case of free dye attention was paid to the transient depletion region as this could yield information on possible excited state behaviour of the dye dimer. Table 3.13 shows the decay-kinetics obtained from the transient absorption traces.

The dye concentration was increased to $6.6x10^{-5}$ M in these experiments, compared to $2.2x10^{-5}$ M in the fluorescence measurements. This was necessary to achieve a reasonable signal to noise ratio.
Figure 3.38
Figure 3.38 Transient absorption decays, measured at 430 nm. Dye concentration $6.6 \times 10^{-6}$ M, buffer = 5 mM phosphate, pH = 6.9. A: free dye; B: MB$^+$/poly[d(A-T)], P/D = 25; C: MB$^+$/poly[d(G-C)] P/D = 12. Excitation wavelength 580 nm.
Figure 3.39 Transient depletion decays, measured at 670 nm. As figure 3.38.
Figure 3.40
Figure 3.40

**WILL6:MBGC/433/AVA**

**WILL6:MBGC/670/AV**

**Δ O.D.**

\[(x \times 10^3)\]

**Residual**

-0.366

-0.170

430 nm

670 nm

(ps)

-0.362

0.237

89

100

20 40 60 80
Figure 3.40A-C Transient decays measured at different time scales. As figure 3.38. Wavelengths are given in each figure.
Unfortunately, this will also result in an increased concentration of dimer in solution. However, it is not believed that the presence of dimeric molecules poses a major problem even though the excitation wavelength (580 nm) implies absorption in the main absorption band of the dimer (Figure 3.1). The transient spectrum recorded 10 ps after the flash shows clearly the dimer absorption band centered at 600 nm (Figure 3.41A). No dimer band could be observed in a complex of dye and CT-DNA and this appears to indicate that binding decreases the amount of dimer in solution (Fig. 3.41B). It is somewhat puzzling that this is in contrast to what has been observed for the results obtained with nanosecond time-resolution (chapter 3.3).

The long lived decay time of 367 ps for free dye is in reasonable agreement with the value obtained from the fluorescence measurements. Free dye in solution does not exhibit a long-lived transient depletion as could be expected if the triplet state of the dye was populated. Furthermore, the depletion, measured at 670 nm, does not follow the exciting laser pulse but grows in with a risetime of ca. 5 ps (Fig. 3.40A). A long-lived transient species, attributable to the triplet state, can be observed in the MB\(^+\)/poly[d(A-T)] sample (Fig. 3.40B). The decay time of ca. 286 ps, measured for the initial transient absorption is in reasonable agreement with the lifetime measured with the streak camera.

The decay of the MB\(^+\)/poly[d(G-C)] complex is qualitatively completely different (Fig. 3.40C). Both transient absorption and transient depletion kinetics are characterized by a very fast initial decay, followed by a slower decay which is however faster than the lifetimes measured in the other complexes.
Figure 3.41 Transient spectra observed 10 ps after exciting (1 ps) laser pulse. Dye concentration 2.2x10^{-3} M. A: free dye, B: MB/CT-DNA, P/D = 100. Note in 3.41A the absorption band in the dimer region (600 nm).
The value of the fluorescence lifetime is similar to the value found for the decay of the transient depletion measured at 670 nm and to literature values [Ohno and Lichtin, 1980b]. This value is thus attributed to decay of the excited $S_1$-state to the ground state $S_0$.

Interestingly, no long-lived transient attributable to the triplet species can be observed in the depletion region. A simple explanation for this is the following. It can be seen (Figure 3.6) that the region around 670 nm is reasonably close to an isosbestic region where the extinction coefficients of the triplet state and the dye monomer are equal. Therefore, no large transient absorption is to be expected although there is population of the triplet state. In agreement with this is the observation of substantial triplet-state population in the MB$^+$-poly[d(A-T)] complex. The ground state absorption spectrum of this is red shifted compared to that of free dye and there is now a substantial difference between the extinction coefficients of the triplet and ground state of the dye, enabling detection of the triplet state population at this wavelength.

The transient depletion kinetics of the free dye exhibit a fast grow-in with a rise-time of ca. 5 ps. A possible explanation for this could possibly be an as yet unidentified excited state absorption of the dimer which decays with a lifetime of 5 ps. The excitation wavelength of 580 nm will selectively excite dye dimers of which there is a relative large concentration. (The percentage of dimer aggregates in this solution is calculated as 23%). The lifetime of 5 ps is in reasonable agreement with the values of ca. 10 ps reported by Sundstrom and Gillbro [1986] for the fluorescence decay of the methylene blue.
dimer. This interpretation has to be regarded as somewhat speculative and further experiments to determine the role of dimer in the ultrafast decay kinetics of methylene blue are required.

The lifetimes of MB⁺-polynucleotide complexes are different from the free dye values. It is believed that this can be explained on the basis of electron-transfer from the nucleotide bases adenine and guanine to the excited state singlet state of the dye. This will be discussed further below.

Electron-transfer between non-equilibrium states such as between the excited state of a molecule and the ground state of another can be described on the basis of of the Marcus-theory for non-adiabatic electron transfer [Marcus, 1956; 1964; 1980]. In this theory the rate constant for electron transfer depends on the respective values of the free energy ΔG of the reaction and is given by equations 3.10 (Fermi's Golden Rule) [Barltrop and Coyle, 1975] and 3.11 [Marcus and Siders, 1982]:

\[
\frac{k_{ET}}{\hbar} = \frac{2\pi V^2}{\hbar} \times (F.C.)
\]

\[
(F.C.) = (\frac{1}{4\lambda kT}) \exp[-(\Delta G + \lambda)^2/(4\lambda kT)]
\]

Equation 3.10 is analogous to that for radiationless decay between electronically excited states (Siebrand's law). V is the matrix element for the exchange interaction between the electronic states of the perturbed reactant and product and (F.C.) is the Franck Condon weighed density of states. The latter is a factor which expresses the vibrational overlap of the wavefunctions of initial and final state. The Franck-Condon factor has been determined by Marcus and Siders [1982] as equation 3.11, where λ is the total reorientation energy (the sum of solvent and molecular contributions), k the Boltzmann constant and T the absolute temperature. ΔG is the free energy.
calculated from equation 3.12.

The equations 3.10 - 3.11 predict a quadratic behaviour of the logarithm of $k_{ET}$ with $\Delta G$, with a decreasing rate constant as $\Delta G$ becomes more exoergic. This is somewhat surprising and the physical background is the decrease in the overlap of the vibrational wavefunctions of the initial and final state as the energy difference between them increases. The results obtained from the transient absorption experiments indicate that the Marcus-theory appears to give an adequate explanation for the observed decay-kinetics of methylene blue in complex with polynucleotides.

The transient absorption of MB$^+$ with poly[d(G-C)] is characterised by a very fast initial decay of $4 \pm 0.5$ ps, followed by a slower decay of $147 \pm 2$ ps. In view of observations made by Irvine et al. [1986] for electron transfer between covalently linked quinone and porphyrin species, it is assumed that the transient decays observed here can be explained on the basis of electron transfer from guanine to the excited state of the dye. Figure 3.42 shows a schematic diagram of the energy levels and electron transfer pathways which are believed to be involved here.

Assuming this hypothesis, then the rate of electron-transfer from nucleotide to excited dye is ca. $2.5 \times 10^{11}$ s$^{-1}$ which is two orders of magnitude larger than a diffusion controlled rate. The slow process, which proceeds with a rate of $6.8 \times 10^9$ s$^{-1}$, could then be attributed to the back transfer of the electron to the nucleotide base. Similarly, the electron-transfer hypothesis could also explain the shortened excited state singlet lifetime of the dye complexed to poly[d(A-T)]. In this case we only observe the forward- and not the back transfer of the electron with a calculated rate constant of ca. $1 \times 10^9$ s$^{-1}$. Table 3.15 summarizes the (hypothetical) rate constants for forward (separation) and backward (recombination) electron-transfer and
Figure 3.42 Energy level diagram for electron-transfer from ground state nucleotides adenine and guanine to singlet excited state methylene blue. Energy levels are in eV. Indicated are absorption of a photon, rate of fluorescence decay, forward electron transfer $k_e$, and recombination (back) electron-transfer $k_{rec}$.
compares these with the free energy $\Delta G$ of each reaction, calculated from equation 3.12:

$$\Delta G^0_{\text{sep}} = E_D - E_A + \Delta G_e - E(S_1)$$  \hspace{1cm} (3.12)$$

$$\Delta G^0_{\text{rec}} = -(E_D - E_A) - \Delta G_e$$

with $E(S_1)$ the energy of the $S_1$ state and $E_D$ and $E_A$, respectively the half-wave potentials for donor oxidation and acceptor reduction. The term $\Delta G_e$ takes account of ion-pair formation and ion-solvation energies and is smallest in a polar solvent. $\Delta G_e$ is assumed to be equal to 0 in an aqueous solution [Beddard, 1986].

<table>
<thead>
<tr>
<th>electron transfer rate $\left( s^{-1} \right)$</th>
<th>$\Delta G$ $\text{(eV)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$G \rightarrow MB^+$</td>
<td>$2.5 \times 10^{11}$</td>
</tr>
<tr>
<td>$G^+ \leftarrow MB^-$</td>
<td>$6.8 \times 10^9$</td>
</tr>
<tr>
<td>$A \rightarrow MB^+$</td>
<td>$1.0 \times 10^9$</td>
</tr>
</tbody>
</table>

Table 3.14 Observed rate constants for electron-transfer from nucleotide base to methylene blue, compared to $\Delta G$ values for the electron transfer separation and recombination.

In figure 3.43 the logarithm of $k_{ET}$ (calculated according to 3.10-3.12) is plotted versus $\Delta G$ (from electrochemical data). The observed rates (attributed to electron-transfer) are reasonably fitted to the calculated parabola with values of $\lambda$ of 0.8 eV and $V$ of 0.005 eV. These values are similar to those found in a linked quinone-porphyrine dimer [Irvine et al., 1986]. It has to be emphasized that values for $\lambda$ and $V$ have not been reported previously for DNA.

Is electron-transfer the only explanation of the observed decay-
Figure 3.43 Observed rate constants obtained from picosecond transient decay experiments, fitted to Marcus theory for non-adiabatic electron-transfer (dotted line: Eq. 3.10 - 3.11). See also text.

1. $k_e : G \rightarrow MB^{**}$
2. $k_{rec} : MB^* \rightarrow G^+$
3. $k_e : A \rightarrow MB^{**}$
kinetics of the MB⁺/polynucleotide complex? Possible involvement of dye-dimer or excited dye singlet S₁ or S₂ states in the excited state kinetics of free dye has already been inferred above and could possibly also be involved in the kinetics of the polymer bound species. It is not thought that this is the case for several reasons: - Contributions from possible free dye to the transient kinetics of the dye/polynucleotide complexes can be ignored as there is no component with the lifetime associated with that of free dye.

- Dimeric [MB⁺]₂ has been shown to be prominent in the transient spectroscopy of poly[d(A-T)] (chapter 3.3). However, the dye does not exhibit very fast kinetics when bound to this polymer. Also the kinetics observed in the case of free dye and in the case of dye/poly[d(G-C)] are qualitatively different which also argues against involvement of dimer species. Finally, the amount of dimer will decrease on addition of the polymers to the dye solution.

- Excited singlet states of the dye could also be involved in the excited state behaviour. Higher excited S₂ dye states are known to have very short lifetimes [Penzkofer and Blau, 1983]. Similar to the argument above, if such states were involved then they should be observed in both polynucleotides.

The conclusion is that the most plausible explanation for the observed kinetic behaviour is a) the observation of dimer excited S₁-state in the case of free dye and b) the observation of electron-transfer in the case of dye/polynucleotide complexes.

An interesting observation is the fact that the back electron transfer occurs in the so-called "inverted region" [Marcus and Siders, 1982]. The rate for this reaction is very much slower than would be expected on classical grounds. It is possible that the observed "direct" cleavage of the nucleic acid as found in anaerobic solutions is due to the apparent slowness of this back reaction (see chapter 5).
The conclusions drawn above have to be considered preliminary due to the paucity of data. Full confirmation of the electron transfer theory as applied to dye/DNA complexes can only be made by investigating a number of compounds, with different redox-potentials.
CHAPTER 4

THE BINDING OF METHYLENE BLUE TO DNA
INTRODUCTION

The binding of methylene blue to DNA has not been studied as extensively as that of similar compounds such as acridine orange and proflavine. A correct interpretation of the results obtained in the study of the photophysical properties of the bound dye as well as of the dye-photosensitized cleavage of DNA, depends crucially on a proper understanding of the modes of binding of the dye to the polynucleotides. Norden and Tjerneld [1982] inferred that the dye is intercalated in the nucleic acid helix, and that the exact mode of intercalation is strongly dependent on ionic strength. It has already been shown that small changes in ionic strength have a substantial effect on the properties of the triplet state of the bound dye (chapter 3) and it was decided to study the effects of variation of ionic strength on the binding of the dye. In addition, the determination of the values of association constants and binding numbers would enable the calculation of the actual amount of dye bound to the polymer.

Topoisomerase unwinding, spectroscopic investigations and thermal denaturation were the experimental procedures used in this study. The results obtained with these methods are reported in the first part of this chapter. In the second part of this chapter a novel method is reported enabling the determination of total binding constants of small ligands to nucleic acids. The method was an off-shoot from a study to investigate the electrochemical behaviour of methylene blue in the presence of DNA.
I. MODES AND STRENGTH OF BINDING OF METHYLENE BLUE TO DNA

4.1 Topoisomerase unwinding

4.1.1. Principle of method

Topoisomerase unwinding is a powerful tool to study the modes of binding of small ligands to DNA and will be discussed here briefly. Closed circular DNA contains, in addition to the normal (Watson-Crick) double helical turns, so-called superhelical turns. The amount of superhelical turns present in a molecule is normally determined by the solvent environment at the time of ring closure. Superhelical turns will relieve the strain imposed on the polymer due to the topological constraints of the closed configuration [Freifelder, 1982].

The number of supercoils $t$ in a topologically constrained helix such as covalently closed circular DNA, is related to the amount of helical turns $b$, via equation 4.1:

$$a = b + t$$

(4.1)

where $a$ = the topological linking number, and is a constant for a given polymer. A decrease in the amount of helical turns will lead to an increase in the amount of supercoiled turns and vice versa. The electrophoretic mobility of closed circular DNA of fixed molecular weight is only dependent on the number of superhelical turns $t$ [Keller, 1975] and thus the number of helical turns can be determined via gel

* The experimental work reported in this section was carried out by Colm OhUigin, Department of Genetics, Trinity College Dublin.
Intercalation of a ligand into the double helix causes unwinding of the helix. This is due to the extension of the helix caused by the insertion of the intercalating molecule (see chapter 2). This decrease in the number of helical turns will result in an increase in the number of supercoils in closed circular DNA and thus the study of the extent of supercoiling in the presence of a ligand can give information on the extent of intercalation of that ligand. Unfortunately it is not always possible to directly measure the extent of unwinding by co-electrophoresis of the ligand and the polymer. Problems are mainly from weak binding and the charge on the ligand [Tossi, 1987]. Instead the technique of topoisomerase unwinding was used.

Topoisomerase is an enzyme that nicks the sugar-phosphate backbone of supercoiled DNA in a single strand and subsequently restores the breakage. Treatment of supercoiled DNA with topoisomerase will initially result in a relief of the strain in the polymer. Subsequent resealing by the enzyme then results in an almost completely relaxed coil (in the solvent conditions employed here, the relaxed condition will have 2 positive supercoils i.e. $t = +2$). If this treatment with topoisomerase is carried out in the presence of an intercalator then negative supercoiling will occur and the number of supercoils will decrease. The value of $t$ will become 0 and subsequently negative. Unavoidably, due to statistical fluctuations, a series of supercoiled polymers will be formed in each case which differ from one another in an integer number of supercoils and thus form a ladder-like electrophoresis pattern. The amount of supercoiling is then determined by an averaging of the respective intensities of each band on the gel [Keller, 1975]. Increasing the amount of ligand in the reaction mixture with the enzyme will thus lead to an increased number of negative supercoils (i.e. an increased negative average). The number of
supercoils and thus the amount of unwinding can be quantified into the characteristic angle which an intercalating ligand causes the DNA to unwind. The unwinding angle for an intercalating compound has been determined as ca. 26° per dye residue [Wang, 1974; Keller, 1975].

The principle of the topoisomerase assay for intercalation is illustrated in figure 4.1.

4.1.2 Experimental

Extensive discussion on the experimental techniques associated with the topoisomerase experiments and DNA purification can be found in the PhD thesis of C. OhUigin (to be published). The closed circular form of DNA used in these experiments was PBR 322, a plasmid DNA of 4362 basepairs length. The DNA was extensively purified on CsCl density gradients. The topoisomerase enzyme was Topoisomerase-1 (Calf thymus, Bethesda Research Laboratories). Unwinding experiments were carried out in a buffer containing 50 mM Tris, 5 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol and 30 μg/ml bovine serum albumen. Initial buffers also contained 10 mM MgCl₂ (see below). Reactions were carried out by incubating ca. 0.5 μg DNA per 50 μl buffer in the presence of known amounts of ligand at 37° for 3 hours and stopped by addition of a phenol/isoamyl alcohol mixture. The DNA is subsequently precipitated using ethanol and redissolved in a Tris/Boric acid buffer (pH=7.4) and loaded onto the gel. Gels were 0.9% agarose and the electric field was 4 V/cm. Gels were visualised by staining with ethidium bromide and observing the fluorescence of the dye.
Figure 4.1 Schematic illustration of topoisomerase unwinding as an assay for intercalation. A: overwound cccDNA. 1: topoisomerase enzyme nicks cccDNA, which in turn relaxes (B). Under normal conditions (2) the enzyme will close the polymer so that it will end up with +2 supercoils. In the presence of intercalating dyes (which unwind the DNA helix) closure will result in added negative supercoiling (3). The more dye is added, the more negative the number of supercoils will be.
4.1.3 Results

Initial experiments on the unwinding of DNA by methylene blue yielded no results. This was surprising as the dye was generally considered to be an intercalator. This absence of any unwinding could be attributed to the concentration of MgCl₂ in the reaction buffer. Similar inhibition of unwinding by MgCl₂ was also found for the intercalators ZnTMPyP⁴⁺, chloroquine and Ru(phen)₃²⁺ [Tossi, 1987].

Figure 4.2 shows a photograph of the unwinding ladders obtained at a P/D of 30 for a series of MgCl₂ concentrations. From such measurements a plot of helical unwinding per dye residue as a function of Mg²⁺ was determined (Figure 4.3). From the figure it can be seen that the unwinding per dye molecule at low salt concentrations is 25°, in good agreement with the value determined for the intercalator ethidium bromide. Our conclusion is that methylene blue intercalates in the double helix at very low concentrations of Mg²⁺. This intercalation is extremely strongly salt dependent, with small amounts of salt reducing unwinding and hence intercalation. The extent of unwinding as a function of [MgCl₂] is similar in shape to that obtained for Ru(phen)₃²⁺ [Tossi, 1987]. In the case of methylene blue a residual unwinding (of 1°) remains present at high magnesium concentrations. At the higher salt concentrations (> 1 mM) little unwinding is present. The dye must however still be considered bound to the polymer as the fluorescence emission of a methylene blue/CT-DNA complex, recorded in a similar solution as the unwinding experiments, still shows considerable quenching (figure 4.4).
Figure 4.2 electrophoresis gel showing the unwinding of pBR322 DNA by MB$^+$ (P/D = 30) as a function of magnesium concentration in the buffer. The negatively supercoiled plasmids become slightly positively supercoiled in the absence of Mg$^{2+}$ (lane 1). The supercoiling induced by 2 µM MB$^+$ becomes increasingly negative as the Mg$^{2+}$ concentration is reduced from 5 mM (lane 2 and 3) to 0.5, 0.2, 0.1, 0.05 and 0 mM (lanes 4 - 8).
Figure 4.3 Magnesium dependence of the unwinding of DNA by MB\(^+\). Shown is the degrees of unwinding induced per input residue of MB\(^+\) in topoisomerisation buffers containing varying Mg\(^{2+}\) concentrations.
Figure 4.4 Fluorescence quenching of 1.1x10⁻⁶ M MB⁺ in a 10 mM Tris/HCl buffer (pH = 7.2) on titration with CT-DNA and subsequent MgCl₂. Excitation wavelength 665 nm and emission wavelength 690 nm.
4.2 Spectroscopic investigation of MB⁺/DNA binding

4.2.1 Introduction

In this section the results of the investigation of the ionic strength dependence of the value of the association constants are reported for the binding of methylene blue to CT-DNA and the synthetic polynucleotides poly[d(A-T)] and poly[d(G-C)]. The aim of this study was to provide additional information for the interpretation of the results obtained in the photophysical investigations of the dye/nucleic acid complexes. Secondly, determination of the association constants $K_b$ would enable a calculation of the actual amount of dye bound under particular experimental conditions. The values of $K_b$ obtained here could also possibly clarify the large discrepancies found for the previously reported values (see Table 2.3).

4.2.2 Experimental method

The experimental method used here is based on the decrease of the fluorescence of the dye by the polynucleotides. Figure 3.4 shows the different decreases in dye fluorescence in the presence of CT-DNA at three different ionic strengths. Similar curves can be obtained for the reduction of the emission of dye in the presence of poly[d(A-T)] and poly[d(G-C)].

Fluorescence quenching curves can be used to determine the amount of free and bound dye in solution [LePecq and Paoletti, 1967]. Consider the intensity of fluorescence in the absence of polymer as $I_o$. For dilute solutions, $I_o$ is proportional to the concentration of dye in
solution. In the presence of polynucleotide, the measured intensity $I_m$ is proportional to the sum of the intensities of bound and free dye i.e.

$$I_m = F \cdot I_b + (1 - F) \cdot I_o$$ (4.2)

with $F$ the fraction of bound dye and $I_b$ the intensity of a fully bound dye molecule. It then follows that:

$$F = \frac{(I_m - I_o)}{(I_b - I_o)}$$ (4.3)

The concentration of bound dye $C_b$ is equal to $F \cdot C_t$, with $C_t$ the total dye concentration. The concentration of free dye $C_f$, is thus equal to $C_t - C_b$. From this data it is then possible to calculate the ratio $r$, i.e. the ratio of $C_b$ and the concentration of sites. In our analysis of the data we have taken this concentration of sites to be equal to the concentration of phosphates.

The thus acquired data can subsequently be analysed in several ways. One method, already discussed in chapter 2, is to plot the data in a Scatchard plot i.e. a plot of $r/C_f$ versus $r$. A Scatchard plot yielding a straight line will yield as slope $K_b$ and as intercept with the $r/C_f = 0$ axis the value of the binding number: $n$. The binding number is the maximum number of dye molecules that can be bound per site ($0 < n < 1$). Additional information on the mode of binding can be gained from a binding curve (a plot of $r$ versus $C_f$) [Van Holde, 1985].

