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TIME-RESOLVED LASER SPECTROSCOPY STUDY
OF
H₂TMPyP/DNA COMPLEXES AND MX/DNA COMPLEXES

by

Yizhong Shen, M.Sc.

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of the requirements of
Doctor of Philosophy

Department of Chemistry

Carleton University

Ottawa, Ontario

April 15, 1993

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**"TIME-RESOLVED LASER SPECTROSCOPY STUDY OF
H₂TMPyP/DNA COMPLEXES AND MX/DNA COMPLEXES"**

submitted by

Yizhong Shen, M.Sc.

in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

Chairman, Department of Chemistry

Thesis Supervisor

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Carleton University
April 15, 1993

ABSTRACT

The mode of binding between two drug/DNA complexes, tetrakis (4-N-methyl-pyridyl) porphyrin/DNA and mitoxantrone/DNA has been determined through time-resolved anisotropic fluorescence and anisotropic absorption spectroscopy.

A theory modelling anisotropic spectroscopy has been developed which extends the current understanding to experimental conditions involving higher pump beam densities. This theory can be used to determine the influence of molecular rotation and excited state life-time.

The experimental results show that the fluorescence decay time of intercalated tetrakis (4-N-methyl-pyridyl) porphyrin/DNA in a buffer solution is 10.0 ± 0.5 nanosecond and 2.0 ± 0.5 nanosecond for the externally bound complexes; and that the fluorescence decay time of the intercalated mitoxantrone/DNA in a buffer solution is 240 ± 50 picosecond.

For tetrakis (4-N-methyl-pyridyl) porphyrin/DNA complexes in a buffer solution, relative orientation of the intercalated tetrakis (4-N-methyl-pyridyl) porphyrin molecules

and the externally bound tetrakis (4-N-methyl-pyridyl) porphyrin molecules is determined by anisotropic absorption spectroscopy. If one assumes that the plane of the intercalated tetrakis (4-N-methyl-pyridyl) porphyrin molecules is perpendicular to the helical axis of DNA, the angle between the normal direction of the externally bound tetrakis (4-N-methyl-pyridyl) porphyrin molecules and the helical axis of DNA is $50^{\circ} \pm 10^{\circ}$.

ACKNOWLEDGEMENTS

It is a pleasure to acknowledge the help of all who made this thesis possible.

I would like to thank my supervisor Dr. J.A. Koningstein for his financial support, academic guidance, advice and consultation during the course of my thesis work over the past four years.

I am grateful to Dr. P. Myslinski and Dr. Y. Liu for their assistance which helped me gain competence and confidence in my abilities, and to Dr. T. Treszczanowicz for her help with my research work and for her friendship.

Also I would like to thank Dr. D. Williams for his kindness in reading the manuscript of my thesis and making suggestions for improvements.

I want to thank Mr. Karl Diedrich and Mr. Robert Kehle for all their help, Dr. Y. Yevdokimov for supplying the DNA samples, Dr. A.G. Szabo, Dr. D.T. Krajcarski and Dr. L. Bramell for their assistance with the iterative deconvolution procedure.

My thanks are also owing to Dr. B.R. Hollebhone, who allowed me to use his equipment for my research work.

I am grateful to Nichole Michelin and Steven Desjardins for their help in improving part of my thesis.

And finally, I am very grateful to my husband and my daughter for their understanding and patience.

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1. INTRODUCTION

Time-resolved anisotropic fluorescence and anisotropic absorption have been used to study liquid phase molecular systems for over 20 years [1-5]. The recent development of ultrashort optical pulse measuring techniques has greatly expanded the capability of time-resolved anisotropic spectroscopy (fluorescence and absorption) such that time-resolved anisotropic spectroscopy can now be performed in the picosecond and femtosecond regimes [6-17].

Spectral parameters probed by anisotropic spectroscopy are dependent upon the rotational relaxation functions of the molecular system under study. The influence of viscosity and temperature on the rotational relaxation function has been studied by many workers [23,27-30,59]. The volume and shape of the molecules can also be derived from the rotational relaxation function [3].

Such measurement techniques have also been used to probe the rotational dynamics of molecules in liquid phase, thus providing information about (i) the interactions between solute and solvent molecules and (ii) the related chemical reactions [4,13,18-25,37,38,46].

Time-resolved anisotropic spectroscopy has also been used to investigate ultrafast

Time-resolved anisotropic spectroscopy has also been used to investigate ultrafast chemical reaction processes. These processes include: (i) the intermolecular energy transfer including singlet-singlet transfer and triplet-triplet transfer [39-42]; (ii) isomerization dynamics of molecules in solution [43-45,60]; (iii) activationless reactions in solution [47,48,51]; (iv) proton transfer and electron transfer [31-33]; and (v) diffusion-controlled chemical reactions [49,50].

The applications of time dependent anisotropic spectroscopy as a tool to study biological systems have increased rapidly. Anisotropic spectroscopy has proven to be valuable in the study of photosynthetic process [42,52-54,67]. Other investigations using this basic experimental approach have targeted research in (i) protein structure and dynamics [25,52,55-58], (ii) the structure and dynamics of deoxyribonucleic acid (DNA) [52,59,61-66], and (iii) the binding modes of drug to DNA [65,68-74].

The underlying principle of time resolved anisotropic spectroscopy is that a pulsed polarized light beam disturbs the equilibrium distribution of ground state molecular orientations by exciting those molecules whose transition dipoles are nearly aligned along the electric field vector of the exciting light, then the relaxation process of the perturbed distribution is observed either through time dependent fluorescence depolarization (anisotropic fluorescence) or by monitoring absorption of a second pulsed polarized probe light beam (anisotropic absorption).

The theory of anisotropic fluorescence for weak optical pump condition has been treated in detail by Tao [1]. Most of the reported anisotropic absorption measurements have been performed in a manner similar to the method reported by Shank and Ippen [2].

The experimental method proposed in this work is different from Shank's method. A comprehensive theoretical model for the new experimental conditions has been developed and is given in this thesis. Using this theory, the angle between any two transition moments in a molecular system can be determined from the experimental results obtained from the new experimental techniques which are also given here.

Complex situations, such as those which arise from (i) effects of the rotation of molecules, (ii) effects of the life-time of the excited state, (iii) the probe light being absorbed by two dipole moments, and (iv) effect of strong pump intensity, can also be treated by the new theory.