4.2.3 Results

Figure 4.5 shows Scatchard plots obtained for the binding of methylene blue to CT-DNA at ionic strengths of ca. 0, 100 mM and 200 mM
Figure 4.5 Scatchard plots for the binding of MB° to CT-DNA at three ionic strengths: 0 mM, 100 mM and 200 mM. The curves were obtained from the fluorescence quenching data shown in Fig. 3.4, using Eq. 4.2 - 4.3.
and figure 4.6 shows similar plots for the binding of MB⁺ to the polynucleotides poly[d(G-C)] and poly[d(A-T)] at an ionic strength of 100 mM. The interpretation of a Scatchard curve is only unambiguous in the case of a straight line. In this case the slope of the line and its intercept with the R-axis can be interpreted in a straightforward manner. This is apparently only the case for the binding of the dye to poly[d(G-C)] \(K_b = 1.5 \times 10^6 \text{ M}^{-1}, n = 0.03\) at an ionic strength of 100 mM and for the binding of the dye to CT-DNA at an ionic strength of ca. 0 mM \(K_b = 6 \times 10^6 \text{ M}^{-1}, n = 0.04\). The Scatchard curve at an ionic strength of 100 mM is biphasic and two different binding constants and n-values have been determined: \(K_b' = 5 \times 10^6 \text{ M}^{-1}, K_b'' = 3 \times 10^5 \text{ M}^{-1}, n' = 0.03\) and \(n'' = 0.13\). The values of these association constants are subject to large uncertainties inherent in both the derivation of the data as in the analysis of the Scatchard plots (see Tossi [1987], for an extensive discussion) and have to be regarded as "order of magnitude" values only. At an ionic strength of 200 mM the Scatchard curve for the MB⁺/CT-DNA complex is upwardly curved for low P/D ratios and a similar plot is observed for the binding of the dye to poly[d(A-T)] (at an ionic strength of 100 mM in the latter case). This type of Scatchard plot has been associated with cooperative stacking of dye molecules on the outside of the polymer [Müller and Crothers, 1975]. Thus at relative high ionic strength and at A-T sites cooperative stacking of dye molecules is indicated. Cooperativity of binding is also indicated by the shape of the binding curves (Figure 4.7). In the case of CT-DNA (at low ionic strength, \(\mu \leq 100 \text{ mM}\)) and of poly[d(G-C)] the binding curves have the general shape of a rectangular hyperbola, indicating non-cooperative binding [Van Holde, 1985]. In the cases where the Scatchard plots are strongly curved, the binding plots have a sigmoidal shape as expected for cooperative binding [Van Holde, 1985]. These results indicate that cooperative stacking plays a role in the
Figure 4.6 Scatchard plots for the binding of MB\textsuperscript{+} to poly[d(G-C)] and poly[d(A-T)], obtained from fluorescence quenching data. Buffer 50 mM phosphate (u = 100 mM), pH = 6.9.
Figure 4.7 Binding plots for the binding of methylene blue to CT-DNA
(A) 1 : $\mu = 0$ mM; 2 : $\mu = 100$ mM; 3 : $\mu = 200$ mM. (B) poly[d(A-T)], $\mu = 100$ mM. (C) poly[d(G-C)], $\mu = 100$ mM.
binding of methylene blue to nucleic acids in contrast to the conclusions by Muller and Crothers.

The values of $K_b$, $K_b'$, and $n$, obtained for the binding of MB$^+$ to CT-DNA (0 and 100 mM) and poly[d(G-C)] are all reasonably similar. This indicates a similar mode of binding. As the mode of binding at low ionic strength has been established as intercalation (see topoisomerase section), the dye is probably intercalated in the DNA helix in these polymers. At the intermediate ionic strength of 100 mM in the case of CT-DNA, two modes of binding are inferred from the Scatchard analysis. One is intercalation whereas the second binding mode is presumably an external form of binding. At the highest concentration of salt used (for CT-DNA) or in the case of poly[d(A-T)] cooperative stacking appears to indicate a third mode of binding of dye to the polymer.

Additional information on the binding of dye to poly[d(A-T)] could be obtained from a thermal denaturation study (Figure 4.8). The binding of methylene blue to poly[d(A-T)] at an ionic strength of 2 mM leads to a large (ca. 20 °C) increase in the thermal denaturing temperature of the complex. This is generally believed to indicate an intercalative binding [Marky et al., 1983]. Increasing the ionic strength to 100 mM results only in a very small (ca. 2 °C) increase in the melting temperature which rules out intercalation to any large extent. Similar experiments with CT-DNA (not shown) demonstrate a similar (20 °C) increase in melting temperature at 2 mM ionic strength but an increase of ca. 10 °C at an ionic strength of 100 mM. This confirms the initial conclusions that at an ionic strength of 100 mM some dye is still intercalated in the nucleic acid. It was not feasible to perform thermal denaturation studies on poly[d(G-C)] in water as its melting temperature (even at low ionic strength) is larger than 90 °C.
Figure 4.8 Thermal denaturation curves for MB+.poly[d(A-T)] complexes at two different ionic strengths. \( \mu = 2 \text{ mM}, \) P/D = 0 (1), 5.8 (2), 9.7 (3), 14.4 (4), 29 (5). \( \mu = 100 \text{ mM}, \) P/D = 0 (1), 7 (2), 16 (3).
4.3 Calculation of amount of bound dye

In the interpretation of the photophysical results it is important to know the amount of dye actually bound to the polymer. This amount can be calculated from the Scatchard equation. Inherent in this calculation is the same assumption which generally applies to the Scatchard analysis i.e. a single mode of binding, with a unique $K_b$. It follows from the Scatchard analysis, with $C_f$ the amount of free dye, $C_b$ the amount of bound dye, $C_t$ the total amount of dye, $P_t$ the concentration of available sites (here taken as concentration of phosphate), $K_b$ the binding constant and $n$ the binding number that:

$$r = \frac{C_b}{P_t} = K_b * C_f * n - K_b * C_f * (C_b / P_t)$$  \hfill (4.4)

Now $C_b = C_t - C_f$ and 4.4 can be rewritten as:

$$(C_b / P_t) * (1 + K_b * C_t - K_b * C_b) = K_b * n * C_t - K_b * n * C_b$$  \hfill (4.5)

Rearranging equation 4.5 this reduces to:

$$K_b * C_b^2 + C_b * (K_b * C_t + 1 + K_b * n * P_t) - K_b * n * P_t * C_t = 0$$  \hfill (4.6)

This quadratic equation in $C_b$ has a unique solution given the boundary condition that $C_b = 0$ when $P_t = 0$. This solution is given in equation 4.7:

$$C_b = \frac{K_b * C_t + 1 + K_b * n * P_t}{(2 * K_b)} - \sqrt{ \frac{(K_b * C_t + 1 + K_b * n * P_t)^2 - 4 * K_b^2 * n * P_t * C_t}{(2 * K_b^2)}}$$  \hfill (4.7)
The values of $C_b$ were calculated using values of $P_t$ similar to those used in the experiments in the previous section. This was done for a series of P/D ratios and for different values of n. In figure 4.9 the percentage of bound dye as a function of total dye concentration is given for a number of different binding constants. The concentration of DNA (in phosphate) is taken to be $2 \times 10^{-3}$ M as being typical of the concentrations used in the laser flash experiments. The value of $K_b$ was varied between $5 \times 10^5$ and $5 \times 10^6$ M$^{-1}$ and the value of the binding number n was varied from 0.02 to 0.1. Figure 4.9 shows that under these conditions and at a P/D-ratio larger than 20 (as used in the laser flash experiments) more than 80% of the dye can be considered bound.

The calculations show that the amount of dye bound to the polymer is strongly dependent on the assumed value of n. Increasing the ionic strength will increase this number (see Scatchard plots) and consequently more dye can be bound to the polymer, although the actual value of the association constant $K_b$ has decreased. As an example the amount of bound dye has been calculated in two situations, both with a DNA concentration of $2 \times 10^{-3}$ M and at a P/D of 10. In the case of $K_b = 5 \times 10^5$ M$^{-1}$ and $n = 0.1$ ca. 98% of the dye is bound; with $K_b = 5 \times 10^6$ M and $n = 0.04$ only 80% of the dye is bound. The P/D ratio is rather low and for a ratio of 20 - 30 the percentages of bound dye will have increased. The conclusion from this is that in the laser flash photolysis experiments most of the dye will be bound to the polymer even when the ionic strength is increased. This aspect of binding mechanism does not seem to be taken account of in ligand-nucleic acid binding studies. The reason for the dependance of n on ionic strength is not very clear and is probably attributed to subtle salt-dependent conformational changes and/or changes in the hydration atmosphere of
% BOUND DYE AS A FUNCTION OF P/D

DNA = 500,000
Kd = 100
C/Ct (%) 50 100

Figure 4.9A
$% \text{BOUND DYE AS A FUNCTION OF } P/D$

$DNA = 0.002 \text{ M}$

$KB = \frac{1000000}{M-1}$

$C_b/C_t$ (%)

$0 \quad 4 \quad 8 \quad 12 \quad 16 \quad 20 \quad 24 \quad 28 \quad 32 \quad 36$ (P/D)

Figure 4.9B
% BOUND DYE AS A FUNCTION OF P/D

$\text{DNA} = \frac{C_b}{C_t} \times 100\%$

Figure 4.9D
% BOUND DYE AS A FUNCTION OF P/D

DNA = 0.002
KB = 5000000
M
M-1

\[ C_b / C_t \times 100 \% \]

Figure 4.9F
Figure 4.9A-F Binding plots determined from a computer simulation (equation 4.7) A-C : n = 0.02; D-F : n = 0.04. DNA concentration was fixed at 2x10^{-3} M.
the polynucleotide. The strong influence of added salt on the binding of MB$^+$ to poly[d(A-T)] could indicate a possible involvement of the water spine (see chapter 1).

4.4 Conclusions

The results presented here indicate that MB$^+$ can bind to DNA in at least two and possibly three ways. The first, which leads to DNA unwinding is intercalative binding. The second, which does not induce DNA unwinding but nevertheless causes fluorescence quenching involves non-intercalative binding of MB$^+$ to the helix. Intercalation will not occur when the DNA phosphate groups are neutralised by the added Mg$^{2+}$.

The effect of the addition of sub-millimolar amounts of Mg$^{2+}$ on the binding of MB$^+$ to DNA was previously reported by Norden and Tjerneld [1982]. They observed a sign reversal in a linear flow dichroism spectrum of the dye bound to the helix. Complete sign reversal was reported at a phosphate/Mg$^{2+}$ (P/M) ratio of 2. They interpreted their results on the basis of a rotation of the dye in its binding site, with the dye moving from a position with its long axis parallel to the basepair hydrogen bonds to one approximately orthogonal to the latter. An important feature of this model is that the dye is considered to be intercalated in both positions. This is not consistent with the results obtained from the topoisomerase experiments. At intermediate P/M ratios (0.2 < P/M < 2), no significant intercalation can be observed but there is still a strong interaction between dye and nucleotides as indicated by the extent of fluorescence quenching (Fig. 4.4). The conclusion is that small amounts of Mg$^{2+}$ change the mode of binding of the dye from an intercalated to a non-intercalated one. This latter binding is then most likely associated with the association constant $K_b'' = 3 \times 10^5 \text{ M}^{-1}$, found for the binding of dye to CT-DNA at $\mu = 100 \text{ mM}$. N.B. the value of
will be ionic strength dependent. The intercalative binding of methylene blue to the synthetic polynucleotide poly[d(A-T)] is most susceptible to variations in ionic strength as shown from the thermal denaturation studies.

The third form of binding is assumed to be a cooperative stacking of dye molecules on the outside of the helix. The similarity between the high CT-DNA Scatchard curve and the poly[d(A-T)] curve indicate that this induced aggregation appears most likely for A-T sites. This aggregation is possibly an explanation for the strong aggregation depletion bands as observed in the laser flash experiments (chapter 3.3). It is not clear why no increased aggregation can be observed in standard ground state UV/VIS absorption spectrometry (chapter 3.1). Finally, the invariance of the protonation of $^3\text{MB}^+$/poly[d(A-T)] with ionic strength (Fig. 3.24) indicates that in both external forms of binding a substantial non-electrostatic component is involved.

The values of the binding numbers $n$, as determined from the Scatchard plots, are remarkably low and are a factor of 10 lower than the values for the comparable dyes acridine orange and proflavine [Peacocke, 1973]. It has been suggested that methylene blue (like other phenothiazines) is not a planar molecule but instead has a dihedral angle of ca. $149^\circ$ along its long axis [McDowell, 1974]. Intercalation of such a "kinked" molecule will cause a larger disturbance in the nucleic acid structure compared to that caused by planar intercalators. This could then prevent other dye molecules intercalating. This explanation is however purely speculative and would await confirmation by X-ray crystallographic studies.

Finally, a calculation of the amount of dye bound to the nucleic acids under the conditions of the laser flash experiments, justifies the assumption that in these experiments more than 80-90 % of the dye can be considered bound.
II. AN ELECTROCHEMICAL METHOD TO DETERMINE BINDING CONSTANTS OF SMALL LIGANDS TO NUCLEIC ACIDS. *

4.5 Introduction

In chapter 5 it is demonstrated that methylene blue can photosensitize cleavage of the phosphodiester backbone of nucleic acids. The mechanism of this cleavage appears to be via an indirect mechanism involving oxygen and partly via a direct \textit{-radical-} reaction. A possible hypothesis for the latter reaction has already been discussed in chapter 3 and could involve a redox (electron-transfer) reaction between the excited singlet state of the dye and the nucleotide base. The fundamental parameters underlying the electron transfer hypotheses are the reduction potentials of the bound dye in ground and excited state. The shifts in the absorption spectra of the dye bound to the nucleic acid provide evidence of an interaction between the electronic wavefunctions of dye and nucleotide bases and this could possibly influence the electron accepting properties of the dye.

In order to obtain more information on the redox properties of the complex a cyclic voltammetry study was performed on MB$^+$ and MB$^+/\text{CT-DNA}$ complexes.

* I would like to acknowledge Dr. M.E.G. Lyons (T.C.D.) for helpful discussions and for allowing the use of his apparatus.
4.6 Results

Figure 4.10 shows a typical cyclic voltammogram obtained for a $2 \times 10^{-5}$ M solution of MB$^+$ with and without added CT-DNA. Buffer was 50 mM phosphate at 25 °C. Addition of CT-DNA markedly reduces the peak currents, both of the anodic oxidation and of the cathodic reduction wave and increases $E_0 ( = 0.5 \times (E_{pA} + E_{pC}))$. This increase in $E_0$ is illustrated in figure 4.11.

Figure 4.11 shows that increasing the DNA concentration leads to only a small decrease in $E_0$. Also the general shape of the cyclic voltammograms for free dye and dye/DNA are similar. It is therefore postulated that the electrochemical properties which are observed in both cases are from the same species i.e. methylene blue. The redox properties of the dye are not changed much on binding to DNA.

MB$^+$ is well known to form aggregate dimers in aqueous solutions (chapter 3.1) and figure 4.11 also shows $E_0$ as a percentage of dimer in solution as determined in the absence of DNA. At a dye concentration of $2 \times 10^{-5}$ M ca. 26% of the dye is in solution as a dimer ($K_d = 1.2 \times 10^4$ M$^{-1}$, chapter 3.1). The small decrease in $E_0$ which is observed on addition of DNA and which parallels a decrease in dimer concentration can then be explained on the basis of binding of monomeric dye to the polymer. This will lead to a decrease of the concentration of dimer in solution. No specific waves are observed which could be contributed to MB$^+$-dimer: [MB$^+$]$_2$. Other effects noted are an increase in the ratio of anodic peak currents over cathodic peak currents on addition of DNA. An exact explanation for this requires more study than was appropriate here but it might be due to a difference in binding to DNA of methylene blue and its reduced form: leuco methylene blue (LMB). The latter is
Figure 4.10 Cyclic voltammogram of \(2 \times 10^{-5} \text{ M}\) MB\(^+\) with and without added CT-DNA (P/D = 43.7) in a pH = 6.9, 50 mM phosphate buffer at 25 °C. Counter electrode Pt-wire and reference electrode Ag|AgCl.

a: \(\text{MB}^+ 2.0 \times 10^{-5} \text{ M}\)

b: \(\frac{\text{phos}}{\text{dye}} = 43.7\)
Figure 4.11 The effect of addition of CT-DNA on the half-wave potential $E_0$ of methylene blue. Dye concentration $2 \times 10^{-5}$ M, buffer 50 mM phosphate. Also shown is $E_0$ as a percentage of dye dimer aggregates. (this was determined separately, without DNA present)
produced in the cathodic wave via a two electron-one proton reduction and is consequently not charged. It is therefore not expected to bind as strongly to DNA as MB⁺. Relatively more LMB could be free in solution and available at the electrode for oxidation to MB⁺.

The main effect of addition of DNA on the observed electrochemistry is a decrease in the anodic and cathodic peak currents and this is illustrated in figure 4.12. This decrease in current is either due to a decrease in diffusion coefficients of the electroactive species or due to a decrease in its apparent concentration. Both explanations are equally valid as bound dye will have the diffusion coefficient of the very large molecular weight polymer, whereas intercalated dye will be shielded from electrode reactions by the polymer environment which appears as a decrease in bulk dye concentration apparent to the electrode.

Other experiments essentially confirm the above observations. Levich plots, obtained from rotating disk electrode experiments, are linear with decreasing slopes on increasing DNA concentration, consistent with either a lower diffusion coefficient or a lower apparent concentration (not shown). The effects observed in the electrochemistry of MB⁺/DNA complexes appear to be solely due to binding of the dye to the polymer. No new electrochemistry is detected.

The decrease in anodic and cathodic peak currents on addition of DNA (Figure 4.12) is similar to the fluorescence quenching curves obtained with the dye and DNA, and suggested the use of an electrochemical method to determine the association constant of the dye with the polymer.

4.7 Electrochemical binding method

Binding constants can be derived from fluorescence quenching and the
Figure 4.12 Anodic ([ ]) and cathodic (0) peak currents of a $2 \times 10^{-5} \text{ M}$ solution of MB$^+$ on addition of calf thymus DNA. Buffer 50 mM phosphate.
most popular method for this is using the Scatchard equation (see above). As was discussed in section I, successful application of the Scatchard formalism depends crucially on an accurate determination of the amount of free dye in solution. This is difficult to achieve when the binding is strong and/or at high P/D ratios. In these cases the results obtained from the Scatchard equation are subject to considerable error.

For the analysis of the electrochemical quenching a different approach has been taken. The analysis is similar to that of Berg et al. [1972] and was slightly modified. First consider the peak currents in the absence and presence of DNA:

\[ i_o = A \times C_t \]  
\[ i = A \times (C_f + qC_b) \]

where \( C_t \) = total dye concentration, \( C_f \) and \( C_b \) respectively free and bound dye, \( A \) a proportionality constant and \( q \) a constant denoting the effect of polymer binding on the current (0 < \( q < 1 \)). The overall association constant of the dye with the polymer is then given by equation 4.9:

\[ K = \text{[complex]} / (C_f \times P_f) \]

with \( P_f \) the concentration of free sites. From 4.8 it follows that:

\[ C_b = (1/(1 - q)) \times (1 - i/i_o) \times C_t \]

Assuming that \( P_t \gg C_t \) then \( P_t = P_f \). Substituting now 4.10 into 4.9 yields, after some manipulation:
\[ P_t = Q * (1 - X) / [K * (1 - Q * (1 - X))] + Q * (1 - X) * C_t \] (4.11)

with \( Q = 1/(1 - q) \), and \( X = i/i_0 \). In general \( q \) and \( X \) are not too small and are of the order 0.5. Then, with \( P_t \gg C_t \) (i.e. a large \( P/D \)) equation 4.11 reduces to:

\[ P_t = Q * (1 - X) / [K * (1 - Q * (1 - X))] \] (4.12)

which can be rewritten as:

\[ 1/P_t = K * \left( (1 - q)/(1 - X) - 1 \right) \] (4.13)

Thus a plot of \( 1/P_t \) vs. \( 1/(1 - X) \) is a straight line with intercept \( -K \) and slope \( K * (1 - q) \).

Figures 4.13-4.15 show binding plots so obtained for the dyes methylene blue, neutral red and cresyl violet in buffers of 100 mM ionic strength. In the case of cresyl violet 5\% EtOH was added to assist in solubility. The values of \( K \) so obtained are respectively 4.7x10^{-3}, 1.2x10^{-3} and 7.3x10^{-3} M^{-1}. The values reported here for the association constant of methylene blue are a factor of 100 smaller than the values derived from the Scatchard equation. The difference is that here the overall binding constant is determined, whereas in the Scatchard analysis the binding constant for binding to a particular site is determined. In the latter analysis account is taken of the binding number \( n \). The limited number of sites available for binding is ignored in the electrochemical analysis. This is not expected to be significant with respect to the values of \( K \) calculated here, due to the imposed condition that \( P_t \gg C_t \). Taking as a rough estimate that the product of \( n \) and the site association constant \( K_b \) (\( n * K_b \)) equals \( K \),
Figure 4.13 Binding curve obtained according to Eq. 4.13 for methylene blue and CT-DNA. Buffer 50 mM phosphate, pH = 6.9.
Figure 4.14 Binding curve obtained according to Eq. 4.13 for neutral red and CT-DNA. Buffer 50 mM phosphate, pH = 6.9.
Figure 4.15 Binding curve obtained according to Eq. 4.13 for cresyl violet and CT-DNA. Buffer 50 mM phosphate, pH = 6.9. 5% EtOH was added to assist in solubility of the dye.
then (for methylene blue) \( n = 0.01 - 0.05 \), and a value of \( K_b \) is determined which is of the order \( 10^5 \, \text{M}^{-1} \). This is of the same order of magnitude as the values determined from Scatchard analysis.

Concluding, an electrochemical method has been described to determine the binding constants of small ligands to nucleic acids. The method is based on the large difference in hydrodynamic properties between bound and free dye. The method of analysis is a rapid and convenient method in determining overall association constants and should be usable for the determination of association constants of ligands to other large polymers such as proteins. It has an advantage over the conventional methods in that it enables the determination of binding constants for compounds who do not exhibit any fluorescence or whose absorption bands overlap those of DNA. The only requirement is that the compounds are electrochemically active in a region where the nucleic acid components are not. The method is easily increased in sensitivity by the use of alternative electrochemical methods such as square wave voltammetry or differential pulse polarography.
CHAPTER 5

DNA PHOTOLYSIS WITH METHYLENE BLUE
Several observations can be made from a survey of the literature on the methylene blue photosensitized damage of nucleic acids and its components (chapter 2.3). Firstly, the exact mechanism of the dye sensitized damage is reasonably well established for the isolated nucleotide and indicates an approximately 60:40 ratio between type II and type I mediated reactions under their experimental conditions. Secondly, the observations made on polynucleotides are not clear cut and sometimes contradictory and do not give a good indication of the importance, if any, of $^{1}O_{2}$ or other oxygen radicals in the reaction. Both alkali induced breaks, indicating apurinic sites, as well as direct breaks have been observed. The large number of contradictory and conflicting reports also indicates the difficulty in the precise determination of the exact reaction mechanism of the strand cleavage (see also Cadet et al. 1986).

Our efforts, described in the following pages, concentrated on the possible elucidation of the reaction mechanism of the sensitized backbone cleavage as well as on its efficiency. Also sequencing was carried out to enable the detection of base-specific cleavage. It was already known that MB$^{+}$ cleaves DNA specifically at G-residues in aerated solutions after treatment with piperidine [Friedman and Brown, 1978] and the effect of different treatments on the base-specificity was investigated. It appeared from literature that singlet oxygen modification of the polymer is guanine specific and it might be conceivable that type I -radical- mechanisms are specific for other nucleotides.
EXPERIMENTAL RESULTS

5.1 Photolysis of pBR322 plasmid DNA

The plasmid DNA used in the photolysis experiments is a covalently closed circular (cccDNA) form of B-DNA and is normally in an overwound configuration. It is obtained as a compound with a well defined molecular weight and will upon electrophoresis give a discrete band. Formation of a single break in one of the phosphodiester backbones of cccDNA will result in release of the strain energy and the molecule will unwind, resulting in open circular DNA (ocDNA) which has a lower electrophoretic mobility. Further strand breaks in ocDNA, close to the initial one, will linearise the molecule. The mobility of linear DNA is between that of cccDNA and ocDNA. The three forms of DNA are easily separated on an electrophoresis gel. This enables an accurate determination of the number of strand breaks and thus the effects of photolysis. It is apparent that this method is more sensitive than methods which depend on the determination of molecular weight or measurement of thermal denaturation temperature. The electrophoretic method enables the detection of a single strand break in a molecule of several thousand basepairs long. Figure 5.1 illustrates the principle of the method.

* The experiments reported here were carried out in conjunction with C. OhUigin (Dept. of Genetics, Trinity College Dublin) who performed most of the molecular biological manipulations.
5.2 Quantum yield of single strand break formation

The quantum yield of strand break formation is defined as the number of breaks formed in DNA per photon absorbed by the dye. The yield was not expected to be very high and was determined using electrophoresis of photolysed pBR322 DNA. Possible $^{1}O_2$ effects were investigated by the addition of NaN$_3$, a well known quencher of $^{1}O_2$ [Hasty et al. 1972]. Additionally, the effects of intercalation of the dye on the photolysis was investigated by the addition of MgCl$_2$. We [chapter 4] and others [Norden and Tjerneld, 1982] have shown that small amounts of Mg$^{2+}$ can change the binding of the dye from intercalative to external binding.

5.2.1 Experimental procedure

The quantum yield was determined in the experimental set up illustrated in figure 5.2. The irradiation source was a 5 mW Helium-
Figure 5.2 Experimental set-up for the determination of the quantum yield of strand-break formation in cccDNA. A: 5 mW HeNe laser. B: f = 35 mm microscope objective. C: glass tube, internal diameter 2.5 mm, length 15 mm. The tube was positioned on an XY-translation stage. D: Detector element Scientech 362 calorimetric power meter. E: thermal shield for detector (styrofoam).
Neon laser (Spectra-Physics model 120 Stabilite) emitting at 632.8 nm. The laser light was focussed through a small horizontal glass tube with a microscope objective (f= 35 mm). Internal tube diameter was 2.5 mm and length 15 mm and the tube was internally silylated to prevent adhesion of the liquid. 50 µl of dye-DNA samples were loaded in using an Gillman micropipette and photolysed for a predetermined time. Light transmission was measured with a calorimetric laser power meter (Scientech 362). The measuring head of the power meter was thermally insulated to increase reproducibility of the readings. Transmission measurements of samples were made relative to blanks, in which the tube was filled with doubly distilled water. By carefully positioning the tube inside the -fairly large- cavity of the measuring head of the power meter, errors due to scattered light are minimised. (In fact, the tube aids light transmission through the sample by acting as a light guide). Excellent sample-to-sample reproducibility was obtained in this set up.

0.5 ml of a stock solution was freshly prepared in doubly distilled water containing $10^{-5}$ M MB+ and pBR322 DNA at a P/D ratio of 15. To 40 µl aliquot of this stock solution was added 10 µl of either H₂O or stock solutions of NaN₃, MgCl₂ or an equimolar mixture of MgCl₂ and NaN₃. This gave final salt concentrations of 0.0, 0.2, 2.0 and 20.0 mM. After rinsing the sample tube with distilled H₂O, samples were loaded into the tube and irradiated. After irradiation the samples were removed from the glass tube and the dye was extracted with a mixture of phenol/chloroform/isoamyl alcohol [Kelly et al. 1985a]. For alkaline hydrolysis, 18 µl of each sample was removed, made up to 0.1 M NaOH and heated to 70 °C for 30 minutes. Samples were loaded on agarose gels (0.8%) and electrophoresed overnight.
5.2.2 Quantification of results

Gels were stained in an ethidium bromide bath (0.5 mg/ml) for 30 minutes and were photographed over an UV transilluminator. With the gel also a linear neutral density wedge was photographed to check and correct any possible non-linearity in film or film processing. Film was Ilford FP4. Prints of the film were scanned on a Joyce-Loebl microdensitometer with an integrator to estimate peak area. Assuming the formation of a strand break to be a random, uniform process, the probability of such a process occurring is described by a Poisson distribution with cccDNA having no breaks and ocDNA having one or more. Then the mean number of strand breaks ($N$) after irradiation in a pBR322 molecule is given by Eq. 5.1:

$$N = -\ln(1 - [Z])$$

(5.1)

with $[Z]$ the fraction of cccDNA left. The initial known concentration of cccDNA is then used to calculate the total number of strand breaks in each sample. Comparison of the energy transmission of the sample relative to the blank enabled the determination of the total amount of energy absorbed over the irradiation time. The energy of a photon at 632.8 nm is $3.13 \times 10^{-19}$ J and thus the total number of absorbed photons could be calculated. The quantum yield of strand break formation is then the number of strand breaks divided by the number of photons absorbed. (Extensive details on the methodology can be found in C.OhUigin [to be published]).
5.2.3 Results

Figure 5.3 shows a print of an electrophoresis gel, produced as described above. Table 5.1 gives the quantum yields for single strand break formation as determined from such pictures.

<table>
<thead>
<tr>
<th>[conc]</th>
<th>Mg$^{2+}$</th>
<th>NaN$_3$</th>
<th>[Mg$^{2+}$ + NaN$_3$]*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>3.1</td>
<td>3.3</td>
<td>2.9</td>
</tr>
<tr>
<td>2.0</td>
<td>3.3</td>
<td>2.8</td>
<td>2.2</td>
</tr>
<tr>
<td>20.0</td>
<td>1.6</td>
<td>2.1</td>
<td>0.9</td>
</tr>
</tbody>
</table>

no salt : 3.1  N.B. All values are x 10$^7$.

Table 5.1. Quantum yields of "overt" single strand formation by methylene blue photosensitised cleavage of pBR322. (* Total salt concentration)

Alkaline cleavage is, in general, associated with the formation of apurinic sites in the polymer i.e. sites where the glycosidic bond between sugar and base has been broken [Piette et al., 1981]. The results obtained here indicate that irrespective of added agent or oxygen the yield of alkali labile sites is always a factor 3 larger than that of sites which produce direct strand breaks. The values reported in the table are for direct ("overt") strand breaks. Control experiments showed that no overt or latent breaks were induced following irradiation of DNA in the absence of dye, or in unradiated samples with dye which had been treated similarly in every other way.

These results show that methylene blue is able to cleave DNA directly i.e. without the need to treat with alkali. It has already
Figure 5.3 Electrophoresis gel of photolysis experiment used in determination of quantum yield of strand breakage. P/D = 15.

Irradiation with the laser photolyses cccDNA (lane 1) to ocDNA (lane 2). This conversion is reduced by addition of NaN₃ (0.2, 2, 20 mM; lane 3-5 respectively), MgCl₂ (0.2, 2, 20 mM; lanes 6-8) and both salts in equimolar concentrations (total salt 0.2, 2, 20 mM; lane 9-11). Lane 12 is identical to lane 2.
been shown that Mg\(^{2+}\) strongly affects the binding of the dye to the nucleic acid [Chapter 4]. By adjusting the Mg\(^{2+}\) concentration it is possible to shift the dye binding from intercalative to external and non-intercalative binding and to determine the effects of this on the photolysis. Mg\(^{2+}\) concentrations over 2 mM prevent intercalation, whereas there is substantial non-intercalative binding up to 10 mM. As shown in table 5.1 and figure 5.3, low Mg\(^{2+}\) concentrations (< 2.0 mM) do not significantly affect the yield of photolysis. At a concentration of 20 mM a 50% reduction in yield is observed. The evidence therefore suggests that, while MB\(^+\) induced photolysis does not require intercalation per se, it is strongly enhanced by binding.

Sodium azide was added to determine possible effects of \(1^{1}\) on the photolysis reaction. Addition of 20 mM azide reduced the yield of strand breaks by ca. 30%. There was no noticeable effect of azide at 0.2 or 2.0 mM both on overt or latent cleavage. The quenching of dye excited states by NaN\(_3\) was investigated as this could compete with the quenching of \(1^{1}\) by NaN\(_3\). The fluorescence emission of methylene blue and MB\(^+/\)DNA in the presence of azide was studied and figure 5.4 shows a Stern-Volmer plot of the observed quenching. It is apparent that N\(_3\) quenches free dye fluorescence with a \(k_q\) of \(1.6 \times 10^{10}\) M\(^{-1}\)s\(^{-1}\), assuming a fluorescence lifetime of 400 ps (chapter 4). Thus a concentration of 20 mM azide, used in the photolysis experiments, will only quench ca. 10% of free dye. Addition of azide to a MB\(^+/\)DNA sample does not show simple Stern-Volmer quenching. However, addition of Na\(^+\) will displace some dye from the polymer and thus increase fluorescence emission. Dye fluorescence is strongly quenched by intercalative binding (Chapter 3) and thus the quenching by NaN\(_3\) can be masked by increased fluorescence due to changes in dye binding or dye release. Correcting the Stern-Volmer plot for the increased dye fluorescence caused by added salt yields a linear plot, with an almost identical \(k_q\) showing that only
Figure 5.4 Stern-Volmer plot for the fluorescence quenching of MB\(^+\) by NaN\(_3\) in the absence of DNA (1), in the presence of DNA (P/D=126) (2). Correction of curve (2) for release of dye from the polymer can be performed, yielding curve 3. The slope of curve 1 yields a \(k_q\) of 1.6x10\(^{-10}\) M\(^{-1}\) s\(^{-1}\).
free dye is quenched.

Possible quenching of dye triplet was investigated in a laser flash photolysis experiment in an aerated solution. Addition of 20 mM azide did not reduce the triplet lifetime, indicating absence of quenching. Hasty et al. (1972) report a rate constant for the reaction of azide with triplet methylene blue of $5 \times 10^5 \text{ M}^{-1}\text{sec}^{-1}$, compared to that of the reaction of azide with $\mathrm{O}_2$ given as $2.2 \times 10^8 \text{ M}^{-1}\text{sec}^{-1}$.

The observations from the fluorescence quenching and the triplet studies indicate that, in our experiments, any reaction of azide can be assumed to be with $\mathrm{O}_2$.

The absence of any effect on the yield of photolysis of the addition of low concentrations of azide indicate that either $\mathrm{O}_2$ is not involved in the photolysis, or that at these low concentrations NaN$_3$ is not able to quench any $\mathrm{O}_2$ produced by the dye. The reason for this is probably not electrostatic repulsion as DNA is practically neutral in solution due to counter-ion condensation (chapter 1). The low quenching could probably be due to a reduced accessibility of the $\mathrm{O}_2$ produced by the intercalated dye. At higher concentrations the salt will make the dye more accessible to quenchers and this could account for the reduced yield, probably by quenching of the $\mathrm{O}_2$ generated by the free dye.

A mixture of MgCl$_2$ and NaN$_3$ in equimolar 10 mM amounts produced greater photolytic inhibition than either alone at 20 mM (Table 5.1). This difference was found consistently in repeated experiments. It suggests that the mechanisms of photolytic protection by MgCl$_2$ and NaN$_3$ are at least partially different.

### 5.3 Anaerobic photolysis

Photolysis in deoxygenated environments would give further information on the possible involvement of $\mathrm{O}_2$-mediated or other
mechanisms on the formation of strand breaks.

5.3.1 Experimental

The removal of oxygen from the -extremely small- samples proved to be a problem. Conventional freeze-pump-thaw cycles could not be used and the following approach was taken: Aqueous solutions of MB+ (10^{-6} M) with and without MgCl₂ (20mM) were carefully deoxygenated by bubbling argon gas through the solution. Then, under a stream of argon, 160 ul of each solution was added to 6 μg of pBR322 in a plastic microcentrifuge container ("Eppendorf"). The DNA had been previously lyophilised and flushed with argon. After resuspension in these tubes, the samples were photolysed under a permanent stream of argon. 40 μl aliquots were removed at fixed time intervals and the dye extracted with the phenol mixture. Further 160 μl aliquots were prepared containing dye and DNA in aerated solutions containing either H₂O or D₂O (97.5%). Photolysis was similar to the deoxygenated solutions. All samples were halved and one half treated with 0.1 M NaOH as described previously.

5.3.2 Results

Figure 5.5 shows an electrophoresis gel obtained according to the procedure described above. Only relative yields are determined in these experiments. Table 5.2 gives relative percentages of strand break formation. The relative contributions of O₂-dependent and -independent contributions to the formation of strand breaks have been estimated and are also shown in table 5.2. This was done by assuming that a) in an deoxygenated solution the contribution of O₂ to the strand break formation is 0, and b) that NaN₃ has no effect on the non-O₂ mechanism.
Figure 5.5 Electrophoresis gel of photolysis products obtained after irradiation of MB⁺/DNA complexes (P/D=12) for 0, 5, 10 and 15 minutes in D₂O (lane 1-4), in H₂O (lane 5-8), in argon saturated aqueous solution with 20 mM MgCl₂ (lanes 9-12) or in argon saturated aqueous solution (lane 13-16). Subsequent treatment of irradiated products with hot alkali is shown in lane 17-20.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Yield (%)</th>
<th>$O_2$-dependent (%)</th>
<th>$O_2$-independent (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>100</td>
<td>61</td>
<td>39</td>
</tr>
<tr>
<td>+ Argon</td>
<td>39</td>
<td>0</td>
<td>39</td>
</tr>
<tr>
<td>+ Argon $\text{MgCl}_2$ (20mM)</td>
<td>25</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>+ $\text{MgCl}_2$ (20mM)</td>
<td>52</td>
<td>27</td>
<td>25</td>
</tr>
<tr>
<td>+ $\text{MgCl}_2$ (10mM) $\text{NaN}_3$ (10mM)</td>
<td>28</td>
<td>3</td>
<td>25</td>
</tr>
<tr>
<td>+ $\text{NaN}_3$ (20mM)</td>
<td>77</td>
<td>38</td>
<td>39</td>
</tr>
</tbody>
</table>

Table 5.2 Relative yields of direct strand break formation in MB$^+$/DNA solutions. Contributions due to oxygen and other mechanisms are estimated assuming that in deoxygenated solutions $O_2$-component $\approx 0$ and that $\text{NaN}_3$ does not influence $O_2$-independent mechanisms.

Removal of oxygen from the solutions does not inhibit production of "overt" or "latent" strand breaks. The relative yield is reduced by about 60% compared to an aerated solution, clearly showing that oxygen does mediate in the cleavage. The 40% residual cleavage must be due to an oxygen independent mechanism in which DNA reacts directly with dye. If so then the reaction can be expected to be more effective if the dye is bound to the polymer. Addition of 20 mM $\text{Mg}^{2+}$ to an deoxygenated solution reduces the quantum yield of cleavage somewhat further. (Figure 5.5, lanes 9 - 12). It appears therefore that the $O_2$ independent mechanism is equally effective for both intercalated as dye which is either free or bound. The topoisomerase experiments and the fluorescence quenching experiments show that even at high $\text{Mg}^{2+}$ concentrations dye remains bound to DNA.

As a final test for the involvement of $^1O_2$, we have investigated the effect of $D_2O$ on the reaction. The lifetime of singlet oxygen in $D_2O$
is approx. 55 us compared to 4 us in H₂O [Frimer, 1985] and generally the increase of yield on replacement of solvent with D₂O is taken to be indicative of the involvement of ¹⁰₂. This assumes that the lifetime of ¹⁰₂ in H₂O is rate limiting. However little difference in strand cleavage was found. Similar to the results reported above, treatment with alkali was found to increase the number of strand breaks by a factor of approximately 3.

5.4 Sequence specificity

The sequence specificity of the methylene blue sensitized photolysis was determined under a variety of conditions.

5.4.1 Experimental *

pBR322 was digested with EcoRl to produce a single fragment of 4363 basepairs long. The 5'-end group was labeled with ³²P ATP using T4 polynucleotide kinase. The labelled fragments were digested with HindIII to produce a small (31bp) and large (4332bp) fragment. These fragments were separated on an agarose gel. A MB⁺/DNA mixture was added to give a final P/D of 15 for photolysis. One sample was dried before addition of a degassed MB⁺ solution. The samples were electrophoresed on polyacrylamide gels according to standard procedures. This electrophoresis was performed alongside samples which were sequenced according to the Maxam and Gilbert method [Maxam and Gilbert, 1977].

5.4.2 Results

Figure 5.6 shows a sequence obtained after photolysis of a dye/DNA

* Performed by C.OhUigin [1987].
Comparison of Maxam and Gilbert sequencing products of the region 4280-4330 of pBR322 with those produced by MB sensitised photolysis of that same region.

Figure 5.6 Sequence specificity of the photolysis reaction is shown by the comparison of the products obtained by the Maxam-Gilbert sequencing reaction of the region 4295 to 4325 of pBR322 with those obtained from MB⁺-sensitized photolysis of the same region. Note lower mobility of the fragments prepared by dye photolysis.
sample in an argon purged sample. The sample is compared with an identical one obtained with the Maxam & Gilbert "G+A" specific reactions.

Photolysis in the presence of dye leads, without alkali treatment, to a discrete set of products. Similar products are observed regardless of the presence of either \( \text{O}_2^{2-}, \text{Mg}^{2+} \) or \( \text{NaN}_3 \). From the known sequence of pBR322 each photoproduct can be unambiguously identified as due to cleavage next to a particular base. In the case of methylene blue all overt cleavages are due to cleavage at a guanine residue in the polymer. However the electric mobility of the 5' end-labelled reaction products is somewhat reduced compared to that of the Maxam & Gilbert products suggesting that these products do not have a free 3' phosphate group but instead have an unidentified adduct attached at that position. Similar observations were made by Blau et al. [1987] investigating the photolysis of DNA upon high intensity laser irradiation at 248 nm wavelength. Treatment with alkali yields products which are exactly identical in mobility to products obtained by the standard Maxam and Gilbert reactions in agreement with the findings of Friedmann and Brown [1978].

5.5 Discussion

5.5.1 Quantum yield determinations

The results from the quantum yield determinations indicate that the production of single strand breaks by photosensitized reactions is not a very efficient process. The value of ca. \( 10^{-7} \) determined in these experiments is about a factor 10 larger than the value of \( 1.7 \times 10^{-8} \) reported by Ciulla et al. (1986) for the photosensitized cleavage of DNA by the dye Rose Bengal. These quantum yields can be compared with
the values reported for the cleavage of phosphodiester bonds by ionising radiation. Hutchinson (1985) reports G-values* of 0.16 to 0.8 for strand breaks induced by ionising radiation in DNA after alkaline treatment in an oxygenated solution.

Similar values are given by Von Sonntag et al. [1981]. The energy of a 632.8 nm photon corresponds to 1.959 eV. Thus a quantum yield of 3x10^{-7} corresponds to a G-value of 1.53x10^{-5}. Ionising radiation appears thus to be a factor of 10^4 more efficient in effecting strand breaks than the dye sensitized cleavage of nucleic acids. The low quantum yield indicates a very low efficiency for formation of the necessary intermediate reaction products or alternatively, a very low probability of any such intermediates reacting with the substrate.

5.5.2 Mechanism of strand cleavage

These results pertain to the mechanism by which methylene blue sensitises phosphodiester backbone cleavage in nucleic acids. Firstly they show that methylene blue sensitized cleavage of DNA leads to direct strand breaks, without the need of treatment with base. Alkaline sensitivity of photosensitized DNA was well known [Friedmann and Brown, 1978; Dubbelman et al, 1985] and here we confirm the "overt" formation of breaks in neutral solution.

The role of bound dye

Both intercalated and externally bound dye are equally effective in the formation of strand breaks as is indicated by the invariance in yield going from 0 to .2 to 2.0 mM MgCl_2. The addition of 20 mM Mg^{2+} reduces the yield by ca. 50%. This concentration of salt displaces

* The G-value is defined as the number of effects (i.e. strand breaks) that occur after exposure to 100 eV of incident radiation.
substantial amounts of dye from the polymer (as indicated by fluorescence, see chapter 4) and we conclude that the dye has to be bound to the DNA for effective cleavage to occur but does not need to be intercalated.

The role of oxygen

MB$^+$ is a good sensitiser of singlet oxygen and the experimental observations indicate that $^1$O$_2$ plays a role in the formation of strand breaks. This is based on the following observation. 20 mM NaN$_3$ significantly reduces the yield of single strand break formation. Reactions between dye excited state and the azide can be ruled out (see section 5.2). Also, the fact that the combination of azide and MgCl$_2$ is more effective than either of them on their own indicates that the reduction in yield is not simply due to release of dye. $^1$O$_2$ is generated via a collisional induced energy transfer between dye triplet and ground state oxygen. Thus, binding of the dye to the polymer implies that most $^1$O$_2$ will be generated at or near DNA. The negatively charged azide ion will be repelled by the negative polyanion and this explains the absence of any quenching at low azide concentrations. Addition of Mg$^{2+}$ will a) release some dye from the nucleic acid and b) change the binding of the remaining dye to an external mode. This will increase the accessibility of the dye to NaN$_3$. Thus a combination of MgCl$_2$ and NaN$_3$ is more effective than azide alone. The conclusion must be that the reduction in yield on addition of 20 mM NaN$_3$ is due to quenching of $^1$O$_2$ by NaN$_3$. $^1$O$_2$ is thus implicated in the cleavage reaction.

While singlet oxygen is apparently involved in the generation of single strand break formation, some evidence suggests that it is not directly responsible for the cleavage. Firstly, addition of D$_2$O has a
negligible effect on the quantum yield (figure 5.5). The lack of any "D$_2$O"-effect on the cleavage yield can be explained in terms of either the absence of any direct involvement of $^1$O$_2$ in the strand break formation or alternatively, that the reaction rate of $^1$O$_2$ with the nucleic acid is much larger than its aqueous decay rate ($2.5 \times 10^5$ s$^{-1}$).

Recently, Lee and Rogers [1987] report a rate of $5.1 \times 10^5$ M$^{-1}$s$^{-1}$ for the reaction of $^1$O$_2$ with DNA, and similarly Bensasson [private communication] did not find any significant change in $^1$O$_2$ decay rate on addition of DNA. The absence of a fast reaction of $^1$O$_2$ with DNA does indicate that singlet oxygen is not directly involved in the strand break formation. Nieuwint et al. [1985] generated $^1$O$_2$ via the thermal decomposition of an endoperoxide and did not find overt strand breaks, suggesting that singlet oxygen itself is not directly responsible for the cleavage.

From our experimental work it is not clear what species are involved in the singlet oxygen mediated cleavage. A possible intermediate could be a product generated by a back reaction of $^1$O$_2$ with the sensitizer. Brabham and Kasha [1974] give evidence for such a species which was not further identified. This species could be responsible for the strand break formation, but further work is required to confirm the structure photochemical reactivity of this compound.

**Anaerobic cleavage**

Single strand breaks occur in the absence of oxygen but at a reduced yield (ca. 30%) compared to aerated solutions (Figure 5.5). Similar observations have been made for porphyrin sensitised cleavage of nucleic acids [Le Doan et al., 1985]. In the absence of oxygen, electron transfer or hydrogen abstraction has been proposed as possible intermediates in the direct reaction between substrate and sensitizer.
[Loeber and Kittler, 1981; Knowles, 1971]. Hydrogen abstraction from
the nucleotide base is accompanied by "fading" of the dye, ie. a
decrease in the visible absorption of the dye [Knowles, 1971] and
formation of the leuco-form of the dye. This does not appear to be the
case with the photolysis of DNA in the presence of methylene blue.