The aim of this work is to demonstrate that time-resolved anisotropic spectroscopy can be used to study binding mechanism of drugs to DNA. This work has important experimental implications, for intercalation of drugs with DNA has been studied as a means of disease control. The drugs studied in this work are tetrakis (4-N-methylpyridyl) porphyrin, H_2TMPyP , and mitoxantrone, MX. The results reveal that the fluorescence decay time of the drug molecules can be used as a spectroscopic characteristic to identify the binding modes. The geometrical structure of the drug/DNA

complexes can be determined by the anisotropic absorption measurements.

The thesis is divided into 6 chapters:

CHAPTER 1 Introduction.

CHAPTER 2 Development of the theoretical model of time resolved anisotropic absorption and fluorescence spectroscopy.

CHAPTER 3 Structure and properties of H₂TMPyP/DNA and MX/DNA complexes.

CHAPTER 4 Experimental procedures:

- (i) picosecond experimental system,
- (ii) nanosecond experimental system,
- (iii) data analysis,
- (iv) sample handling.

CHAPTER 5 Experimental results and discussion.

CHAPTER 6 Summary and conclusion.

2. THEORY

2.1 INTRODUCTION

A large amount of experimental and theoretical work in the area of anisotropic fluorescence and anisotropic absorption has been performed and reported.

The theory of anisotropic fluorescence spectroscopy has been treated in detail by Tao [1]. Most of the experimental and theoretical studies on anisotropic fluorescence are based on Tao's theory derived for a weak optical pump condition (the definition of the weak pump condition is given by equation 2-3-5).

The technique for measuring ultrashort time resolved anisotropic absorption was proposed by Shank and Ippen in 1974 [2]. Many experiments of anisotropic absorption have been carried out in a manner similar to Shank's method (shown at Figure 2-1-1).

In the experimental method proposed and used by Shank, (i) the pump and the probe beams form an angle of 45° between their polarized directions and (ii) the pump and the probe are set at the same wavelength. In the most of the experiments, the weak pump condition is used.

Figure 2-1-1: Experimental set-up for anisotropic absorption measurements proposed by Shank and Ippen [2].

Figure: 2-1-2: Experimental set-up for anisotropic absorption measurements proposed by this work.

The experimental set-up for measuring anisotropic absorption in this work is different from that proposed by Shank and Ippen. For these new studies, the experimental set-up is such that (i) the polarized directions of the pump and the probe pulses form an angle which can be adjusted from 0° to 90° ; and (ii) the pump and the probe are set at the different wavelengths (Figure 2-1-2). Also in the anisotropic fluorescence measurements and in the anisotropic absorption measurements, a strong optical pump condition is used.

The chapter is divided to 4 sections and provides a comprehensive theoretical description to understand the experiments performed in this work.

Section 2.2 presents the theoretical background about absorption spectroscopy and fluorescence spectroscopy.

The theoretical aspects of anisotropic absorption are treated in Section 2.3. Section 2.3.1 considers the basic case of anisotropic absorption in which the effects of (i) the rotation of the molecules and (ii) the life-time of the excited state of the molecules are not considered. Section 2.3.2 considers time-resolved anisotropic absorption, i.e. it deals with the rotation of the molecules and the life-time of the excited state. Section 2.3.3 considers the case where the wavelength of the probe laser beam is tuned between two excited states and the probe light is absorbed by the two transition dipole moments.

The formulae derived in Section 2.3 for anisotropic absorption can be directly used to describe anisotropic fluorescence. Section 2.4 shows how to apply them to the case of anisotropic fluorescence. Section 2.4 also shows that at a weak pump condition, these results agree with the previous theoretical results [1,3,77,78].

2.2 GENERAL SPECTROSCOPIC THEORY: ABSORPTION AND FLUORESCENCE [79]

2.2.1 Absorption Spectroscopy

The quantitative and qualitative property of a molecular system to absorb ultraviolet and visible radiation is measured by absorption spectroscopy. The fundamental law governing absorption is Beer's law given in equation 2-2-1:

$$A = \log \frac{I_0}{I} = \epsilon bc \quad (2-2-1)$$

where A is called the *absorbency*; I_0 is the intensity of incident light which is reduced to I by the absorption; the constant ϵ is called the *molar absorptivity* or *extinction coefficient*; b is the length of the sample and c is *molarity* of the sample.

Another symbol T , *transmission*, is also used to represent the property of absorption:

$$T = \frac{I}{I_0} \quad (2-2-2)$$

The transmission also can be represented by:

$$T = \frac{I}{I_0} = e^{-\sigma NL} \quad (2-2-3)$$

Here the constant σ is the absorption *cross section* of the molecules for the incident light; N is the concentration of the molecules, i.e. the number of molecules at per unit volume; and L is the length of the sample. The discussions in this chapter are based on equation 2-2-3.

The information contained in an absorption spectrum is:

(i) the frequency which represents the energy difference between the two electronic energy levels;

(ii) the intensity of the transition which depends proportionally on the square of the light induced transition dipole moment represented by the cross section of the sample,

concentration of the sample and the length of the sample.

Absorption spectroscopy is widely used in qualitative analysis. The objectives for absorption measurements include (i) identification of a pure compound; (ii) determination of presence or absence of a particular species in a mixture; (iii) determination of the electronic energy level of a molecular system; (iv) finding the concentration or the cross section of a molecular system.

It is impossible to get information about the rotational relaxation of a molecular system from the UV and the visible absorption spectra.

2.2.2 Fluorescence Spectroscopy

Many molecules can be excited by radiation and the molecules then re-emit radiation either at the same wavelength or at a modified wavelength. Fluorescence is concerned with the re-emission process which ceases almost immediately after irradiation is discontinued. A simple theory about fluorescence spectroscopy is introduced below.

In principle, the electronic energy level of a molecule can be derived from the molecular orbital diagram. A molecular orbital is formed from the overlap of the atomic orbitals. There are 3 types of molecular orbital: *bonding*, *antibonding* and *nonbonding* orbital. The bonding orbital has the lower energy and is thus occupied by the electrons

in the ground state.

Most molecules contain an even number of electrons; in the ground state, these electrons exist as pairs in the various molecular orbitals. The Pauli exclusion principle demands that the spins of the two electrons in a given orbital be opposite to one another (the spins are said to be paired). A molecular electronic state in which all of the electron spins are paired is called a *singlet* state. When one of the electrons is excited to a higher energy level, a singlet or a *triplet* state can result. In the excited singlet state, the spin of the electron does not change and it is still paired with the electron in the ground state; in the excited triplet state, the spin of the electron changes to the opposite direction and it is parallel to the electron in the ground state. The properties of a molecule in the excited triplet state differ from those in the excited singlet state. The more important thing is that a singlet-triplet transition (or the reverse) is a significantly less probable event than the singlet-singlet transition.