Figure 5.7 shows the decrease in the visible dye absorption after
photolysis of polynucleotide/dye mixtures in aerated solutions compared
with the bleaching of the dye in the absence of polymer. The bleaching
is reduced most by poly[d(G-C)] and by CT-DNA, followed by poly[d(A-T)].
The protective ability of the nucleic acid appears thus to be inversely
related to the accessibility of the dye to the solvent suggesting that
the formation of the leuco dye proceeds via the solvent and not via
reaction with the bases. Water has been proposed as an intermediate in
the formation of leuco-methylene blue [Somer and Green, 1974]. The
reduced formation of LMB when the dye is bound to DNA indicates
probably that solvent is required for the reduction. It also shows that
the direct reaction of the nucleotides with the dye does not proceed
via hydrogen abstraction. Somer and Temizer [1984] have shown the
evolution of $H_2O_2$ during the photolysis of MB$^+$. This could
disproportionate into OH$^-$ radicals. These radicals are well known to be
able to generate strand breaks [Hutchinson, 1985] and it is possible
that this could be one of the mechanisms for the strand cleavage.

An alternative mechanism for the direct reaction between nucleotide
and dye is electron transfer. Evidence for such electron transfer
between nucleotides and excited state dye has been presented in chapter
3.4. This electron transfer is specific for guanine in agreement with
the results obtained from the sequencing reactions.
Figure 5.7 Decrease in methylene blue optical density under irradiation with light with a wavelength larger than 610 nm. 1: free dye; 2: MB⁺/poly[d(A-T)] P/D-61; 3: MB⁺/poly[d(G-C)] P/D-42; 4: MB⁺/CT-DNA P/D-70. Buffer 50 mM phosphate, pH = 6.9.
Molecular mechanism of cleavage

The results presented here suggest that the dye sensitized cleavage of DNA involves a mechanism similar to that proposed for high intensity laser irradiation [Schulte-Frohlinde et al., 1985; Blau et al., 1987]. In this mechanism, cleavage is initiated by the formation of a oxidized guanine radical $G^+$. This radical will be trapped by oxygen to form a peroxy-radical which can subsequently abstract a hydrogen from the C-4' atom of the ribose-sugar. The involvement of $^{1}O_2$ or of a $^{1}O_2$-dye adduct could be in the formation of the reactive Hydrogen abstracting intermediate. It is well known from studies with ionising radiation that C-4'-sugar radicals can cause strand break formation [Von Sonntag et al., 1981; Schulte-Frohlinde and Von Sonntag, 1985; Hutchinson, 1985]. It should be noted that the products obtained from dye sensitized photolysis (and the excimer laser products) are different from those obtained by $\gamma$-radiation, where the two fragments detected from 5'-end labelled DNA have electrophoretic mobilities identical and greater than the Maxam-Gilbert fragment [Henner et al., 1982 and 1983]. The decreased mobility of the fragment obtained from dye-sensitized photolysis is consistent with a product formed by cleavage of the 3'-phosphodiester bond, but containing a substantial fragment of the -modified- guanine nucleotide. Treatment with alkali will remove this fragment leading to a product identical to the Maxam-Gilbert product, i.e. similar to 5'-phosphodiester bond cleavage. The cleavage reactions are schematically illustrated in figure 5.8.
The exact mechanism in anoxic solutions is less clear. The sequence products are identical to those obtained in aerated solutions and thus it is assumed that the reaction also proceeds via $\text{G}^+$ radicals and an unidentified intermediate which subsequently abstracts a hydrogen atom from the ribose C-4' atom, yielding a result similar to that obtained in aerated solutions. However, the reactive intermediate can now not be a peroxy-intermediate as oxygen was excluded from the solution. It is of course possible that trace amounts of oxygen could be sufficient for the aerobic reaction to occur, but consistent reproducibility in repeated experiments appears to exclude that possibility. A possible mechanism could involve direct hydrogen abstraction by the nucleotide radical but confirmation of this has to await further experimental work. Alternatively, $\text{H}_2\text{O}_2$ could be generated and subsequent OH' radicals could be responsible for the cleavage (see above).

Interestingly, a similar observation of the effect of oxygen on the formation of single strand breaks in DNA is observed on the exposure of DNA to ionising radiation [Schulte-Frohlinde and Von Sonntag, 1985]. Here the reason is also not clear.
CHAPTER 6

ENERGY TRANSFER IN DYE-NUCLEIC ACID COMPLEXES
INTRODUCTION

Studies of excited state energy-transfer has proved fruitful in the analysis of the structure of molecular assemblies. In this chapter, preliminary results are presented on studies of intrinsic energy-transfer in DNA and on extrinsic transfer between molecules which are bound to the polynucleotide.

This chapter consists of two distinct sections. In the first section a numerical computer simulation is presented which predicts fluorescence rise-times for intercalated dye molecules sensitized by energy transfer from DNA nucleotides. The simulation calculates rise-times as a function of the average rate of singlet-singlet energy-transfer between the nucleotides and the dye. The simulation is used to estimate the average distance over which singlet energy can be transferred in the nucleic acid. Similarly, estimates are made of the base-base transfer rate.

In the second part of this chapter preliminary evidence is presented for resonant Forster-type energy transfer between the compounds methylene blue and ruthenium-(phenanthroline)$_3^{2+}$. It is shown that a necessary condition for efficient transfer is that both of the compounds are bound to DNA. Time-resolved measurements of this energy-transfer indicate that static Forster transfer is not applicable. Instead, a combination of molecular diffusion along the polymer and Forster transfer is shown to fit the data.
6.1 Introduction

The study of excited state deactivation processes in DNA continues to attract considerable attention. The study of these processes is of importance for two main reasons [Helene, 1974]: Firstly, it allows for the determination of the relative importance of the diverse deactivation processes in the nucleic acids. This enables the elucidation for the molecular mechanism of photochemically induced damage. Secondly, study of excited state deactivation pathways will yield insight into the short- and long-range interactions between the constituents in the polymer and will lead to a better understanding of its conformation in solution.

One aspect of the excited state decay processes which has received attention is the determination of the possible transfer of electronic energy and/or electrons along the nucleotide. The transfer of excited state energy between nucleotide bases in DNA is of considerable interest as it is implicated in the photochemical degradation of nucleic acids. Understanding of these processes would thus be of significant biological importance. Evidence has been presented for electron and hole transfer in nucleic acids after irradiation with ionizing radiation and high intensity U.V.-laser light pulses [Boon et al., 1984; Magan et al., 1987] as well as for long range triplet exciton interactions in synthetic polynucleotides such as polyadenilic acid at low temperatures (77 K) [Bersohn and Isenberg, 1964; Eisinger
In contrast, evidence for the transfer of singlet excited state energy between nucleotide bases is less secure. Theoretical calculations, based on Forster-type interactions in a Watson-Crick geometry, indicate that the singlet transfer rate is exceedingly fast and of the order of $10^{13} \text{s}^{-1}$ [Gueron et al., 1967; Dee and Bauer, 1974]. This can be compared with observed singlet decay times of less than 80 ps [Georghiou et al., 1985] and with triplet exciton transfer rates (in poly(rA)) of $5 \times 10^9 \text{s}^{-1}$ [Sommer and Jortner, 1968]. The distance over which transfer can occur has been calculated as 10 basepairs [Dee and Baur, 1974].

Experimental determination of the rate of singlet-singlet energy-transfer in DNA and the range over which such transfer can take place yield ambiguous results. The majority of experiments to determine singlet energy-transfer in nucleic acids consists of measurements of the fluorescence of intercalated dye molecules sensitized by light absorbed by the nucleic acid [Weill and Calvin, 1963; Shapiro et al., 1975; Anders, 1978 & 1979; Rayner et al., 1980]. Transfer distances obtained from these experiments range from 2 basepairs [Rayner et al., 1980], to 10 - 20 basepairs [Weill and Calvin, 1963], to several hundred basepairs [Anders, 1978 & 1979; Nikogosyan et al. 1985].

Shapiro et al. [1975] have attempted to measure the singlet transfer rate and transfer distance directly. This was done by measuring the risetime of the fluorescence of the intercalated dye acridine orange after excitating at 266 nm (i.e. in the main DNA absorption band) using a picosecond laser with a 10 ps FWHM. The only result reported by Shapiro et al. is shown in figure 6.1. From this figure they estimated a risetime of 20 ps for the acridine orange fluorescence. They claim that this risetime is due to energy-transfer. This has so far been the only reported attempt to measure directly the rate of transfer of
singlet state energy between nucleotides in DNA.

Figure 6.1 Densitometer trace of risetime of fluorescence emission of a DNA/acridine orange complex (P/D = 400) after excitation with a 266 nm 10 ps laser pulse (from Shapiro et al. [1975]).

Part of the original proposal for the research presented in this thesis was the determination of the extent and magnitude of singlet-singlet energy-transfer in nucleic acids using picosecond laser techniques. The intention was to repeat the experiment of Shapiro et al. using short (ca. 1 ps) laser pulses and modern streak camera techniques. However, considerable problems with the available picosecond laser system prevented such an experimental study to be undertaken.

As an alternative it was decided to model the experiment numerically. Computer simulation of the energy transfer from nucleic acid to dye would enable possible confirmation of the transfer rate as determined by Shapiro et al. as well as enable a prediction of the transfer rate.

The laser system is described extensively in Dennis [1985].
results of future experiments with a functioning picosecond laser system. The simulation was performed for the dyes acridine orange and methylene blue.

6.2 Computer model

The computer model used to perform the simulations is a modification of the program developed by Penzkofer and Blau [1983]. This is a general model to calculate the interaction of laser light pulses with dye molecules. In its original form the dye molecule is assumed to consist of six energy levels. This was reduced to three in the model here, and three energy levels for the biopolymer were added. Figure 6.2 gives a schematic diagram of the energy levels involved. This model of the energy levels with associated rate-constants for inter-system crossing, internal conversion, radiative decay and energy transfer yields the coupled rate equations 6.1a to 6.1f:

\[
\begin{align*}
\frac{dPs0}{dt} &= -\frac{I(t)}{h\nu} \sigma_{pl} Ps0 + \frac{(1-\phi)}{TP_{s}} Ps1 + \frac{Pt1 + k_{tr} Ps1}{TP_{t}} \\
\frac{dPs1}{dt} &= +\frac{I(t)}{h\nu} \sigma_{pl} Ps0 - Ps1 - \frac{k_{tr} Ps1}{TP_{s}} \\
\frac{dPt1}{dt} &= +\frac{\phi}{TP_{s}} Ps1 - Pt1 \\
\frac{dDs0}{dt} &= -\frac{I(t)}{h\nu} \sigma_{dl} Ds0 + \frac{(1-\phi_{d})}{TD_{s}} Ds1 + \frac{Dt1 - k_{tr} Ps1}{TD_{t}}
\end{align*}
\]
In these equations, \( I(t) \) is the time-dependent intensity of the exciting laser pulse. The cross-sections for absorption of light by polymer and dye are respectively \( \sigma_{p1} \) and \( \sigma_{d1} \). \( Ps0, Ps1 \) and \( Pt1 \) are respectively the population of the polymer ground state \( S_0 \), the polymer singlet excited state \( S_1 \) and the polymer triplet state \( T_1 \). \( Ds0, Ds1 \) and \( Dtl \) are those for the dye energy levels. \( TP_s \) and \( TP_t \) are respectively the lifetimes of the polymer excited singlet and triplet state (in sec). \( TD_s \) and \( TD_t \) are those for the dye excited states. \( \phi_p \) and \( \phi_d \) are the yields of triplet state population for polymer and dye. Finally \( k_{tr} \) is the rate of energy transfer from the nucleic acid to the dye.

Several assumptions underlie this model. These will be discussed in the following.

- In this model the nucleic acid is represented as a single entity, with its characteristic lifetimes and triplet yield. In reality the polymer consists of a series of individual nucleotide bases, each with its own excited state properties. Energy transfer will take place between these units. Taking proper account of this would significantly complicate the model. \( N \) nucleotide bases would for instance yield \( 3N + 3 \) coupled rate equations. It was felt that for the preliminary study presented here such extension would not be necessary. Consequently, the rate for energy-transfer represents the average rate (in s\(^{-1}\)) for the transfer of all the nucleotides in the polymer to the dye. This is
Figure 6.2
Figure 6.2 Energy level diagram illustrating the radiative (solid) and non-radiative (wavy) processes in DNA and an intercalated dye. Dye a: acridine orange, b: methylene blue. Heavy arrow indicates direction of postulated (non-radiative) energy transfer.
not equal to the base-base transfer rate. An estimate of the distance over which transfer can occur can be obtained assuming a base-base transfer rate of $10^{+13} - 10^{+12} \text{s}^{-1}$ [Gueron et al., 1967].

- The use of a three level model for the individual molecules implies neglect of excited state absorption. Singlet-singlet ($S_1 \rightarrow S_2$) absorption will only be of importance at high intensities. Such a situation can easily be avoided in an experimental situation. Triplet-triplet ($T_1 \rightarrow T_2$) absorption is ignored as the population of the triplet state will be small on a picosecond time scale.

- Polarization, solvent reorientation and vibrational relaxation effects are ignored. The molecule is assumed to have only sharply defined energy levels. It is a relatively simple matter to incorporate these effects into the model [see Penzkofer and Blau, 1983].
### 6.3 Input variables

In order to solve equations 6.1 - 6.2, it was necessary to determine values for the excited state properties of the "polymer-molecule" and the dye molecule. Table 6.1 summarizes the values used for DNA.

<table>
<thead>
<tr>
<th>Excited state property</th>
<th>Value</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>Singlet lifetime</td>
<td>$T_P^s$</td>
<td>1, 10 and 20 ps (a)</td>
</tr>
<tr>
<td>Triplet lifetime</td>
<td>$T_P^t$</td>
<td>1 μs (b)</td>
</tr>
<tr>
<td>Triplet yield</td>
<td>$\phi_p$</td>
<td>0.02 (b)</td>
</tr>
</tbody>
</table>

Table 6.1 Excited state properties of DNA used in energy-transfer simulation program.

Notes: (a) Widely varying lifetimes for DNA emission have been quoted, ranging from a few picoseconds [Nikogosyan et al., 1985] to 10 ps [Georghiou et al. 1985] and several nanoseconds [Ballini et al. 1983]. The latter emission is presumably due to excimer emission or from impurities. (b) [Salet et al. 1981]. Triplet lifetime is an estimate. $T_P^t$ is much larger than both $T_P^s$ and $k_{tr}$ and results are insensitive to its exact value.

The values for the dye excited states were those found in literature (for acridine orange) or those obtained in this work (methylene blue). The values are (acridine orange): $T_D^{s} = 4.5$ ns, $T_D^{t} = 2$ ms and $\phi_d = 0.2$ [Kubota, 1973; Geacintov et al., 1981]; (methylene blue): $T_D^{s} = 400$ ps, $T_D^{t} = 20$ us and $\phi_d = 0.5$. Additional calculations were performed to determine the influence of the dye absorption at 260 nm. This was done with a fictitious dye which has the excited state parameters of methylene blue except where the extinction coefficient of the dye was varied from 100 to 6,600 to 50,000 $\text{M}^{-1}\text{cm}^{-1}$. These values represent situations in which the dye extinction is significantly less, equal and larger than the DNA extinction coefficient (6,600 $\text{M}^{-1}\text{cm}^{-1}$ at 260 nm).

The time-dependence of the exciting laser was assumed to be Gaussian.
Other parameters of the exciting laser pulse are a wavelength of 260 nm and a Full Width at Half Maximum (FWHM) which was varied from 0.1 to 1 to 10 ps. A final input parameter is the concentration of the intercalated dye. The concentration of the polymer was fixed at $10^{-3}$ M (phosphate) in all calculations. In most calculations a P/D of 400 was used.

The coupled differential equations are solved using simple step-wise integration in the time interval from $-3\times\text{FWHM}$ to $+3\times\text{FWHM}$. The laser pulse is set zero outside this interval. The emission from the dye is assumed proportional to the dye $S_1$ population. This $S_1$-population is calculated in the program in the case of no transfer ($KTR = 0$) and in the case with energy-transfer. Output from the program is a plot of exciting laser pulse profile, DNA $S_1$ and dye $S_1$ population with and without energy transfer. The latter is plotted normalised to the former to enable determination of the extent of the transfer efficiency. Also the risetime of the dye $S_1$ population is given for the two cases. The risetime is defined as the time interval in which the dye excited state population rises from 10% to 90% of its end value.

The program was implemented in GWBASIC on an IBM-PC compatible machine. CPU time of the program is approximately 4 minutes on an AMSTRAD PC1512. A listing of the program is given in Appendix A-3.

6.4 Results

Figures 6.3 - 6.5 show results obtained from the computer simulation in the case of acridine orange. Figure 6.3 assumes a DNA $S_1$-
Figure 6.3
Figure 6.3 Results from computer simulation according to equations 6.1a-f. Conditions were chosen to be similar to those by Shapiro et al. [1975] i.e. DNA/acridine orange, P/D = 400. DNA singlet lifetime is assumed to be 1 ps.
DNA - ACRIDINE ORANGE P/D=400, ENERGY TRANSFER

DNA CONCENTRATION (M) 1.0D-03
DYE CONCENTRATION (M) 2.5D-06
TIME INTERVAL (s) 6.0D-12
LASER FWHM (s) 1.0D-11
TRANSFER RATE (s-1) 1.0D+11

DNA S1 population

DYE S1 population and risetime (ps)
without transfer 0.93D-11
with transfer 0.17D-10
Ok

Figure 6.4
Figure 6.4 As Fig. 6.3. DNA S₁-lifetime 10 ps.
DNA - ACRIDINE ORANGE P/D=400, ENERGY TRANSFER

DNA CONCENTRATION (M) 1.0D-03
DYE CONCENTRATION (M) 2.5D-06
TIME INTERVAL (s) 6.0D-12
LASER FWHM (s) 1.0D-11
TRANSFER RATE (s-l) 1.0D+11

DNA S1 population

DYE S1 population and risetime (ps)
without transfer 0.93D-11 ●
with transfer 0.18D-10 ○

Figure 6.5
DNA - ACRIDINE ORANGE P/D-400, ENERGY TRANSFER

DNA CONCENTRATION (M) 1.0D-03
DYE CONCENTRATION (M) 2.5D-06
TIME INTERVAL (s) 6.0D-12
LASER FWHM (s) 1.0D-11
TRANSFER RATE (s-1) 1.0D+12

DNA S1 population

DYE S1 population and risetime (ps)
without transfer 0.93D-11 *
with transfer 0.97D-11 ♦

Figure 6.5 As Fig. 6.3. DNA S1-lifetime 20 ps.

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lifetime of 1 ps, figure 6.4 one of 10 ps and figure 6.5 one of 20 ps. Each figure shows transfer-rates which range from $10^{+13}$ to $10^{+10}$ s$^{-1}$. The laser FWHM is 10 ps and the dye concentration is 1/400 of the DNA concentration in all cases, in agreement with the values as reported by Shapiro et al.

Figure 6.6 shows a similar simulation for methylene blue, now with a laser of 1 ps FWHM and a P/D equal to 10. Calculations using a fictitious dye with varying extinction coefficients show that the value of the extinction coefficient is not important at a P/D of 400. All yield similar results (not shown) and it was decided not to investigate this any further.
Figure 6.6 As Fig. 6.3. DNA/methylene blue complex. DNA $S_1$ lifetime is assumed to be 20 ps.
6.5 Discussion

Shapiro et al. [1975] obtained a fluorescence risetime of ca. 20 ps for acridine orange sensitized by DNA at a P/D of 400. The simulation shows that the observed emission indeed has to be attributed to the sensitized fluorescence of the dye and not to the directly excited fluorescence of the dye. Although acridine orange has a significantly higher extinction coefficient at 260 nm compared to DNA (57,000 viz. 6,600 M$^{-1}$cm$^{-1}$), at a P/D of 400 the amount of light absorbed by the dye is negligible. It has to be realized that the simulations were performed by firstly calculating the dye S$_1$-population with energy-transfer present and then without such transfer. The latter was then normalized on the former. In the calculation of the dye excited state population without transfer the polymer is still present in solution and absorbs light. Experimentally this would correspond to a situation where dye binding to the polymer is inhibited by the addition of a large excess of salt.

The figures show that a fluorescence risetime of ca. 20 ps as reported by Shapiro et al. can only be achieved with a nucleic acid singlet lifetime of 10 - 20 ps and a transfer rate of $10^{+10}-10^{+11}$ s$^{-1}$. A larger transfer rate will result in a close following of the rise of the laser pulse by the excited state population of the dye. The value for the transfer rate which follows that determined experimentally is surprisingly low. However, it has to be remembered that the rate calculated here is an average transfer rate, weighed over all the nucleotides which can transfer their excited state energy to the dye. It is possible to estimate from this rate the base-base transfer rate. At a P/D of 400 a dye molecule will be surrounded by 200 basepairs on
each side. Assuming an equal probability for forward and backward transfer of the excited state energy, an upper estimate of the number of basepairs that can transfer their energy to the dye molecule is ca. 100. With the average rate constant determined from the computer calculations as $10^{+10} - 10^{+11}$ s$^{-1}$ this yields a value for the base-base transfer rate of $10^{+12} - 10^{+13}$ s$^{-1}$. Alternatively, assuming values of $10^{+12} - 10^{+13}$ s$^{-1}$ as rate constants for base-base singlet energy transfer after Gueron et al. [1967], an estimate of the transfer distance of ca. 100 basepairs is obtained. Both different arguments yield similar results. It does appear that the transfer of excited state singlet energy can take place over a considerable distance. The value of a transfer distance of ca. 100 basepairs agrees reasonably well with the value of 170 as derived by Nikogosyan et al. [1985] in view of the assumptions of the model and its inherent simplification.

Finally, energy transfer is also predicted to increase the fluorescence risetime in the case of methylene blue. This dye could thus also be used as a probe molecule.

6.6 Conclusion

The simulation of the fluorescence rise-time experiment of Shapiro et al. on a computer yields an upper estimate for the transfer of excited state singlet energy in DNA of 100 basepairs. This is in agreement with the most recent report from literature [Nikogosyan et al., 1985], but is in disagreement with others, notably Rayner et al. [1980]. The latter authors claim a maximum transfer distance of 2 basepairs.

Alternatively, the computer simulation suggests a base-base transfer rate of $10^{+12} - 10^{+13}$ s$^{-1}$. The higher rate is in reasonable agreement with values predicted from theoretical considerations [Gueron et al.,
The preliminary simulation study presented here shows that there is considerable scope for more sophisticated computer programs which take specifically account of the individual nucleotide bases. This would also enable an estimation of possible base-specific variations in the transfer possibilities. Possible methods of tackling the problem of a large number of sites with absorbing dye "traps" is given by Rodenstock et al. [1961], Levinson [1962] and Fitzgibbon and Franck [1982] using analytical expressions derived for random walks on a one-dimensional lattice.