Figure 2-2-1 [165] is an energy level diagram for a molecular system. The energies of the ground state and the two electronic excited states are shown by heavy horizontal lines. Note that the energies of the triplet states are slightly lower than those of the corresponding singlet states. A series of closely spaced, excited vibrational states for each electronic state are shown by lighter horizontal lines. The energies required for excitation of the molecule to the two singlet states are represented by λ_1 and λ_2 . When a deactivation process of a molecule occurs by emission (singlet->singlet), the

corresponding fluorescence are represented by λ_1' and λ_2' , and it shows that the energy of a fluorescence is less than the energy required for excitation.

Figure 2-2-1: Partial energy level diagram for a photoluminescence system [165].

An excited molecule can return to its ground state by any of the several mechanistic paths shown at Figure 2-2-1; the favoured route is the one that minimizes the life-time of the excited state. Fluorescence spectroscopy results only to the singlet-singlet transition. Thus, if deactivation by fluorescence is rapid with respect to the other processes, fluorescence is observed. On the other hand, if a radiationless path has a more favourable rate constant, fluorescence is either absent or less intense. Both molecular structure and chemical environment are influential in determining the intensity of fluorescence or quantum yield of fluorescence. The factors that affect the intensity of fluorescence include transition type, temperature, the solvent, pH value of the solution, the concentration, etc..

The most attractive feature of fluorescence spectroscopy is its inherent sensitivity. The lowest limit concentration for fluorescence as compared to that for absorption is a factor 0.1 or better. The fluorescence spectroscopy system is strongly dependent on the structure of the molecular system. The sensitivity and the selectivity of fluorometry make the method particularly valuable in chemical and biological analysis [81]. However it is less widely applicable than absorption spectroscopy because of the relatively limited number of chemical system that can be caused to fluoresce.

2.3 ANISOTROPIC ABSORPTION

2.3.1 Simplified Situation

Two polarized laser beams are required to measure anisotropic absorption. One of the polarized laser beams disturbs the equilibrium distribution to produce an anisotropic spatial distribution, and the second polarized laser beam is used to measure anisotropic absorption. The first laser beam is called the pump beam and the second one is called the probe beam.

Figure 2-3-1 shows the geometry of an anisotropic absorption experiment which is based on the experimental set-up used in this work (Figure 2-1-2). The pump beam with electric vector E_1 is polarized along the Z axis of the fixed laboratory coordinate system, the direction of polarization of the probe beam is E_2 and γ is the angle between E_1 and E_2 . Both beams are propagating along the X axis. μ_1^0 and μ_2^0 are the directions of the transition dipole moments of a molecule for transitions giving rise to a ground state absorption for the pump beam and the probe beam respectively. α^0 is the angle between μ_1^0 and μ_2^0 . Ω is the angle between the Z axis and μ_1^0 . The molecule is situated at the origin of the coordinate system.

Figure 2-3-1: Geometry of anisotropic absorption experiment in XYZ fixed laboratory coordinate system.

The data obtained by the measurements of anisotropic absorption are (i) the transmission of the probe beam with the pump beam on, i.e. $E_1 \neq 0$; (ii) the transmission of the probe beam with the pump beam off, i.e. $E_1 = 0$.

In these discussions (Section 2.3.1), it is assumed that (i) the rotational relaxation time of the molecules is much longer than the duration of the laser pulses of the pump beam and the probe beam and (ii) these two beams arrive at the same time to the origin of the coordinate system.

The electronic structure of the molecule in terms of the singlet levels S_n is shown in Figure 2-3-2 where the wavelength of the pump is thought to be in resonance with the transition of the S_0 and S_1 electronic surface (characterized by the dipole moment μ_1^G and cross section σ_1^G); the probe is tuned to another absorption band (characterized by the dipole moment μ_2^G and cross section σ_2^G ; $S_n \leftarrow S_0$, where $n > 1$).

A large pumping intensity induces an effective population saturation in the excited state. Therefore the excited state absorption cannot be neglected (characterized by the dipole moment μ_2^E and cross section σ_2^E , Figure 2-3-2). The angle between μ_2^E and μ_1^G is α^E . The dipole moment μ_2^E and cross section σ_2^E do not show on Figure 2-3-1.

Figure 2-3-2: Pump (E_1) and probe (E_2) photons related to the energy level of molecules in anisotropic absorption.

According to Beer's law (equation 2-2-3), the transmission of the probe beam is given by

$$T(\alpha^G, \alpha^E, \gamma, a) = e^{(-L \cdot [\sigma_2^G \cdot N_{eff}^G(\alpha^G, \gamma, a) + \sigma_2^E \cdot N_{eff}^E(\alpha^E, \gamma, a)])} \quad (2-3-1)$$

where T is the transmission of the probe beam; L is the length of the sample; σ_2^G and σ_2^E are the cross sections for the ground state and the excited state absorption of the probe beam, respectively; N_{eff}^G and N_{eff}^E are the *effective concentration* of molecules to absorb the probe light in the ground state and the excited state. The parameters α^G , α^E , γ were described earlier (Figure 2-3-1); the parameter a is the *normalized pump intensity* which is defined by equation 2-3-3.

Equation 2-3-1 shows that in anisotropic absorption, the transmission of the probe light is dependent upon (i) the pump intensity (represented by a), (ii) the geometry of the experimental set-up (represented by γ) and (iii) the molecular structure (characterized by α^G and α^E)

Ground state absorption

In some experiments, it may be assumed that the cross section of the excited state absorption of the probe beam can be neglected with respect to the cross section of ground state absorption ($\sigma_2^E \approx 0$, or $\sigma_2^G \gg \sigma_2^E$). Thus the transmission of a sample given by

equation 2-3-1 is only determined by the effective concentration of the ground state, $N_{\text{eff}}^G(\alpha^G, \gamma, a)$.

The spatial distribution anisotropy which is induced by the pump beam can be described by a distribution function of the angular density $n_1^G(\theta, a)$. The angular density refers to the concentration of the molecules in the ground state with the transition moment μ_1^G oriented in the direction (ϕ, θ) which is given by

$$n_1^G(\theta, a) = \frac{N}{4\pi} \frac{1}{1 + a \cdot \cos^2\theta} \quad (2-3-2)$$

Here N represents the molecular concentration and a is the normalized pump intensity.