Besides computer simulations, it is obvious that there is also an urgent need for more experimental data and verification of the results obtained by Shapiro et al.
SECTION II

DYE-DYE ENERGY TRANSFER IN NUCLEIC ACIDS

6.7 Introduction

The previous section has indicated that fluorescence spectroscopy is a powerful tool in the investigation of electronic processes in nucleic acids and can yield information on singlet energy transfer in the polymer. Similarly, energy transfer (as determined with fluorescence spectroscopy) has also been shown to be a powerful tool in the elucidation of the tertiary structure of macromolecules and macromolecular assemblies [reviews: Turro, 1977; Fairclough and Cantor, 1978; Cundall and Dale, 1980; Lakowicz, 1984]. Singlet-singlet energy transfer has proved to be very successful in explaining photophysical and structural phenomena such as excimer formation in synthetic polymer systems [North and Treadaway, 1973; Ng and Guillet, 1982a & b]. Similarly, energy transfer studies have enabled the building up of a detailed picture of the structure of the photosynthetic unit [Schaffner, 1986]. Singlet-singlet energy transfer between the bases in DNA has already been discussed above. In contrast, Paoletti and Le Pecq [1971] used experimental data, combined with computer simulations, to determine the extent of energy transfer between ethidium bromide molecules intercalated in the helix. They obtained thus information about the binding of the dye to the polymer.

In the course of the study presented here, it appeared that a study of energy transfer between dissimilar molecules, both bound to DNA, could yield information on the binding of these molecules to the
polymer as well as on the structure of the polymer. In effect this investigation would be an extension of the well known studies on energy transfer in synthetic polymers [e.g. North and Treadaway, 1973] to nucleic acids. To our knowledge such investigations have not been reported yet. Yamabe [1973] reported that the fluorescence of acridine orange (AO+) intercalated in DNA, was quenched on addition of methylene blue. Both dyes were considered bound. Yamabe considered this effect of MB+ to be due to its "weakening" of the interaction between acridine orange and the polymer. In view of the results presented below it is more likely that the explanation is due to Forster-type energy transfer from AO+ to MB+. Recently Barton et al. [1986] reported that DNA mediates electron-transfer between electron donor and acceptor molecules, both of which were bound to DNA. This enhanced electron-transfer was explained on the basis of a one-dimensional diffusion of the dye molecules along the double helix. It should be possible to determine this diffusion coefficient from singlet-singlet energy transfer measurements. The measurement of the one-dimensional diffusion coefficient along DNA has biological significance. Von Hippel et al. [1984] report a rate of diffusion of promotor proteins along nucleic acids of ca. $10^{-10}$ M$^{-1}$ s$^{-1}$ i.e. faster than a diffusion controlled rate constant. It is conceivable that measurements of diffusion of molecules along the nucleic acid polymer could shed some light on the important area of molecular recognition in the genetic transcription process.

The donor and acceptor molecules which were decided on in this study are Ruthenium(phenantroline)$_3^{2+}$ and Methylene Blue. Both molecules bind to DNA. The binding of Ru(phen)$_3^{2+}$ and associated derivatives has been extensively studied by Barton et al. [1985a,b, 1986], Kelly et al. [1985b] and Tossi [1987] and the binding of methylene blue has been discussed in this work (chapter 4). The luminescence spectrum of Ru(phen)$_3^{2+}$ has a maximum around 600 nm [Tossi, 1987] and overlaps...
strongly with the absorption spectrum of methylene blue. This facilitates a dipole-dipole induced energy-transfer from the excited state of Ru(phen)$_3^{2+}$ to methylene blue according to the mechanism proposed by Forster [1959]. Efficient energy transfer from the excited state of Ruthenium compounds to the singlet state of laser dyes has already been demonstrated [Mandal et al., 1980; Mandal et al., 1983]. The Forster-mechanism is generally invoked for the transfer of excited state energy between singlet states and there is thus indication that the excited state of the Ru-complex (thought to be a metal-ligand charge transfer state [Seddon and Seddon, 1984]) has a considerable amount of singlet character. An additional advantage of using Ru(phen)$_3^{2+}$ as a donor molecule is its long-lived excited state ( > 1 μs) when bound to DNA [Tossi, 1987], which enables an accurate determination of its excited state deactivation in the presence of the acceptor dye. The aim of the experiments presented here was thus firstly to establish whether binding to DNA will increase energy transfer between Ru(phen)$_3^{2+}$ and MB$^+$ and secondly to establish the mechanism of this transfer. Steady state fluorescence spectroscopy and time-resolved luminescence measurements were the experimental techniques used.

6.8 Experimental

6.8.1 Determination of Forster transfer distance

The Forster transfer mechanism involves a coupling of the dipoles of the excited state of a donor D and the ground state of an absorber A. This can be visualised as a simultaneous transition $D^* \rightarrow D$ and $A \rightarrow A^*$. The probability of these reactions occurring is proportional to the emission and absorption probabilities of the donor and acceptor,
respectively. The rate of transfer is furthermore related to the energy of this dipole-dipole interaction and thus proportional to $1/R^6$, with $R$ the distance between donor and acceptor. It is common to express the efficiency of transfer in a distance, the Forster-distance $R_0$, which is that distance at which the rate of energy-transfer equals the decay rate of $D^*$ in the absence of $A$ [Turro, 1977]. The parameter $R_0$ is a useful parameter in energy-transfer studies. An analytical expression for $R_0$ has been given in equation 6.2 [Forster, 1959; Lakowicz, 1984]:

$$R_0^6 = \frac{9000 \ln(10) k^2 \psi_d}{128 \pi^4 N_a n^4} \int_0^\infty \frac{F_d(\nu) E_a(\nu)}{\nu^4} d\nu$$

or

$$R_0^6 = 9.79 \times 10^3 (k^2 \psi_d n^{-4} J)^{1/6}$$

In these equations $N_a$ is Avogadro's number, $n$ is the refractive index of the solvent, $\psi_d$ is the fluorescence quantum yield of the donor in the absence of acceptor, $F_d(\nu)$ is the emission spectrum of the donor, normalised by setting $\int_0^\infty F_d(\nu) d\nu$ equal to 1, $E_a(\nu)$ is the absorption spectrum of the acceptor (in $M^{-1} cm^{-1}$) and $k$ is a factor which expresses the relative orientation of the transition dipoles of donor and acceptor. $J$ is the total spectral overlap integral.

Emission yields were determined as described in chapter 3.1, using Ruthenium-trisbipyridyl as a reference compound. Its quantum yield in a deoxygenated aqueous solution has been determined as 0.042 [Kalyanasundaram, 1982]. The emission yield for Ru(phen)$_3^{2+}$ has so been determined as 0.062 and for a Ru(phen)$_3^{2+}$/CT-DNA complex (P/D=300) as 0.137. In a solution without DNA, the transition dipoles will be
randomly oriented with respect to each other and $k^2$ is equal to $2/3$ [Turro, 1977]. In the case of polymer bound molecules, the transition dipoles are assumed to be in parallel planes, but randomly oriented in these planes (Figure 6.7). In this case $k^2$ is equal to .476 [Lakowicz, 1984 page 309]. Finally, the values for the refractive indices were taken as 1.332 for the aqueous solution and 1.6 for the DNA-complex [Hakim et al., 1984].

Figure 6.8 shows the overlap integral for the dyes free in solution and bound to DNA and table 6.2 gives the values of the overlap integrals and the Forster transfer distances.

<table>
<thead>
<tr>
<th></th>
<th>integral</th>
<th>$R_o$ (in Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>in solution</td>
<td>$6.4 \times 10^{-13}$</td>
<td>44</td>
</tr>
<tr>
<td>bound to DNA</td>
<td>$5.55 \times 10^{-13}$</td>
<td>41</td>
</tr>
</tbody>
</table>

Table 6.2 Overlap integrals and Forster transfer distances for the system Ru(phen)$_3^{2+}$-MB$^+$, in H$_2$O and bound to DNA.

The excellent overlap between the luminescence spectrum of Ru(phen)$_3^{2+}$ and the absorption spectrum of MB$^+$ yields a fairly large transfer distance. The distance found here is in good agreement with similar values reported for transfer between Ru(bipy)$_3^{2+}$- and dyes like Nile Blue A and Oxazine 1 [Mandal et al., 1983].

6.8.2 Energy transfer in CT-DNA

Figure 6.9 shows the fluorescence emission of a $2 \times 10^{-6}$ M solution of Ru(phen)$_3^{2+}$ in a 1 mM phosphate buffer (pH=6.9) on addition of different amounts of methylene blue in solutions with and without CT-DNA. Excitation was at 460 nm and the emission was measured at 595 nm. It was checked that direct absorption by methylene blue of the
Figure 6.7 Schematic illustration of position of intercalated MB$^+$ and Ru(phen)$_3^{2+}$ in DNA. The double helical structure is simplified to a cylinder. Transition dipole moments of the molecules are drawn as solid black lines. Distance between the molecules is denoted with $R$. 
buffer

CT-DNA
Figure 6.8 Diagrams illustrating extent of overlap between fluorescence spectrum of Ru(phen)$_3^{2+}$ and absorption spectrum of MB$^+$. Vertical units in diagram are arbitrary.
Figure 6.9 Fluorescence emission of Ru(phen)$_3^{2+}$ (2x10$^{-6}$ M) in a 1 mM phosphate buffer on addition of MB$^+$. a: no DNA present. b: In the presence of CT-DNA (F/Ru=40). c: in the presence of poly[d(G-C)] (F/Ru=40). d: in the presence of poly[d(A-T)] (F/Ru=40). Excitation wavelength 460 nm and emission wavelength 595 nm.
Ru(phen)$_3^{2+}$ emission could be neglected at all methylene blue concentrations used. The ratio P/[Ru] was 40 in all solutions and the maximum overall P/D ratio was 14. Both compounds are assumed to be bound to DNA. This was verified by determination of the absorption spectrum of the complex and both dyes exhibit the characteristic changes in their absorption spectra associated with binding to DNA [This work, chapter 3; Tossi, 1987] (not shown).

The figure shows that in buffer, at the dilute concentrations of both compounds used, no quenching occurs. However, when both dyes are bound to the biopolymer a strong decrease in donor emission can be observed.

Figure 6.9 also shows the quenching of donor emission by methylene blue in the presence of the synthetic polynucleotides poly[d(A-T)] and poly[d(G-C)]. The very much diminished quenching at low methylene blue concentrations presumably indicates the basepair specific nature of the binding of both Ru(phen)$_3^{2+}$ (to A-T basepairs [Tossi, 1987]) and methylene blue (stronger to G-C basepairs, chapter 4).

The steady state emission quenching experiment was repeated with different DNA concentrations. In all cases the absorption at the emission wavelength (595 nm) was measured to exclude the possibility of an inner-filter absorption effect due to the addition of methylene blue. The absorption at this wavelength was in all cases smaller than 0.08 and necessitated a small correction in the emission of Ru(phen)$_3^{2+}$. The fact that according to the absorption spectra both compounds are bound to the polymer and the relative absence of an inner-filter effect appears to secure the fact that the observed quenching is caused by transfer of excited state energy from

* The fluorescence of methylene blue is quenched by DNA. Also its emission is very much weaker than that of Ru(phen)$_3^{2+}$. It was therefore more feasible to determine the extent of energy-transfer by measuring the quenching of the donor luminescence than the determination of the sensitized fluorescence of MB$^+$. 262
An attempt was made to determine the mechanism of this luminescence quenching. Figure 6.10 shows representative Stern-Volmer plots of the quenching of the donor fluorescence at different P/D ratios. These plots are clearly not-linear. The significance of this will be discussed in the discussion.

Luminescence kinetics of Ru(phen)$_3^{2+}$ were measured on the laser spectrometer with as excitation source the Oxford EDL-1 dye laser, pumped by an excimer laser. Excitation was in the MLCT-band of Ru(phen)$_3^{2+}$ with a wavelength of 450 nm (Coumarin 1 in methanol). Figure 6.11 shows a representative decay of the luminescence of Ru(phen)$_3^{2+}$/CT-DNA measured at 605 nm, on addition of methylene blue. From plots of log(intensity) versus time it is clear that addition of methylene blue causes the excited state of Ru(phen)$_3^{2+}$ to decay in a non-exponential manner. Analysis of kinetic traces as shown in figure 6.11 was performed using an adapted version of the standard program LUMDEC (see chapter 7 and below). In agreement with the results obtained form the steady state fluorescence experiments, the decay of Ru(phen)$_3^{2+}$ is not affected by addition of methylene blue when no DNA is present (not shown).

6.9 Discussion

The results indicate that efficient energy-transfer occurs between Ru(phen)$_3^{2+}$ and Methylene Blue when both dyes are bound to DNA. Under the experimental conditions used here, no transfer occurs when DNA is absent from the solution.

The Stern-Volmer plots of the quenching of the luminescence are not linear, indicating that the no diffusional mixing of donotr and acceptor occurs over the lifetime of the excited state of the donor...
DNA: $6.25 \times 10^{-5}$ M

$\frac{I_0}{I}$

$\ln\left(\frac{I_0}{I}\right)$

MB $\times 10^6$ (M)
Figure 6.10 Stern-Volmer (●) and Perrin plots (■) of the emission quenching of Ru(phen)$_3^{2+}$ by methylene blue. A: DNA phosphate $= 6.25 \times 10^{-5}$ M, B: DNA $= 2.5 \times 10^{-4}$ M.
A upwardly curved Stern-Volmer plot is what is expected for Forster-transfer in which the distribution of donor and acceptor molecules is random and where the molecules are immobile during the decay of the donor. [Forster, 1959; Lakowicz, 1984]. It has however been suggested that other (non-random) distributions and/or mobility of donor and acceptor molecules will give similar curves [Lakowicz, 1984]. It should be noted that, if the donor and acceptor are effectively stationary during the lifetime of the donor excited state, the exponential decay of the transient donor luminescence will follow Forster kinetics [Ng and Guillet, 1982b].

Subsequently the data was fitted to the Perrin formalism. The Perrin formalism involves a plot of log(I_0/I) versus acceptor concentration. If this plot is linear then the quenching of donor emission by the acceptor can be envisaged as taking place in a sphere with volume V in which the quenching efficiency of the acceptor equals 1. Outside the sphere the quenching efficiency is considered to be 0 [Turro, 1977]. Energy transfer is thus only supposed to take place if donor and acceptor are inside the "active sphere".

The DNA molecule is fairly rigid (chapter 1) and as both donor and acceptor are bound to the polymer, the concentration of acceptor in solution has now to be treated as one-dimensional. This one-dimensional concentration was approximated as follows. At a P/D of X, 1/X dye molecules are bound per phosphate and thus 2/X dye molecules per basepair. This yields then a concentration A' of 7.2/X dye molecules per angstrom. The Perrin-plot can then be written as (equation 6.3):

$$\text{Log}(I_0/I) = L \ [A']$$  \hspace{1cm} (6.3)

The value of L (in Å) is then the length of the effective quenching cylinder. Inside the cylinder the quenching is assumed to take place.
with unity efficiency; outside the cylinder no quenching takes place.
It has to be understood that this is a rather crude approximation.
Analysis of the data in figure 6.10 shows that the quenching data
accurately fits the Perrin formalism. Table 6.3 gives the values of L
derived for a series of DNA concentrations from figures as 6.10. The
Ru(phen)$_3^{2+}$ concentration was kept constant in all the experiments at
5.6x10$^{-6}$ M.

<table>
<thead>
<tr>
<th>[DNA] (M)</th>
<th>initial P/[Ru]</th>
<th>L (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 10$^{-4}$</td>
<td>44.6</td>
<td>16</td>
</tr>
<tr>
<td>1.9</td>
<td>33.4</td>
<td>15.2</td>
</tr>
<tr>
<td>1.3</td>
<td>22.3</td>
<td>10.8</td>
</tr>
<tr>
<td>6.3 10$^{-5}$</td>
<td>11.2</td>
<td>6.8</td>
</tr>
</tbody>
</table>

Table 6.3. One-dimensional Perrin length, determined for the
quenching of the emission of Ru(phen)$_3^{2+}$ by Methylene Blue in the
presence of CT-DNA.

As a caveat it has to be remarked that logarithms tend to "smooth"
out non-linearities. The interpretation of the data in terms of the
Perrin-formalism has therefore to be done with caution.

Analysis of the kinetics of the donor luminescence can yield
additional information on the transfer mechanism. The analysis was
firstly performed by comparing the experimental data to calculated
decays according to the standard Forster kinetics [Lakowicz, 1984].
Forster kinetics is applicable when the donor and acceptor are
effectively "frozen" on the time-scale of the decay of the donor and
appears applicable in view of the curved Stern-Volmer plots. The
equations for Forster decay are given in 6.4-6.8. Analysis of the
kinetic quenching data was performed manually and examining the fit of
the decay (as judged by eye and the value of chi-squared). Figure 6.11
shows an example of a best fit according to the Forster kinetics. The
**Figure 6.11** Representative kinetic trace of decay of $2 \times 10^{-6}$ M $\text{Ru(phen)}_3^{2+}$ in the presence of $\text{MB}^+ (4.6 \times 10^{-6}$ M) and $\text{DNA} (9 \times 10^{-4}$ M) after excitation with a 10 ns, 460 nm laser pulse from the Oxford EDL-1 laser. The trace is fitted to decay according to Forster. The fit is not good.
analysis indicates that no satisfactory fit could be obtained by simple Forster-kinetics.

It was found subsequently that a theory which takes account of a limited diffusion of donor and acceptor gave better fits. This theory was developed by Yokota and Tanimoto [1967] to explain decays of donor molecules in the presence of energy-transfer and in the presence of diffusion. Diffusion is however not so fast that Stern-Volmer kinetics are applicable [North and Treadaway, 1973; Ng and Guillet, 1982b]. The decays according to both the Forster and the Yokota-Tanimoto formalism have the general form:

$$I_D^\ast(t) = I_o \exp\left(-t/\tau_o\right) - 2B \left(R_o/R_{\text{dis}}\right)^3 \left(t/\tau_o\right)^{0.5}$$

with $R_o$ the Forster distance and $R_{\text{dis}}$ the average distance of donor and acceptor. In the case of the analysis by Yokota and Tanimoto, $B$ equals:

$$B = \left(\frac{1 + 10.87X + 15.5X^2}{1 + 8.743X}\right)^{3/4}$$

$$X = D \alpha_{da}^{-1/3} t^{2/3}$$

with

$$\alpha_{da} = \frac{R_o^6}{\tau_o}$$

Equation (6.4) reduces to standard Forster kinetics when $B = 1$, which is the case when the diffusion coefficient $D = 0$.

Analysis was performed as described previously. It was now necessary to specify a diffusion coefficient to get a reasonable fit of the data to the calculated curve. Figure 6.12 shows a fit of the same data as shown if figure 6.11 according to Yokota-Tanimoto kinetics. A best fit was found by assuming an average distance between donor and acceptor of $70 \pm 5 \text{ Å}$ and a diffusion coefficient of $(300 \pm 50) \times 10^6 \text{ Å}^2 \text{s}^{-1}$. This $\tau_o = $ lifetime of donor in absence of acceptor.
Figure 6.12 As Fig. 6.11. Trace is fit to decay according to Yokota-Tanimoto (see text). A reasonable fit is obtained.
value for the diffusion coefficient is between typical values for a low viscosity organic solvent ($10^{11} \text{ Å}^2 \text{s}^{-1}$) and of those of a viscous, nearly rigid solvent ($10^6 \text{ Å}^2 \text{s}^{-1}$) [Turro, 1977]. Diffusion will probably be that of the Ruthenium-compound. The binding of Ru(phen)$_3^{2+}$ to DNA is only partially intercalative and mainly external, compared to the binding of methylene blue which fully intercalates under the conditions of ionic strength used here.

It is somewhat surprising that the transient luminescence quenching can be fitted by an analysis which takes account of diffusion of the donor, while on the other hand the steady state emission quenching is characterised by a -modified- Perrin-formalism which excludes diffusion [Turro, 1977]. It has to be realised that the Perrin-formalism is a rather simplified model and the two apparently contradicting theories could possibly be reconciled by assuming that diffusion takes place within the Perrin-length. On the other hand it is possible that alternative theories could also explain the observed effects. It might be conceivable that the distribution of donor and acceptor molecules is not random but instead is centered around an average distance between them. Both the steady state emission as the kinetic decays would then be dependent on the actual distribution of the donor-acceptor pairs. If all the donor and acceptor pairs have the same distance to each other then the decay would revert back to a single-exponential one.

The implication of the results of the analysis of the transient decay of Ru(phen)$_3^{2+}$ in the presence of methylene blue and DNA indicates that the binding of such small molecules to DNA does not necessarily mean that these molecules are fixed rigidly to the polymer. This is in agreement with the conclusions by Geacintov et al. [1981] based on the observations of the decay of the triplet state of acridine orange and proflavine bound to DNA. Those observations were interpreted in terms of a rapid equilibration between free and bound
dye. In view of the much shorter lifetime of the excited state of Ru(phen)$_3^{2+}$ the equilibrium could possibly between dye intercalated in DNA and dye in the DNA hydration sphere. It is conceivable that dye in this H$_2$O-layer could move along the polymer. These conclusions have however to be regarded as preliminary.

The observed diffusion of a small molecule along the nucleic acid helix confirms the observations reported by Von Hippel et al. [1984] on the movement of polymerase enzymes along the non-specific regions of DNA. Von Hippel et al. assume a rate-constant for the random-walk of the enzyme along the polymer of $10^8 \text{ M}^{-1}\text{s}^{-1}$. According to the Einstein-Smoluchowski equation this rate-constant yields a diffusion constant of $1.7 \times 10^8 \text{ A}^2\text{s}^{-1}$. This value is of the same order of magnitude as the value found here for the diffusion of Ru(phen)$_3^{2+}$.

These preliminary results indicate the great scope that energy-transfer measurements have in nucleic acid research. Further experiments would be required to confirm the diffusion hypothesis. Other extensions to the above experiments would be to perform the experiments at different solvent conditions and extend the experiments with synthetic polynucleotides. It would also be interesting to label genetically important molecules such as transcription enzymes and measure their diffusion along the polynucleotide directly.

6.10 Conclusion

Effective transfer of electronic excited state energy takes place between Ru(phen)$_3^{2+}$ and MB$^+$ when both compounds are bound to DNA. This transfer is probably due to a combination of a Forster-type transfer and molecular diffusion. The value of this diffusion coefficient is comparable to values found for the diffusion of an RNA polymerase enzyme along DNA.
CHAPTER 7

MATERIALS AND METHODS
**MATERIALS**

Methylene blue (Fluka "puriss" grade) was purified on Sephadex LH20 using methanol as eluent. Purity was confirmed using thin layer chromatography (Silica, methanol:acetic acid 9:1) and UV/VIS spectroscopy. Stock solutions of dye were made up in the desired buffer and stored at 4 °C in the dark. Concentration of the stock solutions were determined using an extinction coefficient of 91000 M\(^{-1}\)cm\(^{-1}\) at 665 nm [Kamat and Lichtin, 1982b] in good agreement with Bergman and O'Konski [1963]. It was verified that such solutions could be used up to several months after purification without noticeable degradation.

Calf Thymus DNA (Sigma Corp., high molecular weight, Na-salt) was made up in the required phosphate buffer. Histone proteins were removed using standard procedures [Maniatis et al., 1982; Kelly et al., 1985a; Murphy, 1985; Tossi, 1987]. Concentrations were determined spectrophotometrically using an extinction coefficient of 6600 M\(^{-1}\)cm\(^{-1}\) (phosphate) at 260 nm [Maniatis et al., 1982]. Solutions were made up in small vials and stored at -20 °C where they remained stable for months.