The value for a can be derived from

$$a = \frac{I_1}{I_s} \quad (2-3-3)$$

where I_1 is the pump intensity and the *saturation intensity* I_s is given by [80]

$$I_s = \frac{h\nu}{Q \cdot \sigma_1^G \cdot \tau_1} \quad (2-3-4)$$

Here $h\nu$ is the energy of the pump photons (E_1); σ_1^0 is the cross section for absorption of these photons by the ground state; τ_1 is the radiative lifetime of the S_1 state; and Q is the quantum yield of fluorescence which originates in S_1 and terminates on S_0 .

With a weak pump condition, i.e. $a \ll 1$, the angular density becomes

$$n_1^0(\theta, a) = \frac{N}{4\pi} (1 - a \cos^2 \theta) \quad (2-3-5)$$

In the absence of the pump ($a = 0$), the angular density is isotropic and equal to $N/4\pi$.

If the probe beam E_2 corresponds to the transition moment μ_2^0 , the spatial anisotropy which is induced by the pump beam E_1 can be described by the angular density $n_2^0(\theta, \alpha^0, a)$. Here $n_2^0(\theta, \alpha^0, a)$ refers to the concentration of the ground state molecules with the transition moment μ_2^0 oriented in the direction (ϕ, θ) . $n_2^0(\theta, \alpha^0, a)$ is dependent on $n_1^0(\theta, a)$ and α^0 .

Consider a direction (ϕ, θ) in Figure 2-3-1. The concentration of the molecules with μ_2^0 orientated in this direction is given by the molecules with μ_1^0 orientated in a cone. This cone is determined by the angle α^0 . The probability of the transition moment μ_2^0 being oriented in the direction (ϕ, θ) is $1/2\pi$ for one molecule in the cone.

Thus $n_2^G(\theta, \alpha^G, a)$ can be represented as

$$n_2^G(\theta, \alpha^G, a) = \frac{N}{4\pi} \times \frac{1}{2\pi} \int_0^{2\pi} d\varphi' \frac{1}{1 + a \cos^2 \Omega} \quad (2-3-6)$$

Here Ω is the angle between the Z axis and μ_1^G in the cone and $\cos^2 \Omega$ is given by

$$\cos^2 \Omega = |Z \cdot \mu_1^G|^2 \quad (2-3-7)$$

In the X'Y'Z' coordinate system, Z and μ_1^G can be written as

$$Z = \begin{pmatrix} 0 \\ -\sin\theta \\ \cos\theta \end{pmatrix}$$

and

$$\mu_1^G = \begin{pmatrix} \sin\alpha^G \cos\varphi' \\ \sin\alpha^G \sin\varphi' \\ \cos\alpha^G \end{pmatrix} \quad (2-3-8)$$

Hence, the effective concentration of molecules absorbing the probe light in the ground state, N_{eff}^G , is

$$N_{eff}^G(\alpha^G, \gamma, a) = \int_0^{2\pi} d\varphi \int_0^\pi \sin\theta d\theta n_2^G(\theta, \alpha^G, a) |E(\gamma) \cdot r|^2 \quad (2-3-9)$$

where n_2^G is defined by equation 2-3-6. In the XYZ coordinate system, $E_2(\gamma)$ and r can be represented by

$$E_2(\gamma) = \begin{pmatrix} 0 \\ \sin\gamma \\ \cos\gamma \end{pmatrix}$$

and

$$r = \begin{pmatrix} \sin\theta \cos\varphi \\ \sin\theta \sin\varphi \\ \cos\theta \end{pmatrix} \quad (2-3-10)$$

$N_{eff}^G(\alpha^G, \gamma, a)$ also can be written as

$$N_{eff}^G(\alpha^G, \gamma, a) = \frac{N}{3} \cdot g(\alpha^G, \gamma, a) \quad (2-3-11)$$

where

$$g(\alpha^G, \gamma, a) = \frac{3}{8\pi} \int_0^\pi d\theta \int_0^{2\pi} d\varphi' \frac{2\cos^2\gamma \cos^2\theta \sin\theta + \sin^2\gamma \sin^3\theta}{1 + a\cos^2\Omega} \quad (2-3-12)$$

and $\cos^2\Omega$ is determined by equation 2-3-7. The value of $g(\alpha^G, \gamma, a)$ can be obtained by

numerical methods. The subroutines used to calculate the value of $g(\alpha^G, \gamma, a)$ are given in Appendix 1.

In the absence of the pump ($a = 0$, i.e. $E_1 = 0$), the effective molecular concentration in the ground state is given by

$$N_{eff}^G(\alpha^G, \gamma, 0) = \frac{N}{3} \quad (2-3-13)$$

From an experimental point of view it is advantageous [75,82] to measure the intensity of the transmitted probe beam with the pump light on [I(on)] and the pump light off [I(off)] as a function of the angle γ . By combining of equations 2-3-1, 2-3-11 and 2-3-13, it is formed that

$$\ln \frac{I(on)}{I(off)} = \ln \frac{T(\alpha^G, \gamma, a)}{T(\alpha^G, \gamma, 0)} = -A \cdot [g(\alpha^G, \gamma, a) - 1] \quad (2-3-14)$$

where

$$A = \sigma_2^G \cdot L \cdot \frac{N}{3} \quad (2-3-15)$$

A convenient quantity for discussion of results given by equation 2-3-14 is the logarithm of the transmitted intensity ratio divided by the optical density of the sample, $\ln [I(on)/I(off)] / A$. Its value can be determined experimentally and is referred to as the *signal intensity*.

Figure 2-3-3(a) shows the dependence of the signal intensity on the angle γ for different angles α^G between two ground state absorption dipoles. The following conclusions can be drawn from Figure 2-3-3(a):

(i) The signal intensity is always positive when excited state absorption can be neglected;

(ii) In the case of the parallel transition moments μ_1^G and μ_2^G ($\alpha^G = 0^\circ$), the ratio of the signal intensities for $\gamma = 0^\circ$ to that of $\gamma = 90^\circ$ is > 1 ; for perpendicular transition moments ($\alpha^G = 90^\circ$), the ratio is < 1 ;

(iii) Isotropic absorption takes place if the angle between the two transition dipole moments is $\alpha^G = 54.7^\circ$ and different values of the angle α^G do not change the strength of the signal when $\gamma = 54.7^\circ$.

The value 54.7° , "magic angle" (for both α^G and γ), arises because the ratio of the number of the undisturbed (by the pump) directions in space (X and Y) to the disturbed direction (Z) is equal to 2 ($\tan 54.7^\circ = 2$).

The dependence of signal intensities on γ is similar for different pumping levels, and is shown in Figure 2-3-3(b). Stronger pumping induces a larger observed signal.

Figure 2-3-3 (a): The ratio of the intensities of the transmitted probe light (equation 2-3-14) as a function of the angle γ for different angle α^G for $a = 1$ pumping level.

Figure 2-3-3 (b): The ratio of the intensities of the transmitted probe light (equation 2-3-14) as a function of the angle γ for different angle α^d for different pumping level.

Excited state absorption

The effective concentration, $N_{\text{eff}}^E(\alpha^E, \gamma, a)$, of the molecules in the excited state to absorb the probe beam can be derived from the following considerations: if the ground state transition moment μ_2^G is parallel to the excited state transition moment μ_2^E (i.e. $\alpha^G = \alpha^E = \alpha$), the effective concentration of the molecules can be written as

$$N_{\text{eff}}^G(\alpha, \gamma, a) + N_{\text{eff}}^E(\alpha, \gamma, a) = \frac{N}{3} \quad (2-3-16)$$

Then the effective concentration $N_{\text{eff}}^E(\alpha^E, \gamma, a)$ can be represented in the form

$$N_{\text{eff}}^E(\alpha^E, \gamma, a) = \frac{N}{3} \cdot e(\alpha^E, \gamma, a) \quad (2-3-17)$$

where

$$e(\alpha^E, \gamma, a) = 1 - g(\alpha^E, \gamma, a)$$

and $g(\alpha, \gamma, a)$ is the function previously defined.

Combining equations 2-3-1, 2-3-11 and 2-3-17, the signal intensity is represented by equation 2-3-18. Equation 2-3-18 considers both the ground state absorption and the excited state absorption.

$$\begin{aligned} \ln \frac{I(on)}{I(off)} &= \ln \frac{T(\alpha^G, \alpha^E, \gamma, a)}{T(\alpha^G, \alpha^E, \gamma, 0)} \\ &= -A \left\{ [g(\alpha^G, \gamma, a) - 1] + \frac{\sigma_2^E}{\sigma_2^G} \cdot e(\alpha^E, \gamma, a) \right\} \end{aligned} \quad (2-3-18)$$

The dependence of the signal intensity on γ at different values of σ_2^E/σ_2^G for parallel transition moments ($\alpha^E = 0^\circ$) is shown in Figure 2-3-4(a). Weak excited state absorption reduces the signal intensity, but the shape of the curves is the same as that of the ground state absorption (comparing with Figure 2-3-3). Strong excited state absorption induces a negative signal and the isotropic absorption for $\alpha^G = 54.7^\circ$ is destroyed. In the case where $\sigma_2^E > \sigma_2^G$, the molecules with the transition moments oriented parallel to the polarization of the excitation are highly excited; the absorption of these molecules in the excited state is stronger than that in the ground state. This gives rise to the phenomenon that the dependence of the signal intensity on γ reverses.

Numerical results for the dependence of the signal intensity on γ for different cross sections are also shown in Figure 2-3-4(b) ($\alpha^E = 54.7^\circ$) and Figure 2-3-4(c) ($\alpha^E = 90^\circ$). For $\alpha^E = 54.7^\circ$ the contribution from excited molecules is isotropic and the signal intensity dependence on angle γ is similar to that of the ground state absorption but weaker (comparing with Figure 2-3-3(a)). The signal induced by the excited state absorption for $\alpha^E = 90^\circ$ is opposite to that for $\alpha^E = 0^\circ$ (comparing with Figure 2-3-4(a)).

The dependence of the signal intensity on the different parameters can be

determined from experimental results. The optical density A and σ_2^E/σ_2^G can be measured from separate experiments [75]. It is usually hard to determine the pumping level, a , and its value should be fitted to the experimental results.

Figure 2-3-5 shows the set of simulated curves for the pump intensity $a = 0.3$, the other parameters are equal to the curves shown at Figure 2-3-4(a). By comparing these two figures, one can conclude that the shape of a simulated curve does not change with the different pump levels. A change in the pump level can only affect the amplitude of the signal intensity. Therefore, any large uncertainty in the normalized pump intensity does not affect conclusions about the internal structure of the molecule (α^G and α^E) drawn from the observed signal.

Figure 2-3-4 (a): The ratio of the intensities of the transmitted probe light with the contribution from excited state absorption (equation 2-3-18) as a function of the angle γ for different angle α^G : $\alpha^E = 0^\circ$, $a = 1$.

Figure 2-3-4 (b): The ratio of the intensities of the transmitted probe light with the contribution from excited state absorption (equation 2-3-18) as a function of the angle γ for different angle α^G : $\alpha^E = 54.7^\circ$, $a = 1$.

Figure 2-3-5: The ratio of the intensities of the transmitted probe light with the contribution from excited state absorption (equation 2-3-18) as a function of the angle γ for different angle α^G : $\alpha^E = 0^\circ$, $a = 0.3$.

2.3.2 Time resolved anisotropic absorption

Not considered in the discussions of Section 2.3.1 are (i) the rotation of the molecules and (ii) the life-time of the excited state. Section 2.3.2 presents the theoretical aspects of time-resolved anisotropic absorption which deals with the rotation of the molecules and the life-time of the excited state. Here the life-time of the excited state is considered as an average life-time of the molecules for the relaxation process. This work does not consider the photophysical relaxation processes which can be studied by resonance Raman saturation spectroscopy [84-86].

The general expression for the signal intensity of anisotropic absorption can be represented by equation 2-3-18 which considers both the ground state and the excited state absorption. Equation 2-3-18 also can be written as

$$\ln \frac{I(on)}{I(off)} = -\sigma_2^G L \cdot (N_{eff}^G(\alpha^G, \gamma, a) - \frac{N}{3}) - \sigma_2^E L \cdot N_{eff}^E(\alpha^G, \gamma, a) \quad (2-3-19)$$

From equation 2-3-19, it can be seen that the time dependent factors in the signal intensity expression are the effective molecular populations, N_{eff}^G and N_{eff}^E . From a theoretical point of view, the effective molecular populations in the ground state and the excited state need to be considered in the time-resolved anisotropic absorption. Equation

2-3-16 gives the relationship between N_{eff}^G and N_{eff}^E , so the only parameter discussed in this section is the time dependent effective population of the ground state, $N_{\text{eff}}^G(\alpha^G, \gamma, a, t)$. For simplicity, the concentration of the molecules, N , is set to 1.