Synthetic polynucleotides poly[d(A-T)] and poly[d(G-C)] (Pharmacia/PL Biochemicals Ltd.) were used as received. Stock solutions of the polynucleotides were made up directly in the required buffer in which they were to be used. Concentrations were determined spectrophotometrically using published extinction
coefficients [poly[d(G-C)]: 8400 (254 nm); poly[d(A-T)]: 6600 (262 nm), PL Biochemicals, 1979].

Covalently closed circular plasmid pBR322 DNA was obtained from E.coli bacterial DNA by extensive ultracentrifugation on a CsCl/ethidium bromide density gradient [performed by C.OhUigin, Dept. of Genetics, Trinity College Dublin].

2-Aminopyridine (A) was recrystallized from hot n-hexane. May and Baker, research grade. The crystals were dried under vacuum and dissolved in 1 N H$_2$SO$_4$ to make up stock solutions.

3-Aminophthalimide (B) Kodak Chemicals. Used as received. Dissolved in 0.05 N H$_2$SO$_4$.

4-dimethylamino-4'-nitrostilbene (C) Kodak Chemicals. Used as received. Dissolved in O-dichlorobenzene [see M. Murphy, 1985]. (A), (B) and (C) used as calibration compound in determination of fluorimeter correction curve [White and Argauer, 1970; Melhuish, 1975].

Laser dyes The laser dyes Rhodamine B, Cresyl Violet and Coumarine 120 were obtained from Lambda Physik (Lambdachrome). They were used as received. Solvent was Analar Grade Methanol.

Buffers All buffers in the photophysical experiments were phosphate buffers made up of the appropriate amounts of K$_2$HPO$_4$ and KH$_2$PO$_4$. (B.D.H.) Buffers used in the unwinding and photolysis experiments are described in the respective chapters.

Solution preparation

All experimental samples were made up by dissolving appropriate aliquots of stock solutions of dye and polynucleotide in the reaction
buffer. In experiments with varying DNA concentration either small amounts of DNA were added (taking account of appropriate dilution factors) or alternatively a series of dye:polymer was made up by withdrawing a known amount of polymer/dye mixture from the sample cell and replacing it with a similar amount of dye (in buffer) at the same concentration, thus keeping the initial dye concentration constant.

Solutions for the laser flash experiments were deoxygenated by at least 20 minutes bubbling with water-saturated argon gas. It was verified that this yielded the same results as five freeze-pump-thaw cycles under high vacuum.

METHODS

UV/VIS spectrophotometers

Absorption spectrophotometry was performed on a Pye-Unicam SP8-200 UV/VIS spectrophotometer. Spectra were generally obtained with the baseline correction facility of the machine. Occasionally, a Shimadzu UV-240 spectrophotometer was used. Fused quartz cells of varying thickness were used.

Spectrofluorimeter

Fluorescence spectra were mostly obtained on a Perkin-Elmer MPF 44B spectrofluorimeter, equipped with an R928 photomultiplier tube. The relative wavelength sensitivity of the emission side of the machine was calibrated using standard compounds according to the procedure described by White and Argauer [1970]. 2-Amino pyridine (320 - 430 nm), 3-Aminophthalimide (430 - 600 nm) and 4-dimethyl-4'-nitrostilbene (600
- 750 nm) were used. The emission spectra of the reference compounds in dilute solutions were determined and compared to the published values.

This yielded wavelength dependent correction factors by which the measured emission has to be multiplied to take account of the wavelength sensitivity of the detection system. The values of the correction factors are given in table A.1 in the Appendix.

Occasionally use was made of a Perkin-Elmer LS5 spectrofluorimeter with an Sl response photomultiplier. Data was corrected in a similar method as described above.

**Electrochemical equipment**

The electrochemical apparatus consisted of a Metrohm E612 sweep generator, a Thompson "Ministat" potentiostat and a homebuilt voltage follower. Cyclic voltammograms were recorded on a Linseis X-Y recorder. The working electrode was glassy carbon which was polished between each measurement. The counter electrode was a platinum wire and the reference electrode was Ag|AgCl. Sweep rates in all experiments were 20 mV s\(^{-1}\) and solutions were deoxygenated by vigorous bubbling with nitrogen. Solutions were thermostatted at 25 °C.

**Laser Flash Photolysis**

The installation and commissioning of a laser flash spectrometer and the system will be described in some detail below.

The system is set up as a kinetic spectrometer with nanosecond time resolution with excitation and monitoring light in a conventional right angle geometry. The system could also be used for time-resolved emission measurements. A block diagram of the system is given in figure 7.1 and a summary description of its most important components follow.
Figure 7.1 Schematic overview of kinetic spectrometer.

1 - Lambda Physik EMG50 Excimer laser
2 - Quartz planoconvex lens, $f = 300$ mm.
3 - SiO coated Ag. mirror
4 - Quartz biconvex lens, $f = 75$ mm.
5 - Water bath with thermostat controller.
6 - Thermostatted cell holder.
7 - Quartz biconvex lens, $f = 75$ mm.
8 - Electronic shutter controller.
9 - 250 Watt, water cooled, Xenon arc lamp.
10 - Lamp power supply.
11 - Lamp pulsing unit.
12 - $f:3/5$ High radiance monochromator. Grating blazed for 500 nm.
13 - Photomultiplier powersupply.
14 - Hamamatsu R 928 photomultiplier.
15 - Philips PM3311C 125 Mhz digital oscilloscope.
16 - Acorn IEEE interface.
17 - BBC B+ 64K microcomputer with high resolution graphics monitor.
18 - Twin double sided disk drives.
19 - Epson FX80 printer.
20 - OXFORD EDL-1 dye laser. Can replace 2 and 3 as indicated in diagram.
21 - Variable iris diaphragm.
Excitation source

The excimer laser is a Lambda Physik EMG 50 laser, operating with a mixture of Xenon (partial pressure 75 mbar), Helium/HCl (5%) (100 mbar) and Helium to a total pressure of 2600 mbar. The XeCl mixture lases at 308 nm. Maximum operating voltage of the laser was 35 kV. At this voltage the maximum pulse energy of the laser (with a fresh gas mixture) was ca. 35 mJ as measured with a Scientech 352 laser power meter. This would drop to ca. 25 mJ after prolonged operation. Normal operating voltage was 28 - 30 kV. The laser pulse length was determined indirectly by measuring the response of the photomultiplier system and was found to be ca. 15 ns. The rectangular beam of the laser (26 x 10 mm) is transformed via a Galilean telescope to 10.8 x 4.2 mm which is directed on the side of a standard 10 x 10 mm quartz cuvette in a thermostatted cell holder.

The alternative excitation source was a Oxford EDL 1 dye laser, pumped by the excimer laser. This laser consists of an orthogonally pumped Hansch-type cavity. The laser was adjusted to give maximum energy output at the expense of wavelength resolution. The dye laser output beam was focussed on the side of the sample cuvette at right angles with the monitoring beam. The dyes used in the laser were Rhodamine B, Cresyl Violet and Coumarin 120, all dissolved in Analar grade methanol at a concentration of ca. $5 \times 10^{-3}$ M. Lasing wavelengths were determined by scattering light into the monochromator of the spectrometer. Wavelengths so obtained were accurate to within 0.1 nm. This was considered adequate for the purposes of the research.

Finally, use was made of a Q-switched Ruby laser with a pulse duration of ca. 15 ns and a pulse energy of up to 1.5 Joule*. This laser was used in the determination of triplet extinction coefficients (chapter 3.2). Excitation with this laser was at right angles with the
monitoring light beam. The laser beam was unfocussed.

Transient Detection System

The detection system consists of a monitoring light source, sample housing, monochromator and photomultiplier. The light source was a water-cooled 250 Watt Xenon short arc lamp (Osram XBO 250). The light from the lamp is collimated and focussed with quartz lenses into the cell. The focal point of the optical system was adjusted to be just on the inside of the cuvette. A variable iris diaphragm was used to limit the beam diameter. Care was taken to ensure proper overlap of the monitoring and the exciting light beams. The monitoring light beam is refocussed after passing through the sample cuvette onto the entrance slit of the monochromator. A facility existed to increase the intensity of the monitoring light by pulsing a current through the lamp. This is done by discharging an LRC-circuit through the lamp which increases the operating current from 15 A to ca. 130 A for about a millisecond, with a corresponding increase in light intensity. It was found that the pulsed lamp proved only useful in cases of strong ground state absorption. This is however not near the wavelength of the major triplet absorption bands of methylene blue and the unit was only used in the assessment of excitation wavelength on the formation of dye dimers (chapter 3.3). The pulse unit proved indispensible in the analysis of transient absorption of the strongly fluorescent Ruthenium-polypyridyl samples [Tossi, 1987].

The monochromator was a high radiance F/3.4 Applied Photophysics model. Wavelength calibration was routinely performed with a "Pen-Ray" low pressure mercury lamp.

The light detector was a Hamamatsu R928 photomultiplier tube wired for nanosecond response times [Porter and West, 1974]. The linearity of the photomultiplier response was determined for pulsed and steady state.*

*We acknowledge Professor E.T.Kennedy (N.I.H.E.D.) for allowing us to use this laser.
illumination. The light intensity was varied with Neutral Density filters which had previously been calibrated on the Pye-Unicam SP8-200 spectrophotometer. The response of the photomultiplier to steady state illumination is shown in figure 7.3 for an operating voltage of 850 Volt. The output current of the photomultiplier was linear to a current of 1.4 mA. Higher currents were not investigated as the limits of the power supply were reached and in practice steady state currents were kept below 1 mA to ensure linearity of response. In the same figure the response for pulsed illumination (at 850 Volt) is shown. In this case linearity is maintained up to ca. 4 mA. Higher supply voltages gave a higher signal level but also decreased the linearity limits. The operating voltage was kept at 850 Volt in all transient absorption experiments. In the case of luminescence (lower signal levels) the power supply voltage was sometimes increased to 950 Volt to increase the signal/noise ratio. The photomultiplier power supply was manufactured by AEI. It was found that this power supply gave significantly less noise (x10) than a Farnell power supply which was used originally.

The current output of the photomultiplier was passed through a variable load resistor $R_L$. Consequently, a high load resistor will result in a high signal. The time-constant of the detection system is given by $R_LC_p$ with $C_p$ the parasitic and cable capacitance of the detection system and a large value of $R_L$ will result in a large time-constant. Detection of fast transients involves thus a trade-off between between a large signal (large $R_L$) and high time resolution (small $R_L$). The value of $C_p$ of the detection system was measured by determining the decay times of scattered laser light at large values of $R_L$ where $R_LC_p \gg T_{laser}$ (15 ns). The value of $C_p$ so determined was 230 ± 20 pF. Table 7.1 gives the response-times of the system as a function of the value of the load resistor $R_L$. 

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Table 7.1 Table of electronic time constants of detection system of nanosecond kinetic spectrometer as a function of load resistor.

<table>
<thead>
<tr>
<th>R&lt;sub&gt;nominal&lt;/sub&gt;</th>
<th>R&lt;sub&gt;measured&lt;/sub&gt;</th>
<th>T (nsec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>51.5</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>100</td>
<td>99.4</td>
<td>23 ± 1</td>
</tr>
<tr>
<td>500</td>
<td>485.7</td>
<td>112 ± 10</td>
</tr>
<tr>
<td>1000</td>
<td>993.3</td>
<td>228 ± 20</td>
</tr>
<tr>
<td>5000</td>
<td>4941</td>
<td>1136 ± 100</td>
</tr>
<tr>
<td>10000</td>
<td>9992</td>
<td>2298 ± 200</td>
</tr>
</tbody>
</table>

Experimentally, a load resistor was chosen which would give the largest signal with a corresponding time constant less than 10% of the decay time to be measured [Demas, 1983].

The transient signal analyzer was a Philips PM 3311C digital oscilloscope with a sampling speed of 125 MHz. The signals are stored into the 256 channels of the device and subsequently processed. Maximum time resolution is 8 nsec/channel. Maximum signal resolution is 8 bit i.e. 1 in 256. The oscilloscope was provided with a IEC/IEEE computer interface and could be controlled from a host computer. In the early stages of the project no computer was available and data analysis was performed manually by tracing data recorded on an X-Y recorder.

Computer interfacing was initially attempted with a Commodore 3032 32K microcomputer with associated disk drives and printer, connected via the IEEE interface of the computer. The lack of a suitable graphical display and recurrent hardware unreliability precluded full development of this system. Replacement of the Commodore by a BBC model B+ 64K microcomputer proved more successful. This computer possesses full graphics capabilities and enabled automatic storage and analysis of the data, both from transient absorption as from transient luminescence experiments. The software developed for this computer will be described
in detail in the next section.

**Software**

The BBC B+ computer was interfaced to the oscilloscope via an Acorn IEEE unit and its 1 MHz bus. The computer was fitted with a "Printmaster" EPROM chip enabling fast graphics dumps from the monitor screen to the printer.

The software package for data handling and analysis consists of two programs, a program to store the data from oscilloscope onto disk, and a program to analyze the data. Separation of the programs was done to save memory space and speed up program execution. Table 7.2 gives an overview of the programs used in connection with this research project.

<table>
<thead>
<tr>
<th>TRABS</th>
<th>Data storage, absorption.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LUMEN</td>
<td>Data storage, luminescence.</td>
</tr>
<tr>
<td>ABSDAT</td>
<td>Data analysis, absorption. 1-exponent.</td>
</tr>
<tr>
<td>LUMDEC</td>
<td>Data analysis, luminescence. 1-exponent.</td>
</tr>
<tr>
<td>ABSDUB</td>
<td>Data analysis, absorption. 2-exponents.</td>
</tr>
<tr>
<td>DUB2</td>
<td>Data analysis, luminescence. 2-exponents.</td>
</tr>
<tr>
<td>YOKOTA</td>
<td>Data analysis, luminescence. See chapter 6.</td>
</tr>
</tbody>
</table>

*Table 7.2 Suite of programs developed for transient spectroscopy with BBC B+ computer.*

Listings of representative programs are given in the Appendix and the programs TRABS, ABSDAT and ABSDUB are briefly described there. The storage programs have the provision to perform ensemble averaging over a number of shots. This proved to be invaluable in the analysis of the extremely weak transient absorptions in MB⁺/poly[d(G-C)]. Furthermore data could be smoothed. The routine is based on a weighed linear regression method as developed by Savitsky and Golay [1963]. Kinetic
analysis in the programs ABSDAT and ABSLUM is via a simple linear regression method and only yields first order kinetics. Subsequently the programs ABSDUB and DUB2 were developed which can analyze a trace in up to two arbitrary exponential decay constants and two amplitudes. The mathematical analysis is based on the Marquard-algorithm as described by Demas [1983]. The program developed here is a modification of the program "EXPFIT" in the book by Demas. ABSDUB and DUB2 were extensively tested with artificial data generated with the program DUBTEST.

A problem which sometimes plagues the system is the extreme sensitivity of the ACORN IEEE-device to transient surges. The HV-starting pulse of the xenon arc lamp is sufficient to destroy the sensitive electronics of the device. Care has to be taken to electrically insulate every electronic device present in the same room as the lamp.
CHAPTER 8

CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK
The main aims of the research described in this thesis have been described in the preface. They were 1) the study of the methylene blue photosensitized cleavage of the nucleic acid backbone with a view to sequencing the genetic code and 2) the use of methylene blue as a probe in the determination of excited state energy transfer in DNA. The main conclusions that follow from the research are presented below and will be discussed.

1. The MB$^+$ photosensitized cleavage of DNA phosphodiester-bonds always takes place at a Guanine base. Both oxygen-dependent as O$_2$-independent pathways can be identified.

2. The oxygen dependent pathway involves the reactive oxygen species singlet oxygen, $^1$O$_2$. It is however not likely that $^1$O$_2$ reacts directly with the nucleotide base. Binding of the dye to the polymer reduces the yield of production of singlet oxygen.

3. The oxygen independent pathway involves electron-transfer from the nucleotide to the excited state of the dye. Guanine has the lowest oxidation potential of all the nucleotides and is thus preferentially oxidized. Laser flash photolysis experiments on a picosecond timescale give evidence for electron-transfer from guanine. The observed rates can be fitted to the Marcus theory for non-adiabatic electron-transfer.

4. The theory of electron-transfer also explains the base-pair dependent quenching of the fluorescence emission of the dye. Electron-transfer competes with fluorescence emission for deactivation of the
excited $S_1$-state of the dye. Quantum yields of methylene blue fluorescence are lowest for polymers with a large G-C content.

5. The decrease in triplet yield that is observed on binding of the dye to the polymer has been shown to be concomittant with the decrease in the fluorescence yield. The triplet state of the dye is photochemically not reactive with respect to the nucleotides.

6. Topoisomerase experiments show that the binding of methylene blue to DNA is intercalative, but only at very low ionic strengths ( <20 mM). At higher ionic strengths the binding shifts to a more external form of binding. The conclusion of Norden and Tjerneld [1982] that at the higher ionic strengths methylene blue is still intercalated appears to be wrong.

7. The binding of methylene blue to poly[d(A-T)] is different in character from that to poly[d(G-C)]. The binding of the dye to the former is relatively salt-insensitive in contrast to the observations for the latter.

8. Electrochemical methods can be used fruitfully to determine binding parameters of electrochemically active ligands (such as methylene blue) to DNA.

9. Kinetic spectroscopy of the triplet state of methylene blue indicates that a dimer of methylene blue binds to poly[d(A-T)] and possibly also to CT-DNA and poly[d(G-C)]. Cooperativity of the binding process, which would lead to the binding of higher oligomers is not observed under the experimental conditions used in these experiments. The dimer can be selectively excited with a dye laser.
10. Computer simulation of excited singlet state energy-transfer from DNA to intercalated dye molecules suggest a distance over which transfer can take place of ca. 100 basepairs.

11. Energy transfer experiments between different intercalated dye molecules suggest a one dimensional diffusion of such molecules along the polymer.

The salient points of the above observations will be discussed below.

- The photosensitized cleavage of DNA proceeds via the guanine base and this is irrespective of environmental conditions such as oxygen concentration. A possible explanation for this would be that the strand cleavage starts with an oxidative reaction of the nucleotide bases. As guanine has the lowest oxidation potential of all the four bases, it is the easiest to oxidise. The mechanism of the cleavage reaction is thought to be similar to that found for strand cleavage by high intensity UV laser radiation [Blau et al., 1987], but proceeds via a somewhat different mechanism from that of ionizing radiation. Guanine radical cations appear to initiate the cleavage reaction. The conclusion of these experiments must be that it will be extremely difficult to obtain cleavage at nucleotides other than guanine using a straight forward photosensitizer. Even photosensitizers which bind with a 100% selectivity to A-T basepairs will most likely yield some cleavage at neighbouring G-C basepairs. It is thus necessary to add additional specificity to the photochemical reactivity. Possibly two-photon excitation mechanism such as proposed by Andreoni et al. [1980] could yield required specificity. Their reaction scheme involves
excitation from either the singlet or the triplet state of the dye. Photochemical selectivity is achieved utilising the difference in the excited state properties of these states on binding to different regions of the polymer. In the work here it has been shown that both the yield of fluorescence and of triplet state population are unchanged when the dye is bound to poly[d(A-T)] and considerably decreased on binding to poly[d(G-C)]. Excitation of the triplet state of the dye could thus possibly lead to novel photochemistry of the dye, which would then possibly be base-specific for A-T basepairs.

- Picosecond kinetic spectroscopy of methylene blue/polynucleotide complexes appears to give direct evidence for fast electron-transfer from guanine to the excited state of the dye. This interpretation is in excellent agreement with a theory which explains the base-specific quenching of a series of DNA intercalators. Performing picosecond experiments on a wide range of different compounds would give a better verification of the present interpretation of the picosecond results. These experiments have however to be regarded as preliminary ones which need verification in the future. This is the first time that these experiments have been performed. The electron-transfer theory could explain the formation of guanine cation radicals leading to strand cleavage in the absence of oxygen.

- The role of oxygen in the cleavage reaction is complicated. Inhibition of strand cleavage by addition of NaN₃ suggests involvement of singlet oxygen. In contrast, replacement of the water by D₂O did not increase the yield of the photochemical reaction. This suggests that O₂ does not react directly with the polymer. Flash spectroscopy also suggests that the formation of O₂ will be inhibited by binding of the dye to the polymer. The role of O₂ in the cleavage reaction can not
really be resolved from the observations reported in this work. It has been suggested that singlet oxygen intermediates of the photosensitizer itself could be responsible for the observed effects [Niewint et al., 1985; Brabham and Kasha, 1975]. It is clear that the presence of oxygen increases the yield of the cleavage reaction.

The binding of the dye to the different polymers is intriguing. The observations on the protonation of the dye triplet state and the observations on the oxygen quenching indicate that the dye is considerably protected from bulk solvent when bound to poly[d(A-T)], even at ionic strengths of up to 200 mM. The relative insensitivity of the binding of the dye to poly[d(A-T)] indicates a binding mode which is most likely not electrostatic but could be composed of hydrogen bonding or hydrophobic effects. The insensitivity of the dye binding in this polymer is in contrast to that found for poly[d(G-C)], where the observations point to a decrease in binding on increasing ionic strength. This strong dependence of binding on ionic strength in poly[d(G-C)] could possibly be the explanation for the somewhat anomalously high values of the fluorescence quantum yield as found for this polymer at an ionic strength of 100 mM. At lower ionic strengths the emission of the dye with this polynucleotide is much lower. The lower quantum yield that is found for MB+ with CT-DNA can be attributed to the dye binding to (non-salt dependent) A-T basepairs which are next to G-C basepairs.

The dimer aggregate of the dye appears to bind to poly[d(A-T)]. This is based on observations of the transient depletion in the region of dimer absorption on excitation in the main dimer absorption band. Binding of dimer would also give an explanation of the double exponential lifetimes found for the bound dye on excitation at either
308 nm or 600 nm. In contrast no double exponential decay is observed when the complex is excited at 665 nm ie. in the dye monomer band. Somewhat contradictory to this conclusion is the absence of a large dimer band in the UV/VIS ground state absorption of dye/polynucleotide complex and an absence of a dimer band in the picosecond transient spectra. Dimer aggregate bands are observed in the absorption spectrum of acridine orange/DNA complexes. The appearance of such a band in the spectrum of this dye in the presence of the polymer has been interpreted as caused by an electrostatic, sandwich-like stacking of the dye molecules along the polymer. The conclusion with respect to the binding of the methylene blue dimer must be that the dimer binds without the possibility of such stacking occurring. It is conceivable that the dimer binds as an intact entity in one of the grooves of the polymer. Currently there is no supporting evidence for this hypothesis. The absence of a dimer band in the ground-state absorption spectrum is puzzling. If the dimer binds, which is what the laser-flash results appear to show, then the 600 nm should also be clearly visible. It does appear clear however that the large dimer bands seen in acridine orange/DNA under certain conditions is almost certainly due to a cooperative stacking of the dye molecules on-top of one another. This is not observed for methylene blue.

- Simulation of the energy transfer in DNA leads to the conclusion that such transfer could possibly be over distances of 100 basepairs. It has to be realised that these calculations are a considerable simplification on the true physical situation. Within the assumptions it is believed that the results give a reasonable interpretation of reality. It has however to be realised that the conclusions from these calculations are based on only one reported, experimental observation. The simulation does show the scope of these calculations and will be of
considerable help in the interpretation of future experiments which
will undoubtedly be performed.