The rotation of molecules will first be included in the calculations, then life-time of the excited state.

It is assumed that the three rotational relaxation times corresponding to the rotational diffusion tensor of the molecules are equal.

The time dependent angular density is represented by $n_1^G(\phi, \theta, a, t)$. Equation 2-3-2 represents the time dependent angular density at $t = 0$. Considering rotation of the molecules, at time t , the time dependent angular density is given by [1]

$$n_1^G(\phi, \theta, a, t) = \int_0^{2\pi} d\phi_0 \int_0^\pi d\theta_0 \sin\theta_0 n_1^G(\phi_0, \theta_0, a, 0) G(\phi_0, \theta_0 | \phi, \theta, t) \quad (2-3-20)$$

Here $G(\phi_0, \theta_0 | \phi, \theta, t)$ is Green's function of the differential equation which the angular density satisfies.

For a weak pump condition ($a \ll 1$), at $t=0$, the angular density is

$$n_1^G = \frac{1}{4\pi} (1 - a \cos^2 \theta) \quad (2-3-21)$$

Expanding the Green's function in terms of the surface harmonics gives [1]:

$$G(\varphi_0, \theta_0 | \varphi, \theta, t) = \sum_{l=0}^{\infty} \sum_{m=-l}^{m=l} c_{l,m}(t) Y_{l,m}^*(\theta_0, \varphi_0) Y_{l,m}(\theta, \varphi) \quad (2-3-22)$$

From the boundary condition

$$G(\varphi_0, \theta_0 | \varphi, \theta, 0) = \delta(\cos \theta - \cos \theta_0) \delta(\varphi - \varphi_0) = \sum_{l=0}^{\infty} \sum_{m=-l}^{m=l} Y_{l,m}^*(\theta_0, \varphi_0) Y_{l,m}(\theta, \varphi)$$

and the normalization condition

$$\int_0^{2\pi} \int_0^\pi d\varphi \sin \theta d\theta G(\varphi_0, \theta_0 | \varphi, \theta, t) = 1 \quad (2-3-23)$$

it can be seen that

$$c_{0,0}(t) = 1$$

and

$$c_{1,m}(0) = 1 \quad (2-3-24)$$

respectively.

Substituting equations 2-3-21 and 2-3-22 into equation 2-3-20 and using the relations of equation 2-3-24, one obtains

$$n_1^G(\varphi, \theta, a, t) = \frac{1}{4\pi} \left(1 - \frac{a}{3} - \frac{2a}{3} c_{2,0}(t) Y_{2,0}(\theta, \varphi) \right) \quad (2-3-25)$$

The angular density satisfies the diffusion function [87]

$$\frac{\partial n_1^G(\varphi, \theta, a, t)}{\partial t} = D \nabla^2 n_1^G(\varphi, \theta, a, t) \quad (2-3-26)$$

D in equation 2-3-26 is given by Einstein's law

$$6D = kT / V\eta \quad (2-3-27)$$

where k is Boltzmann constant; T is the temperature; V is the volume of rotational diffusion of an Einstein sphere; and η is the viscosity of the medium.

The solution of equation 2-3-26 in terms of its Green's function is [88]

$$G(\varphi_0, \theta_0 | \varphi, \theta, t) = \sum_{l,m} e^{-l(l+1)Dt} Y_{l,m}^*(\theta_0, \varphi_0) Y_{l,m}(\theta, \varphi) \quad (2-3-28)$$

Therefore

$$c_{2,0}(t) = e^{-6Dt} \quad (2-3-29)$$

and the time dependent angular density can be written as

$$n_1^G(\varphi, \theta, a, D, t) = \frac{1}{4\pi} \left(1 - \frac{a}{3} + \frac{a}{3} e^{-6Dt} - a \cos^2 \theta e^{-6Dt} \right) \quad (2-3-30)$$

where D represents the diffusion parameter (equation 2-3-27). Now the angular density, n_1^G , is also dependent on D and t .

The time dependent effective population is determined by equation 2-3-9, but n_2^G

in equation 2-3-9 is replaced by

$$n_2^G(\varphi, \theta, \alpha^G, a, D, t) = \frac{1}{4\pi} \times \frac{1}{2\pi} \int_0^{2\pi} d\varphi' \left(1 - \frac{a}{3} + \frac{a}{3} e^{-6Dt} - a \cos^2 \Omega e^{-6Dt} \right) \quad (2-3-31)$$

where $\cos^2 \Omega$ is defined by equation 2-3-7. From this relationship, the time dependent effective population is given by:

$$N_{eff}^G(\alpha^G, \gamma, a, D, t) = \frac{1}{3} \left(1 - \frac{a}{3} + \frac{a}{3} e^{-6Dt} \right) - \frac{a}{5} e^{-6Dt} N_g(\alpha^G, \gamma) \quad (2-3-32)$$

where $N_g(\alpha^G, \gamma)$ is

$$N_g(\alpha^G, \gamma) = \frac{2}{3} \sin^2 \alpha^G \sin^2 \gamma + \frac{1}{3} \sin^2 \alpha^G \cos^2 \gamma + \frac{1}{3} \cos^2 \alpha^G \sin^2 \gamma + \cos^2 \alpha^G \cos^2 \gamma \quad (2-3-33)$$

The parameter which can be obtained in time-resolved anisotropic absorption is the rotational relaxation time, τ_D . The rotational relaxation times of a globular molecule (assuming the three rotational relaxation times corresponding to the rotational diffusion tensor of the molecules are equal) can be estimated from equation 2-3-34.

$$\tau_D = \frac{1}{6D} \quad (2-3-34)$$

Now the life-time of the excited state is taken into account. If the life-time of the excited state is τ and the pump intensity is weak ($a \ll 1$), the angular density, n_1^G , is

$$n_1^G(\varphi, \theta, a, \tau, t) = \frac{1}{4\pi} (1 - a \cos^2 \theta e^{-\frac{t}{\tau}}) \quad (2-3-35)$$