- Preliminary experiments on energy-transfer experiments between
  Ru(phen)$_3^{2+}$ and MB$^+$ appear to indicate that the dye molecules can
diffuse along the polymer. This would be analogous to the diffusion of
transcription enzymes such as RNA-polymerases. This interpretation of
the experimental data is still very much preliminary. If it is correct
then such energy-transfer experiments would open the way of monitoring
directly the mechanism of genetical recognition and transcription
processes. These processes are of extreme importance in molecular
biology and the mechanism underlying the recognition processes is still
not very well understood. It has to be realised that more experiments
need to be performed to rigorously exclude the possibility of other
interpretations.

**Future experiments**

Experiments which would be worthwhile to carry out in the future have
occasionally already been mentioned. Laser flash spectroscopy on the
picosecond timescale of a range of compounds with different redox
potentials would give an opportunity to verify the electron-transfer
hypothesis. The effect of singlet oxygen on the photosensitized
cleavage of the dye could be measured using time-resolved luminescence
of $^{1}O_2$ in the presence of DNA and dye.

Multi-photon excitation of dye-DNA complexes could lead to novel
photochemistry. This could be the method to avoid cleavage solely at
guanine and to effect strand breaks at other nucleotides as well.

The laser flash experiments indicated the complications caused by
the involvement of the dye dimer in the binding process. It has become
apparent during the cause of this study that present knowledge of the photophysical properties of such dimers is at the most scanty. There is considerable scope for a thorough investigation on the photophysics of dimers.

The energy transfer calculations show the urgent need for experimental data. The only direct measurement of energy-transfer processes in DNA was reported in 1975. Such data should then be fitted to a more sophisticated version of the computer program.

The dye-dye energy-transfer experiments need to be extended to rigorously define the exact mechanism of the transfer process. This can shed light on important areas in molecular biology.

- Concluding, it is clear that the binding of an apparent simple molecule as methylene blue to DNA can be fraught with complications. It has become apparent that simple intercalation is rarely ever achieved and that a multitude of binding modes are possible. Base-sequencing with methylene blue currently would be a reasonable alternative for the G-specific Maxam-Gilbert reaction. Dye molecules, like methylene blue are eminently suitable as probes for DNA structure and function.
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A.1 WAVELENGTH SENSITIVITY CORRECTION VALUES

PERKIN-ELMER MPF-44B SPECTROFLUORIMETER / R928 PHOTOMULTIPLIER

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<th>mult. fact.</th>
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<td>0.538</td>
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N.B. Multiplication factors are normalised to 600 nm = 1.
Calibration compounds: 333-440 nm: 2-aminopyridine; 450-600 nm: 3-aminophthalimide; 610-750 nm 4-dimethylamino-4'-nitrostilbene.
A.2 DATA CAPTURE, STORAGE AND ANALYSIS SOFTWARE

TRABS

Function of program: Capture of data from Philips PM3311C oscilloscope and subsequent storage of data on disk. Related programs: ABSDEC and ABSDUB. TRABS is similar to LUMEN.

Line 10-320: Presentation of options i.e. menu
Line 280: Function call to "Printmaster" EPROM on BBC expansion board. Performs screen dump of data on Epson printer.
Line 500-850: Measurement of $I_0$. Steady state light intensity can either be D.C. or can be obtained using arc lamp pulsing unit. The value (in mV) can also directly be put in.
Line 860-1180: Capture of transient decays. Input is number of shots that have to be averaged. Also scale factor of transient decay (in mV) as well as timebase (in seconds/div) has to be given.
Line 1370-1470: Storage of data on disk.
Line 1190-1360: Restore and display data from disk (is option not
normally used in this program).

Line 1470-1590: Return to main menu and error trapping routine.

PROCDISPLAY: Display transient data on screen.

PROCADATAIN: IEEE procedure to take data from oscilloscope.

FNDEC: Function to transform reversed decimal ASCII
(output from oscilloscope) to number from -128 to +127.

PROCBOX: Draws frame for display of transient data.
Program TRABS

10 ON ERROR GOTO 1520
20MODE128
30DIMZ$(268):DIMY%(268):DIMW%(256):DIMS(256)
40CONT=0
50TELLER=0
60CLS
70PRINT" PROGRAM FOR TRANSIENT LASER SPECTROSCOPY"
80PRINT
90PRINT" ** MAIN MENU **"
100PRINT" ** NEW MEASUREMENT :1""
110PRINT" ** DATA TO DISK :2""
120PRINT" ** DATA FROM DISK :3""
130PRINT" ** SCREEN DUMP :4""
140PRINT" ** DECAY ANALYSIS :5""
150PRINT" ** QUIT PROGRAM :6""
160INPUTF%
170IF F% = 1 GOTO 330
180IF F% = 2 GOTO 1370
190IF F% = 3 GOTO 1170
200IF F%= 4 GOTO 250
210IF F% = 5 GOTO 230
220IF F% = 6 GOTO 1500
230*DISK
240CHAIN"ABSDEC"
250IF CONT=0 GOTO 300
260VDU 2
270*FX6,0
280*GDUMP 0 0 11
290VDU3
300CLS
310PRINT"NO TRACE ON SCREEN"
320 GOTO 70
330*IEEE
340cmd%=OPENIN("COMMAND")
350data%=OPENIN("DATA")
360PRINT$cmd%,"BBC DEVICE NO",0
370PRINT$cmd%,"CLEAR"
380PRINT$cmd%,"REMOTE ENABLE"
390PRINT$cmd%,"DEVICE CLEAR"
400DEL$=CHR$(10)
410PRINT$cmd%,"END OF STRING",DEL$
420scope%=OPENIN("5")
430PRINT$cmd%,"UNLISTEN"
440PRINT$cmd%,"LISTEN",scope%,"EXECUTE"
450D$=CHR$(27)+"ODDO"+DEL$
460PRINT$data%,D$
470PRINT$cmd%,"UNLISTEN"
480PRINT$cmd%,"GO TO LOCAL",scope%,"EXECUTE"
490CLS
500PRINT" IO MEASUREMENT : SET SCOPE TO DC, RECURRENT AND AUTO"
510TELLER=1
520CONT=0
530PRINT
540PRINT" MEASURE IO ? Y/N"
550IF GET$ = "N" GOTO 840
560CLS
570PRINT
580PRINT" IO MEASUREMENT, FIRST CLOSE SHUTTER"
590PRINT" PRESS R WHEN READY"
600IF GET$ = "R" GOTO 620
610 GOTO 600
620 PROC DATA IN
630 FOR I%=1 TO 256
640 AA%=AA%+Y%(I%+12)
650 NEXT I%
660 AA%=AA%/256
670 PRINT "IO MEASUREMENT, NOW OPEN SHUTTER"
680 PRINT "PRESS R WHEN READY"
690 IF GET$="R" GOTO 710
700 GOTO 690
710 PROC DATA IN
720 FOR I%=1 TO 256
730 BB%=BB%+Y%(I%+12)
740 NEXT I%
750 BB%=BB%/256
760 PRINT
770 PRINT
780 INPUT "GIVE OSCILLOSCOPE SCALE IN mV/div ", SCL
790 INUL=(AA%-BB%)*(SCL*10)/256
800 PRINT
810 PRINT
820 PRINT "I-zero IS "; INUL; PRINT " millivolt"
830 GOTO 850
840 INPUT " GIVE I-zero VALUE IN MILLIVOLT ", INUL
850 PRINT
860 PRINT "SET SCOPE FOR TRANSIENT MEASUREMENTS 
870 PRINT
880 PRINT "TRIGGER TO RECURRENT AND AC, DISPLAY TO AC"
890 PRINT
900 PRINT "PRESS R WHEN READY"
910 IF GET$="R" GOTO 930
920 GOTO 900
930 CLS
940 INPUT "HOW MANY SHOTS DO YOU WANT TO AVERAGE ", QQ%
950 FOR I%=1 TO QQ%
960 PRINT
970 PRINT
980 PRINT "OPEN SHUTTER AND FIRE LASER 
990 PRINT "PRESS R WHEN SHOT IS OK 
990 IF GET$="R" GOTO 1010
1000 GOTO 980
1010 PRINT "SHOT NUMBER "; I%
1020 PROC DATA IN
1030 FOR L%=1 TO 256
1040 W%(L%)=Y%(L%+12)+W%(L%)
1050 NEXT L%
1060 NEXT I%
1070 PRINT
1080 PRINT "FINISHED TAKING REQUIRED £ OF TRACES, CLOSE SHUTTER"
1090 PRINT
1100 INPUT "GIVE OSCILLOSCOPE SCALE IN mV/div ", SCL
1110 FOR I%=1 TO 256
1120 S(I%)=W%(I%)/QQ%
1130 NEXT I%
1140 PRINT
1150 INPUT "GIVE OSCILLOSCOPE TIMEBASE IN microseconds/div ", TMB
1160 *DISK
1170 CLS
1180 IF TELLER=1 GOTO 1280
1190 INPUT "GIVE FILENAME ", A$
1200 *DRIVE 1
1210 X=OPEN IN A$
1220 INPUT$X, BB$, INUL, TMB, SCL
1230 FOR I%=1 TO 256
1240 INPUT$X, S(I%)
1250 NEXT I%
1260 CLOSE$X
1920 L% = L%-48+(L%>64)*7
1930 DC=DC*16+L%
1940 L%=ASC(RIGHT$(Q$,1))
1950 NEXT J%
1960 IF DC>127 THEN DC=DC-256
1970 DC=DC+128
1980 =DC
1990 DEF PROCBOX
2000 VDU28,55,10,79,0
2010 MOVE 0,0
2020 DRAW 1279,0
2030 DRAW 1279,1023
2040 DRAW 0,1023
2050 DRAW 0,0
2060 MOVE 0,511
2070 DRAW 1279,511
2080 FOR I%=1 TO 7
2090 MOVE 160*I%,481
2100 DRAW 160*I%,511
2110 MOVE 160*I%-140,471
2120 VDU5:PRINT 32*I%
2130 NEXT I%
2140 VDU4
2150 ENDPROC
ABSDEC

Function of program: Performs simple unweighted linear least square regression analysis of transient absorption data and subsequently plots data and fitted decay on screen or printer. Related programs: TRABS and ABSDUB. ABSDEC is similar to LUMDEC.

Line 10-130: Input of data from disk.
Line 140-220: Display of raw data on screen and/or screen dump
Line 220: Function call to "Printmaster" EPROM on BBC expansion board. Performs screen dump of data on Epson printer.
Line 230-310: Data smoothing using 5-point Savitsky-Golay procedure. Display of smoothed data.
Line 320-370: Baseline determination.
Line 380-410: Start and stop channels of analysis.
Line 420-470: Scaling of data and transforming data into transient absorbances.
Line 480-500: First or second order analysis.
Line 510-590: First order manipulation of data. Determination of scaling factors for plot.
Line 690-760: Plot of transformed data,
Line 770-920: Least square analysis, plotting and printing of data.
Line 930-960: Screen dump of data and analysis.

Line 970-1020: Chain program TRABS for new data or return for more analysis.

PROCBOX: Procedure to draw frame for data display.

PROCDISPLAY: Procedure to display transient data.

PROCANALYSIS: Unweighted least square analysis of transient data.
Program : ABSDEC

LIST
10 MODE 128
20 DIMS(256): DIMS1(256): DIMOD(256): DIMX(256): DIMCHI(256)
30 CLS
40 PRINT "** DECAY ANALYSIS **"
50 PRINT
60 INPUT "NAME OF FILE TO ANALYSE ", A$  
70 *DRIVE 1
80 X=OPENIN A$
90 INPUT£X, BB$, INUL, TMB, SCL
100 FOR I% = 1 TO 256
110 INPUT£X, S(I%)
120 NEXT I%
130 CLOSE£X
140 *DRIVE 0
150 PROCBOX
160 PROCDISPLAY
170 IF GET$ = "" GOTO 180
180 PRINT "press R for graphics dump"
190 IF GET$ = "R" GOTO 210
200 GOTO 230
210 *FX6, 0
220 *GDUMP 0 0 1 1
230 PRINT " SMOOTHING OF THE TRACE Y/N"
240 IF GET$ = "N" GOTO 320
250 FOR I% = 3 TO 254
260 S(I%) = (-3*S(I% - 2) + 12*S(I% - 1) + 17*S(I%) + 12*S(I% + 1) - 3*S(I%))/35
270 NEXT I%
280 PROCBOX
290 PROCDISPLAY
300 IF GET$ = "" GOTO 320
310 GOTO 230
320 GOTO 340
330 FOR I% = 0 TO 1279: PLOT7, I%, BS%: NEXT I%
340 INPUT "BASELINE AT CHANNEL", BS%
350 MOVE0, BS%: DRAW 1279, BS%
360 PRINT "BASELINE OK Y/N"
370 IF GET$ = "N" GOTO 330
380 GOTO 230
390 PRINT
400 INPUT "STOP CHANNEL ANALYSIS", SP%
410 PRINT
420 FOR I% = ST% TO 256
430 S1(I%) = (BS%/4 - S(I%))* (SCL*10)/256
440 NEXT I%
450 FOR I% = ST% TO SP%
460 OD(I%) = ABS(LOG(INUL/(INUL + S1(I%)))
470 NEXT I%
480 INPUT "1ST OR 2ND ORDER ANALYSIS? 1,2", RDR%
490 IF RDR%=1 GOTO 510
500 IF RDR%=2 GOTO 600
510 PRINT "FIRST ORDER ANALYSIS"
520 XMAX = -10
530 XMIN = 0
540 FOR I% = ST% TO SP%
550 X(I%) = LN(OD(I%))
560 IF XMAX<X(I%) THEN XMAX=X(I%)
570 IF XMIN>X(I%) THEN XMIN=X(I%)
580 NEXT I%
590 GOTO 680

322
600 PRINT "SECOND ORDER ANALYSIS"
610 XMAX=0
620 XMIN=10
630 FOR I% = ST% TO SP%
640 X(I%) = 1/OD(I%)
650 IF XMAX<X(I%) THEN XMAX=X(I%)
660 IF XMIN>X(I%) THEN XMIN=X(I%)
670 NEXT I%
680 IF RDR%=2 GOTO 710
690 IF P=1000/(X(ST%)-XMIN) : QQ=1000*XMIN/(X(ST%)-XMIN)
700 GOTO 720
710 IF P=1000/(X(ST%)-XMAX) : QQ=1000*XMAX/(X(ST%)-XMAX)
720 MOVE 5*ST%,1000
730 FOR I% = ST% TO SP%
740 PLOT 69,5*I%, P*X(I%)-QQ
750 NEXT I%
760 PRINT
770 PRINT "FITTING CURVE"
780 PRINT "press R for hardcopy"
790 PRINT
800 INPUT "START CHANNEL", CST%
810 INPUT "STOP CHANNEL ", CSP%
820 SUM=0: SUMX=0: SUMY=0: SUMXY=0
830 SIGMA=0: CORR=1
840 PROC ANALYSIS
850 CLS
860 PRINT "I-zero=", INUL
865 SIGMA+%1-", SIGMA
870 PRINT "TIMEBASE microsec/channel ", TMB/25
880 PRINT "SCALE mV/channel =", SCL/25.6
890 PRINT "DECAY microseconds", (1/TAU)*TMB/25
900 PRINT "SIGMA +/-", SIGMA
910 PRINT "CORRELATION ", CORR
920 IF GET$="R" GOTO 950
930 IF GET$="R" GOTO 950
940 GOTO 970
950 FX6,0
960 GDUMP 0 0 1 1
970 PRINT "main program press R"
980 IF GET$="R" GOTO 1000
990 GOTO 1010
1000 CHAIN "TRABS"
1010 VDU26: CLG
1020 PRINT "***** END OF PROGRAM; REMOVE DISKS *****"
1030 END
1040 DEF PROCBOX
1050 CLS
1060 MOVE 0,0
1070 DRA\.\ 1270,0
1080 DRA\.\ 1279,1023
1090 DRA\.\ 0,1023
1100 DRA\.\ 0,0
1110 DRA\.\ 0,511
1120 DRA\.\ 1279,511
1130 FOR I% =1 TO 7
1140 MOVE 160*I%,481
1150 DRA\.\ 160*I%,511
1160 MOVE 160*I%-140,471
1170 VDU5: PRINT 32*I%
1180 NEXT I%
1190 FOR I% =1 TO 15
1200 MOVE 64*I%-1
1210 DRA\.\ 40,64*I%-1
1220 MOVE 1239,64*I%-1
1230 DRAW 1279.64*I%-1
1240 MOVE 1075.64*I%+10
1250 PRINT 64*I%-1
1260 NEXT I%
1270 VDU4
1280 ENDPROC
1290 DEF PROC DISPLAY
1300 VDU28,40,7,79,0
1310 PRINT "SAMPLE: ", BB$
1320 PRINT "I-ZERO (mV): ", INUL
1330 PRINT "microseconds/channel", TMB/25
1340 FOR I%=1 TO 256
1350 DRAW 5*(I%-1), S(I%)*4
1360 NEXT I%
1370 ENDPROC
1380 DEF PROC ANALYSIS
1390 SUM=CSP%-CST%+1
1400 FOR I%=CST% TO CSP%
1410 SUMX=SUMX+I%
1420 SUMY=SUMY+X(I%)
1430 SUMX2=SUMX2+I%*I%
1440 SUMY2=SUMY2+X(I%)*X(I%)
1450 SUMXY=SUMXY+X(I%)*I%
1460 NEXT I%
1470 RA=SUM*SUMXY-SUMX*SUMY
1480 RB=SQR(SUM*SUMX2-SUMX^2)
1490 RC=SQR(SUM*SUMY2-SUMY^2)
1500 CORR=RA/(RB*RC)
1510 DELTA=SUM*SUMX2-SUMX^2
1520 ICP=(SUMX2*SUMY-SUMX*SUMXY)/DELTA
1530 TAU=(SUM*SUMXY-SUMX*SUMY)/DELTA
1540 FOR I%=CST% TO CSP%
1550 SIGMA=SIGMA+(X(I%)-ICP-TAU*I%)^2
1560 NEXT I%
1570 SIGMA=SIGMA/(CSP%-CST%-1)
1580 MOVE 5*CST%, PP*(ICP+TAU*CST%)-QQ
1590 DRAW 5*CSP%, PP*(ICP+TAU*CSP%)-QQ
1600 ENDPROC
ABSDUB

Function of program: Performs unweighed non-linear regression analysis of up to two-exponential transient absorptions. Determines both amplitude of components as decay-times. Algorithm used is Marquardt analysis [Bevington, 1969; Demas, 1984]. Program is adaption from program EXPFIT form Demas. Related programs: TRABS and ABSDEC. ABSDUB is similar to DUB2.

Line 10-240 : Choice of disk-drive and input of transient data.
Line 250-320 : Plot data on screen; optional screen dump to printer.
Line 430-510 : Set baseline and calculate signal in millivolt.
Line 520-570 : Set zero time.
Line 580-620 : Give start and stop channels for analysis.
Line 630-650 : Calculate optical density data.
Line 660-720 : Set boundary limits for iteration programs.
Line 730-780 : Fill time channels. Calculate number of points.
Line 790-980 : Choice of single or double exponential decay.
Line 990-1050 : Give input parameters. Program requires A(1), A(2) and A(4). A(3) is set = 100 - A(1).

Line 1050-1400: Main program. Iterative solving of matrix. See Demas.

Line 1410-1550: Check on limits for convergence and accuracy.
Line 1560-1900: Output of data on screen; optional printing of data to printer.
Line 1930-2010: Choice of repeat of analysis or loading of new trace.

PROCBOX: Procedure to draw frame for display of raw transient data.

PROCDISPLAY: Display of raw data.

PROCDISP2: Display of raw data with fitted decay curve. Also plot of unweighed residuals. Option for screen dump.

PROCCHICALC: Calculation of unreduced and unweighed CHI-square.

PROCFILLMAT: Calculation of matrix elements for Marquardt procedure.

PROCSOLVE: Solving equation $AX = B$ with $A$ matrix and $X$ new changes in values of variables $A(1)$ to $A(4)$.

Algorithm is that of moving pivot [Dr. J. Carroll, N.I.H.E.D., private communication].
Program: ABSDUB