The time dependent effective molecular population also can be obtained by equation 2-3-9, if n_2^G is replaced by

$$n_2^G(\varphi, \theta, a, D, \tau, t) = \frac{1}{4\pi} \times \frac{1}{2\pi} \int_0^{2\pi} d\varphi' (1 - \frac{a}{3} e^{-\frac{t}{\tau}} + \frac{a}{3} e^{-\frac{t}{\tau}} e^{-6Dt} - a \cos^2 \Omega e^{-\frac{t}{\tau}} e^{-6Dt}) \quad (2-3-36)$$

where $\cos^2 \Omega$ is defined by equation 2-3-7. The time dependent effective population of the ground state is

$$N_{eff}^G(\alpha_G, \gamma, a, D, \tau, t) = \frac{1}{3} (1 - \frac{a}{3} e^{-\frac{t}{\tau}} + \frac{a}{3} e^{\frac{t}{\tau}} e^{-6Dt}) - \frac{a}{5} e^{-\frac{t}{\tau}} e^{-6Dt} N_g(\alpha^G, \gamma) \quad (2-3-37)$$

where $N_g(\alpha^0, \gamma)$ is from equation 2-3-33.

Equations 2-3-32 and 2-3-37 represent the time dependent effective molecular population of the ground state in the anisotropic absorption measurements. Equation 2-3-32 is only concerned with the rotation of the molecules; and equation 2-3-37 is concerned with both the rotation of the molecules and the life-time of the molecules.

The dependence of the time dependent effective population on γ is shown in Figure 2-3-6. These curves are based on equation 2-3-32 which only deals with the rotation of the molecules. The conclusions which can be drawn from Figure 2-3-6 are:

(i) The effective population of the ground state for the probe beam with the pump light on is always less than for pump light off, $1/3$, which is obtained from equation 2-3-13.

(ii) The effects of the molecular rotation push the effective population to isotropic distribution on γ , but the shape of the distribution curve at $6Dt > 0$ is similar to the curve at $6Dt = 0$. If the rotation of the molecules is very fast (meaning D is large) or it is slow but takes a longer time (meaning t is large), $6Dt = 10$ for example, an isotropic effective population distribution on γ takes place for any value of α^0 .

(iii) An isotropic effective population distribution on γ takes place if $\alpha^0 = 54.7^\circ$

or $\gamma = 54.7^\circ$.

Figure 2-3-7 is based on equation 2-3-37. Here $6Dt$ is fixed at 0.5, the normalized pump intensity, $a = 0.3$ and the angle $\alpha^0 = 0^\circ$. The figure shows the life-time of the excited state in the four regions: t/τ is 0 ($\tau = \infty$ or $t = 0$), 0.05, 0.5, 5.0.

The conclusions which can be drawn from the figure are:

(i) The effective population for the probe beam with the pump light on is always less than the effective population with the pump light off ($1/3$).

(ii) If the life-time of the excited state is short (it means that τ is small) or it is long but takes longer time (it means t is large), $t/\tau = 5$ for example, the effective population will be close to $1/3$ (= effective population with pump light off) and become an isotropic distribution.

(iii) If t/τ is smaller or equal to $6Dt$, the shape of effective population curve is dependent on $6Dt$, i.e. is dependent on the rotation relaxation process of the molecules.

Figure 2-3-6: The dependence of the time dependent effective population on the angle γ
 (equation 2-3-32): $a=0.01$; $\alpha^G = 0^\circ, 54.7^\circ, 90^\circ$; $6Dt = 0, 0.5, 1.0, 10.0$.

Figure 2-3-7: The dependence of the time dependent effective population on the angle γ

(equation 2-3-37): $6Dt = 0.5$; $\alpha^0 = 0^\circ$; $a = 0.3$; $t/\tau = 0, 0.05, 0.5, 5.0$.

2.3.3 Two transition moment absorption

The probe light is not always absorbed only by one transition dipole moment in a chemical system. Here one considers the situation where the probe laser beam is tuned between the two excited states S_a and S_b (Figure 2-3-8), and the probe light is absorbed by the two dipole moments, μ_a^0 and μ_b^0 .

Figure 2-3-8: Schematic of two transition moment absorption.

The discussions below assume that

(i) the rotational relaxation time of the molecules is longer than the duration of the laser pulses of the pump beam and the probe beam;

(ii) the life-time of the excited state (S_{pump}) is longer than the duration of the laser pulses of the pump beam and the probe beam;

(iii) both pump and probe beams arrive at the same time at the origin of the coordinate system;

(iv) excited state absorption can be neglected.

With a normalized pump intensity, the angular density of the molecular system can be represented by equation 2-3-2. The effective population for absorbing the probe light is

$$N_{eff}^G(\alpha_a^G, \alpha_b^G, \gamma, a) = N_{eff}^{Ga}(\alpha_a^G, \gamma, a) + N_{eff}^{Gb}(\alpha_b^G, \gamma, a) \quad (2-3-38)$$

where α_a^G is the angle between the dipole moment of the excited state S_{pump} and the dipole moment of the excited state S_a ; α_b^G is the angle between the dipole moment of S_{pump} and S_b (Figure 2-3-8). The effective populations N_{eff}^{Ga} and N_{eff}^{Gb} are determined by equations 2-3-9 and 2-3-11

$$N_{eff}^{Ga}(\alpha_a^G, \gamma, a) = \frac{N}{3} g(\alpha_a^G, \gamma, a)$$

and

$$N_{eff}^{Gb}(\alpha_b^G, \gamma, a) = \frac{N}{3} g(\alpha_b^G, \gamma, a) \quad (2-3-39)$$

where $g(\alpha_a^G, \gamma, a)$ and $g(\alpha_b^G, \gamma, a)$ are same as $g(\alpha^G, \gamma, a)$ in equation 2-3-12.

From equation 2-3-39, the expression for the signal intensity can be derived as

$$\begin{aligned} \ln \frac{I(on)}{I(off)} &= \ln \frac{T(\alpha_a^G, \alpha_b^G, \gamma, a)}{T(\alpha_a^G, \alpha_b^G, \gamma, 0)} \\ &= -\sigma_a^{GL} \cdot N_{eff}^{Ga}(\alpha_a^G, \gamma, a) L \cdot \frac{N}{3} \\ &\quad -\sigma_b^{GL} \cdot N_{eff}^{Gb}(\alpha_b^G, \gamma, a) L \cdot \frac{N}{3} + (\sigma_a^G + \sigma_b^G) L \cdot \frac{N}{3} \end{aligned} \quad (2-3-40)$$

where σ_a^G and σ_b^G are the cross sections for the probe beam corresponding to the excited states S_a and S_b (Figure 2-3-8); L is the length of the sample and N is the concentration of the sample.