10MODE128
40 DIM YT(256):DIM S1(256)
50CLS
60VDU2:PRINT:PRINT:PRINT:PRINT:VDU3
70PRINT "** TRANSIENT ABSORBANCE ANALYSIS **"
80PRINT
90 INPUT " DRIVE NUMBER OF DATA DISK ",DRDK
100 IF DRDK=0 THEN *DRIVE 0
105 IF DRDK=1 THEN *DRIVE 1
110 IF DRDK=2 THEN *DRIVE 2
115 IF DRDK=3 THEN *DRIVE 3
120 IF DRDK=3 THEN *DRIVE 3
130PRINT
140 INPUT " NAME OF FILE TO ANALYSE ",A$
150X=OPENIN A$
160 INPUT£X,BB$,INUL,TMB,SCL
170FOR I% = 1 TO 256
180 INPUT£X, S1(I%)
190 NEXT I%
200 CLOSE£X
210 FOR I%=1 TO 256
220 S(I%) = S1(I%)
230 NEXT I%
240 *DRIVE0
250 PROCBOX
260 PROCDISPLAY
270 IF GET$ = " ", GOTO 330
280 IF GET$ = "R" GO TO 300
290 GOTO 330
300 VDU2:PRINT":PRINT":PRINT":PRINT":VDU1
310 *FX6,0
320 *GDUMP 0 0 1 1
330PRINT
340 PRINT " SMOOTHING OF THE TRACE Y/N"
350 IF GET$ = "N" GOTO 430
360 FOR I% = 3 TO 254
370 S(I%) = (-3*S(I%-2)+12*S(I%-1)+17*S(I%)+12*S(I%+1)-3*S(I%)/35
380 NEXT I%
390 PROCBOX
400 PROCDISPLAY
410 IF GET$ = " ", GOTO 430
420 GOTO 340
430 GOTO 450
440 FOR I% = 0 TO 1279: PLOT7, I%, BS%: NEXT I%
450 INPUT " BASELINE AT CHANNEL %", BS%
460 MOVE0, BS%: DRAW 1279, BS%
470 PRINT " BASELINE OK Y/N"
480 IF GET$ = "N" GOTO 440
490 FOR I%= 3 TO 254
500 S(I%) = (BS%/4-S(I%))/(SCL*10)/25
510 NEXT I%
520 GOTO 540
530 FOR I%= 0 TO 1023: PLOT7, ZT%*5, I%: NEXT I%
540 INPUT " SET ZERO-TIME AT CHANNEL %", ZT%
550 MOVEZT%*5, 0: DRAW ZT%*5, 1023
560 PRINT " ZERO-TIME OK Y/N"
570 IF GET$ = "N" GOTO 530
580 INPUT " START CHANNEL ANALYSIS ", ST%
590PRINT
INPUT "STOP CHANNEL ANALYSIS", SP%
NP = SP% - ST% + 1
PRINT
FOR I% = ZT% TO 256
S(I%) = ABS(LOG(INUL / (INUL + S(I%))))
NEXT I%
NP = SP% - ST% + 1
PRINT FOR 1% = ZT% TO 256
S(I%) = ABS(LOG(INUL / (INUL + S(I%))))
NEXT 1%
T = 1
L9 = 1
T(1) = 0
E1 = .00001
E2 = .00001
ZAM = .001
LIMIT = 50
FOR J% = ZT% TO SP%
T(J% - ZT% + 1) = (J% - ZT%) * T
NEXT J%
NUH% = SP% - ST% + 1
PRINT "** TRANSIENT ABSORPTION ANALYSIS **"
PRINT:
PRINT "** EXPONENTIAL DECAY ANALYSIS **"
PRINT
INPUT "** SINGLE (1) OR DOUBLE EXPONENTIAL (2) DECAY **", N1
PRINT
CLS
IF N1 = 2 GOTO 910
PRINT ** GIVE GUESSES FOR PARAMETERS **
PRINT ** FUNCTION = A(1)*EXP(-T/A(2)) **
GOTO 970
PRINT ** GIVE GUESSES FOR PARAMETERS **
PRINT ** FUNCTION = A(1)*EXP(-T/A(2)) + A(3)*EXP(-T/A(4)) **
PRINT ** A(1) + A(3) = 100 **
PRINT
NEQN = 2 * N1
INPUT " A(1) = ?", A1
A(1) = A1
A(1) = A(1) * S(ZT% + 2) / 100
INPUT " SHORT LIFETIME (in nanoseconds)?", A2
A2 = A2 / (40 * TMB)
IF N1 = 1 GOTO 1050
A3 = (100 - A1) * S(ZT% + 2) / 100 : A(3) = A3
A1 = A1 * S(ZT% + 2) / 100
INPUT " LONG LIFETIME (in nanoseconds)?", A4
A4 = A4 / (40 * TMB)
REM VDU26
PROCCHICALC
PRINT "CHISQUARE = ", CHN
CHT = CHN
PROCFLILLMAT
FOR J% = 1 TO NEQN
V(J%) = B(J%, J%)
Y(J%) = A(J%)
NEXT J%
FOR J% = 1 TO NEQN
B(J%, J%) = B(J%, J%) * (1 + ZAM)
NEXT J%
PROC SOLVE
1180 A(1) = A(1) - X(1): A(2) = A(2) - X(2): A(3) = A(3) - X(3): A(4) = A(4) - X(4)
1190 PRINT "*******************************************************************************/
1200 PRINT
1210 PROCCHICALC
1220 PRINT " CYCLE £", L9, "; LAMBDA = ", ZAM
1230 PRINT "A1=", A(1), " A3=", A(3)
1240 PRINT
1250 PRINT "A(2) =", A(2) * 40 * TMB, " A(4) = ", A(4) * 40 * TMB
1260 PRINT
1270 PRINT "CHI-new =", CHN, " ; CHI-old =", CHT
1280 IF CHT > 100000 THEN GOTO 1300
1290 GOTO 1320
1300 PRINT "DIVERGING ITERATIONS"
1310 GOTO 1510
1320 IF CHN < CHT THEN GOTO 1400
1330 ZAM = ZAM * 10
1340 IF ZAM <= 10000 THEN GOTO 1360
1350 PRINT "LARGE LAMBDA"
1360 FOR J% = 1 TO NEQN
1370 B(J%, J%) = V(J%): A(J%) = Y(J%)
1380 NEXT J%
1390 GOTO 1140
1400 ZAH = ZAH / I0
1410 F = 0
1420 FOR J% = L TO NEQN
1430 IF ABS(X(J%)/A(J%)) > E1 THEN F = 1
1440 NEXT J%
1450 IF ABS(CHT - CHN) < E2 THEN GOTO 1540
1470 CHT = CHN
1480 L9 = L9 + 1
1490 IF F = 0 AND ABS(CHT - CHN) / CHN < .05 THEN GOTO 1540
1500 GOTO 1090
1510 INPUT "TRY NEW GUESSES? Y/N", A$
1520 IF A$ = "Y" THEN GOTO 850
1530 END
1540 CLG
1550 VDU26
1560 PRINT "=================================================================
1570 PRINT
1580 PRINT
1590 PRINT ** "; PRINTBB$; ; PRINT" **
1600 PRINT
1620 PRINT
1630 PRINT
1640 PRINT " LAMBDA = ", ZAM
1650 PRINT "END OF ITERATION; CHISQUARE = ", CHN/(NP-NEQN-1)
1660 PRINT "DEGREES OF FREEDOM = ", NP-NEQN-1
1665 PRINT "INITIAL GUESS A(1) = ", A1, " A(2) = ", A2
1670 PRINT
1680 PRINT "AMPLITUDE = ", A(1)
1690 PRINT
1700 PRINT "LIFETIME = ", A(2) * 40 * TMB, " nanoseconds"
1710 IF N1 = 1 GOTO 1780
1720 PRINT
1730 PRINT "INITIAL GUESS A(3) = ", A3, " A(4) = ", A4
1740 PRINT
1750 PRINT "AMPLITUDE = ", A(3)
1760 PRINT
1770 PRINT "LIFETIME = ", A(4) * 40 * TMB, " nanoseconds"
1780 PRINT: PRINT" FOR HARDCOPY PRESS R RTN"  
1790 INPUT SS$: IF SS$ = "R" GOTO 1810
1800 GOTO 1910
1810 VDU26
1820 CLG: PRINT: PRINT: PRINT" ** "; BB$; ; PRINT" **
1830 PRINT: PRINT" CHISQUARE = ", CHN/(NP-NEQN-1): PRINT
1840 PRINT "AMPLITUDE = ", A(1)
1850 PRINT: PRINT" LIFETIME = ", A(2) * 40 * TMB, " ns"
1860 IF N1 = 1 GOTO 1900
1870 PRINT: PRINT" AMPLITUDE = ", A(3)
1880 PRINT: PRINT "LIFETIME = ", A(4) * 40 * TMB, " ns"
1890 PRINT
1900 VDU3
1910 CLG
1920 PROC_DISP2
1930 IF GET$ = "R" GOTO 1950
1940 GOTO 1960
1950 *GDUMP 0 0 1 1
1960 GOTO 1990
1970*FX6,0
1980*GDUMP 0 0 1 1
1990 PRINT "MORE ANALYSIS? , press R"
2000 IF GET$ = "R" GOTO 210
2001 PRINT "ANOTHER TRACE? , press R"
2002 IF GET$ = "R" GOTO 50
2010 END
2020 DEF PROC_BOX
2030 CLG
2040 VDU5
2050 MOVE 0,0
2060 DRAW 1270,0
2070 DRAW 1279,1023
2080 DRAW 0,1023
2090 DRAW 0,0
2100 MOVE 0,511
2110 DRAW 1279,511
2120 FOR I% = 1 TO 7
2130 MOVE 160*I%,481
2140 DRAW 160*I%,511
2150 MOVE 160*I%-140,471
2160 PRINT 32*I%
2170 NEXT I%
2180 FOR I% = 1 TO 15
2190 MOVE 1239,64*I%-1
2200 DRAW 1279,64*I%-1
2210 MOVE 1075,64*I%+10
2220 PRINT 64*I%-1
2230 NEXT I%
2240 VDU4
2250 ENDPROC
2260 DEF PROC_DispLAY
2270 VDU28,40,7,79,0
2280 PRINT "LUMINESCENCE DECAY"
2290 PRINT "SAMPLE: , BB$
2300 PRINT "microseconds/channel", TMB/25
2310 FOR I% = 1 TO 256
2320 Draw 5*I%,4*S(I%)
2330 NEXT I%
2340 ENDPROC
2350 DEF PROC_DISP2
2360 MOVE 15,50
2370 DRAW 1270,50
2380 MOVE 5*ZT%,800
2390 FOR I% = ZT% TO 254
2400 DRAW 5*I%,800*S(I%)/S(ZT%+2)+50
2410 NEXT I%
2420 MOVE 5*ZT%,800*A(1)/S(ZT%+2)+50
2430 FOR I% = ZT% TO 256
2440 IF N1 = 2 GOTO 2470
2450 YT(I%-ZT%+1) = A(1)*EXP(-(I%-ZT%)/A(2))
2460 GOTO 2480
2470 YT(I%-ZT%+1) = A(1)*EXP(-(I%-ZT%)/A(2))+A(3)*EXP(-(I%-ZT%)/A(4))
2480 NEXT I%
2490 FOR I% = ZT% TO 256
2500 DRAW 5*I%,800*YT(I%-ZT%+1)/S(ZT%+2)+50
2510 NEXT I%
2520 MOVE 550,600
2530 DRAW 1150,600
2540 MOVE 1150,500
2550 DRAW 1150,700
2560 MOVE 550,500
2570 DRAW 550,700
2580 MOVE 550,600
2590 FOR I%=ST% TO SP%
2600 DRAW 550+(600/(SP%-ST%+1))*(I%-ST%+1),600+5E3*(YT(I%-ZT%+1)-S(I%))
2610 NEXT I%
2620 ENDPROC
2630 DEF PROC CALC
2640 CHN=0
2650 FOR K%=1 TO N1:E(K%,1)=1:Z=EXP(-T/A(2*K%))
2660 FOR J%=ZT%+1 TO SP%:E(K%,J%-ZT%+1)=E(K%,J%-ZT%)*Z
2670 NEXT J%
2680 NEXT K%
2690 FOR J%=ST% TO SP%
2700 Z=0
2710 FOR K%=1 TO N1
2720 Z=Z+A(-1+2*K%)*E(K%,J%-ZT%+1)
2730 NEXT K%
2740 FOR J%=ST% TO SP%
2750 Z=Z=Z+T(J%-ZT%+1)*Z
2760 NEXT J%
2780 ENDPROC
2790 DEF PROC FILLMAT
2800 LOCAL Z1, Z2, Z3, Z4, Z5, Z6, Z7
2810 FOR I%=1 TO NEQN
2820 FOR J%=1 TO NEQN
2830 B(1%,J%)=0:C(1%)=0
2840 NEXT J%:NEXT I%
2850 FOR J%=ST% TO SP%
2860 Z1=E(1,J%-ZT%+1)*E(1,J%-ZT%+1):B(1,1)=B(1,1)+Z1
2870 B(1,2)=B(1,2)+T(J%-ZT%+1)*Z1
2880 B(2,2)=B(2,2)+T(J%-ZT%+1)*T(J%-ZT%+1)*Z1
2890 Z2=E(1,J%-ZT%+1)*R(J%-ST%+1):C(1)=C(1)+Z2
2900 C(2)=C(2)+T(J%-ZT%+1)*Z2
2910 NEXT J%
2920 IF N1=1 THEN GOTO 3220
2930 FOR J%=ST% TO SP%
2940 Z3=A(1)/(A(2)*A(2))
2950 B(1,2)=B(1,2)*Z3
2960 B(2,2)=B(2,2)*Z3
2970 IF N1=1 THEN GOTO 3220
2980 FOR J%=ST% TO SP%
2990 Z4=E(2,J%-ZT%+1)*E(2,J%-ZT%+1)
3000 Z5=E(2,J%-ZT%+1)*R(J%-ZT%+1)
3010 Z7=E(1,J%-ZT%+1)*E(2,J%-ZT%+1)
3020 B(2,3)=B(3,3)+Z4
3030 B(4,4)=B(4,4)+T(J%-ZT%+1)*T(J%-ZT%+1)*Z4
3040 B(1,3)=B(1,3)+Z7
3050 B(1,4)=B(1,4)+T(J%-ZT%+1)*Z7
3060 B(2,4)=B(2,4)+T(J%-ZT%+1)*T(J%-ZT%+1)*Z7
3070 B(3,4)=B(3,4)+T(J%-ZT%+1)*Z4
3080 C(3)=C(3)+Z5
3090 C(4)=C(4)+T(J%-ZT%+1)*Z5
3100 NEXT J%
3110 B(2,3)=B(1,4)
3120 Z6=A(3)/(A(4)*A(4))
3130 B(4,4)=B(4,4)*Z6
3140 B(1,4)=B(1,4)*Z6
3150 B(2,3)=B(2,3)*Z3
3160 B(2,4)=B(2,4)*Z3
3170 B(3,4)=B(3,4)*Z6
3180 C(4)=C(4)*Z6
3190 FOR J%=1 TO NEQN:FOR K%=2 TO NEQN
3200 B(K%,J%)=B(J%,K%)
3210 NEXT K%
3220 NEXT J%
3220ENDPROC
3230DEF PROCSOLVE
3240FOR I%=1 TO NEQN:W(I%)=0
3250FOR J%=1 TO NEQN
3260IF ABS(B(I%,J%))>W(I%) THEN W(I%)=ABS(B(I%,J%))
3270NEXTJ%:NEXTI%
3280FOR K%=1 TO NEQN-1:ISTAR=K%
3290RATIO=ABS(B(K%,K%))/W(K%)
3300FOR I%=K%+1 TO NEQN
3310TEMP=ABS(B(I%,K%))/W(I%)
3320IF TEMP>RATIO THEN RATIO=TEMP:ISTAR=I%
3330NEXTI%
3340IF ISTAR<=K% THEN GOTO 3400
3350T9=C(K%):C(K%)=C(ISTAR):C(ISTAR)=T9
3360T9=W(K%):W(K%)=W(ISTAR):W(ISTAR)=T9
3370FOR J%=1 TO NEQN
3380T9=B(K%,J%):B(K%,J%)=B(ISTAR,J%):B(ISTAR,J%)=T9
3390NEXT J%
3400FOR I%=K%+1 TO NEQN
3410RATIO=B(I%,K%)/B(K%,K%)
3420C(I%)=C(I%)-RATIO*C(K%)
3430FOR J%=K%+1 TO NEQN
3440B(I%,J%)=B(I%,J%)-RATIO*B(K%,J%)
3450NEXT J%
3460NEXTI%
3470NEXTK%
3480X(NEQN)=C(NEQN)/B(NEQN,NEQN)
3490FOR I%=NEQN-1 TO 1 STEP -1
3500SUM=0
3510FOR J%=I%+1 TO NEQN
3520SUM=SUM+B(I%,J%)*X(J%)
3530NEXT J%
3540X(I%)=(C(I%)-SUM)/B(I%,I%)
3550NEXT I%
3560ENDPROC
TRANSFER

Function of program: calculates excited state populations of both dye and DNA, according to equations 6.1a-f. Integration of differential equations is by simple stepwise addition.

- Line 200 - 290: Calculation of absorption cross sections.
- Line 300 - 410: DNA and dye rate constants.
- Line 420 - 530: Input transfer rate and path length of cell (not used in calculations).
- Line 540 - 640: Calculation of laser parameters.
- Line 650 - 740: Concentration of compounds (in M)
- Line 800 - 910: Set initial population levels and laser pulse.
- Line 920 - 1230: Print and plot output routines.
- Line 1240-1440: DNA and dye rate equations.
- Line 1450-1520: Vary laser pulse with absorption (not used in present calculations).
- Line 1570- END: print and plot routines.
Program: TRANSFER

10 REM
20 REM******************************************************************************
30 REM
40 REM      CALCULATION OF DYE S1 POPULATION LEVELS
50 REM      WITH AND WITHOUT ENERGY TRANSFER FROM DNA S1 LEVEL
60 REM      INPUT - ENERGY TRANSFER RATE (in s⁻¹)
70 REM
80 REM******************************************************************************
90 REM
100 REM
110 KEY OFF
120 DIM DNA5O#(500):DIM DNAT1#(500):DIM DNAT1#(500)
130 DIM DYESO#(500):DIM DYET1#(500):DIM DYET1#(500)
140 DIM D2SO#(500):DIM D2S1#(500):DIM D2T1#(500)
150 DIM LASER#(500)
160 REM
170 XS="INTERCALATED DYE"
180 REM
190 REM******************************************************************************
200 REM
210 REM      VALUES OF EXTINCTION COEFFICIENTS
220 REM
230 REM
240 DEF FNSIGMA#(X#)=LOG(10#)*1000*X#/6.022045D+23
250 EXDNA#  = 6600#
260 EXDYE#  = 6600#
270 DNASIG# =FNSIGMA#(EXDNA#)
280 DYESIG# =FNSIGMA#(EXDYE#)
290 REM
300 REM******************************************************************************
310 REM
320 REM      DNA RATE CONSTANTS
330 DNATFS#=.00000000001#
340 DNATFT#=.00001#
350 PHIDNA#=.02#
360 REM      DYE RATE CONSTANTS
370 DYETFS#=.0000000004#
380 DYETFT#=.00002#
390 PHIDYE#=.5#
400 REM
410 REM
420 REM******************************************************************************
430 REM      ENERGY TRANSFER RATE
440 REM
450 INPUT" ENERGY TRANSFER RATE (double precision)";ETR#
460 REM
470 PATH#=1#
480 NZ#=50
490 DZ#=PATH#/NZ#
500 REM
510 REM
520 REM******************************************************************************
530 REM
540 REM      PARAMETERS OF EXCITING LASER PULSE
550 REM
560 REM
570 REM INPUT" LASER PULSE FWHM (double precision)";TLASER#
580 REM
590 TO#=TLASER#/2*SQR(LOG(2#)))
600 LAMBDA#=260#
610 HNU# =1.986305D-16/LAMBDA#
620 POWER#=1000000000#
630 REM
640 REM

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CONCENTRATIONS OF COMPOUNDS

CDNA#=.001#

CDYE#=.00004#

DNATOT#=CDNA#*6.022045D+20

DYETOT#=CDYE#*6.022045D+20

START OF CALCULATIONS

NT%=500

DT#-6*TO#/NT%

SET INITIAL BOUNDARY CONDITIONS

FOR I%-1 TO NT%

DNASO#(I%)=DNATOT#

DYESO#(I%)=DYETOT#

D2S0#(I%)=DYETOT#

LASER#(I%)=POWER#*EXP(-((I%*DT#/TO#-3)^2))

NEXT I%

DNA Sl population":LINE(180,60)-(220,60),1

DYE Sl population":

DNA EQUATIONS

DNAS#(I%+1)=DNAS#(I%)+(-LASER#(I%)*DNASIG#*DNAS#(I%)jHNU#+DNASI#(I%)*(1-PHIDNA#)/DNATFS#+DNAT1#(I%)/DNATFT#+ETR#*DNASI#(I%))*DT#
1280 DNA1#(I%+1)=DNA1#(I%)+(LASER#(I%)*DNA1#(I%)/HNU#-DNA1#(I%)/DNAFS#-DNA1#(I%)*ETR#)*DT#
1290 DNA1#(I%+1)=DNA1#(I%)+(PHIDNA#*DNA1#(I%)/DNAFS#-DNA1#(I%)/DNAFT#)*DT#
1300 REM
1310 REM
1320 REM ***** DYE EQUATIONS *************
1330 REM
1340 REM
1350 IF CDYE#=O# THEN GOTO 1420
1360 DYESO#(I%+1)=DYESO#(I%)+(-LASER#(I%)*DYESIG#*DYESO#(I%)/HNU#+DYES1#(I%)*(1-PHIDYE#)/DYTEFS#-DYESI#(I%)/DYTEFT#-ETR#)*DT#
1370 DYES1#(I%+1)=DYES1#(I%)+(LASER#(I%)*DYESIG#*DYESO#(I%)/HNU#-DYES1#(I%)/DYTEFS#+DYES1#(I%)*ETR#)*DT#
1380 DYE1#(I%+1)=DYE1#(I%)+(PHIDYE#*DYES1#(I%)/DYTEFS#-DYE1#(I%)/DYTEFT#)*DT#
1390 D2SO#(I%+1)=D2SO#(I%)+(-LASER#(I%)*DYESIG#*D2SO#(I%)/HNU#+D2S1#(I%)*(1-PHIDYE#)/DYTEFS#)*DT#
1400 D2S1#(I%+1)=D2S1#(I%)+(LASER#(I%)*DYESIG#*D2SO#(I%)/HNU#-D2S1#(I%)/DYTEFS#)*DT#
1410 D2T1#(I%+1)=D2T1#(I%)+(PHIDYE#*D2S1#(I%)/DYTEFS#-D2T1#(I%)/DYTEFT#)*DT#
1420 REM
1430 REM
1440 REM ***** VARY LASER PULSE WITH ABSORPTION ************
1450 REM
1460 REM
1470 LASER#(I%)=LASER#(I%)+(-LASER#(I%)*(DNA1#(I%)+DYES1#(I%)+DYE1#(I%)))*DZ#
1480 NEXT I%
1490 IF K%-l GOTO 1590
1500 GOTO 1800
1510 REM
1520 REM
1530 REM
1540 REM
1550 REM
1560 REM
1570 REM
1580 REM
1590 PSET(100,180)
1600 FOR M%-1 TO NT%-1
1610 IF Z# < DYES1#(M%+1) THEN Z#=DYES1#(M%)
1620 IF X# < D2SO1#(M%+1) THEN X#=D2SO1#(M%)
1630 NEXT M%
1640 IF CDYE#=O# THEN Z#=1 AND X#=1
1650 FOR L%-1 TO NT% STEP 10
1660 CIRCLE(L%+99,165-165*(DYES1#(L%)/Z#)+15),4
1670 NEXT L%
1680 PSET(100,180)
1690 FOR L%-1 TO NT% STEP 10
1700 PSET(L%+99,165-165*(D2SO1#(L%)/Z#)+15)
1710 DRAW "BU2R2D4L4U4R2"
1720 NEXT L%
1730 PSET(100,180)
1740 FOR M%-1 TO NT%-1
1750 IF Y# < DNA1#(M%+1) THEN Y#=DNA1#(M%)
1760 NEXT M%
1770 FOR L%-1 TO NT%
1780 LINE-(L%+99,165-165*(DNA1#(L%)/Y#)+15),1
1790 NEXT L%
1800 NEXT K%
1810 K%-1:Q#-=0
1820 WHILE Q#<.1*Z#
1830 Q#=DYES1#(K%):K%-K%+1
1840 WEND
1850 L%-1:R#-=0
1860 WHILE R# < .9*Z#
1870 R# = DYES1#(L#): L# = L# + 1
1880 WEND
1890 RISE1# = (L# - K#) * DT#
1900 K# = 1: Q# = 0
1910 WHILE Q# < .1*X#
1920 Q# = D2S1#(K#): K# = K# + 1
1930 WEND
1940 L# = 1: R# = 0
1950 WHILE R# < .9*X#
1960 R# = D2S1#(L#): L# = L# + 1
1970 WEND
1980 RISE2# = (L# - K#) * DT#
1990 PRINT" DYE S1 population and risetime (ps)";
2000 PRINT" without transfer ": PRINT USING"#.##"; RISE2#: PSET(230, 83): DRAW"
2010 BU2R2D4L4U4R2"
2020 PRINT" with transfer ": PRINT USING"#.##"; RISE1#: PSET(230, 92), 0: SI RCLE(230, 92), 4
2030 K# = 1
2040 END
ERRATA

page 10, line 6 : "figure 1.1" should be "figure 1.2".

page 23, line 8 : "sites the" should be "sites in the".

page 38, line 10 : $^{3}\text{MB}^{2+}$ should be $^{3}\text{MBH}^{2+}$.

page 39, line 13 : $(\text{KH}_{2}\text{PO}_{4})$, add $\text{K}_{2}\text{HPO}_{4}$.

page 70, line 14 : 690 nm should be 684 nm.

page 111, line 8 : "$k_Q$" should be "$k_q$".

page 124, figure 3.33 : 600 nm should be 595 nm.

page 142, legend figure 3.13 : lifetimes are in picoseconds.

page 158, line 16 : "of the dye", should be "of the dye monomer".

page 174, line 9 : "Scatchard curve", should be "Scatchard curve for CT-DNA"

page 189, legend figure 4.9 : A-C : n increases from 0.02 to 0.1 in steps of 0.02; D-F : n increases from 0.04 to 0.2 in steps of 0.04.

page 255, line 10 : insert "(MLCT)" after "charge transfer".

page 294, line 1 : "cause" should be "course".