If $\alpha_a^G = \alpha_b^G = \alpha^G$, i.e. the angle between the dipole moment of the excited state S_{pump} and of S_a is equal to the angle of the dipole moment of the excited state S_{pump} with S_b , equation 2-3-40 can be represented by equation 2-3-14, if the parameter A is replaced by

$$A = (\sigma_a^G + \sigma_b^G) \cdot L \cdot \frac{N}{3} \quad (2-3-41)$$

When $\alpha_a^G = \alpha_b^G$, the signal intensity dependence on the angle γ is the same as that where the probe beam is absorbed only by one dipole moment. In this situation, it is impossible to determine if the probe beam is absorbed by one or two dipole moments.

If α_s^G is not equal to α_b^G , then α_s^G and α_b^G can be determined by fitting experimental data with equation 2-3-40.

2.4 Anisotropic Fluorescence

Anisotropic fluorescence or the rotation correlation function is defined as [1]

$$r(t) = \frac{I_{\parallel}(t) - I_{\perp}(t)}{I_{\parallel}(t) + 2I_{\perp}(t)} \quad (2-4-1)$$

Figure 2-3-1 also can be used to represent the geometry of anisotropic fluorescence. Here the excitation laser beam with electric vector E_1 is polarized along the Z axis of the fixed laboratory coordinate system and this beam is propagating along the X axis. μ_1^G is the direction of the transition dipole moments absorbing the excitation laser beam at the ground state. μ_2^E (replacing μ_2^G in Figure 2-3-1) is related to the fluorescence emission dipole moment. α^E (replacing α^G in Figure 2-3-1) is the angle between μ_1^G and μ_2^E . The molecule is situated at the origin of the coordinate system. In equation 2-4-1, $I_{\parallel}(t)$ represents the time dependent fluorescence intensity in a direction parallel to the electric field E_1 , i.e. $\gamma = 0^\circ$; $I_{\perp}(t)$ represents the time dependent fluorescence intensity in a direction perpendicular to the electric field E_1 , i.e. $\gamma = 90^\circ$.

The time dependent fluorescence intensity emitted by μ_2^E polarization at the direction of E_2 (represented by γ) is proportional to the time dependent effective population, $N_{eff}^E(\alpha^E, \gamma, a, t)$. Therefore, the time-resolved anisotropic fluorescence is

given as

$$r(t) = \frac{N_{eff}^E(\alpha^E, 90, a, t) - N_{eff}^E(\alpha^E, 0, a, t)}{N_{eff}^E(\alpha^E, 90, a, t) + 2N_{eff}^E(\alpha^E, 0, a, t)} \quad (2-4-2)$$

Equation 2-4-2 can be used at both the strong pump condition and the weak pump condition. $N_{eff}^E(\alpha^E, \gamma, a, t)$ can be obtained by a similar procedure to that used in Section 2.3.2.

The theory for anisotropic fluorescence spectroscopy in the weak pump condition has been derived by Tao [1]. In Tao's theory, the rotational correlation function $r(t)$ is represented as

$$r(t) = \frac{2}{5} Y_{2,0}(\theta, \varphi) e^{-6Dt} \quad (2-4-3)$$

where the detected rotation relaxation time $\tau_r = 1/6D$. When $t = 0$, $r(0)$ is called the *no-motion anisotropy value* for the particular chromophore [77]

$$r(0) = \frac{2}{5} Y_{2,0}(\theta, \varphi) = \frac{1}{5} (3\cos^2\alpha^E - 1) \quad (2-4-4)$$

Here α^E is the angle between the absorption dipole moment and the emission dipole

moment.

The time dependent effective population of excited state, $N_{\text{eff}}^E(\alpha^E, \gamma, a, D, \tau, t)$ can be represented by equation 2-4-5 (set $N = 1$)

$$N_{\text{eff}}^E(\alpha^E, \gamma, a, D, \tau, t) = \frac{1}{3} \left(\frac{a}{3} e^{-\frac{t}{\tau}} - \frac{a}{3} e^{-6Dt} e^{-\frac{t}{\tau}} \right) + \frac{a}{5} e^{-6Dt} e^{-\frac{t}{\tau}} N_g(\alpha^E, \gamma) \quad (2-4-5)$$

where $N_g(\alpha^E, \gamma)$ is defined by equation 2-3-33.

N_{eff}^E is replaced in equation 2-4-2 by equation 2-4-5, the rotational correlation function $r(t)$ transforms into equation 2-4-6 which agrees with equation 2-4-3.

$$\begin{aligned} r(t) &= \frac{N_{\text{eff}}^E(\alpha^E, 0, a, D, \tau, t) - N_{\text{eff}}^E(\alpha^E, 90, a, D, \tau, t)}{N_{\text{eff}}^E(\alpha^E, 0, a, D, \tau, t) + 2N_{\text{eff}}^E(\alpha^E, 90, a, D, \tau, t)} \\ &= \frac{1}{5} (3 \cos^2 \alpha^E - 1) e^{-6Dt} \end{aligned} \quad (2-4-6)$$

It is known that the rotational correlation function is dependent on the effective population N_{eff}^E (equation 2-4-2). An isotropic effective population of ground state occurs if $\gamma = 54.7^\circ$ or $\alpha^G = 54.7^\circ$. Because $N_{\text{eff}}^G + N_{\text{eff}}^E = N/3$ (equation 2-3-16), then an isotropic population for excited state also takes place for $\gamma = 54.7^\circ$ or $\alpha^E = 54.7^\circ$.

This means the rotation of the molecules does not affect the intensity of the fluorescence which polarizes at the direction of the "magic" angle (54.7°). Thus experimentally, the decay time of fluorescence is measured at the direction of magic angle to avoid effects of the rotation of the molecules.

3. THE STRUCTURE AND PROPERTIES OF H₂TMPyP/DNA COMPLEXES AND MX/DNA COMPLEXES

3.1 THE STRUCTURE AND PROPERTIES OF H₂TMPyP/DNA COMPLEXES

3.1.1 Tetra (4-N-methyl-pyridyl) porphyrin: H₂TMPyP

The structure of tetrakis (4-N-methyl-pyridyl) porphyrin abbreviated below as H₂TMPyP is shown in Figure 3-1-1(a). At its centre, H₂TMPyP has a 18 member ring with approximate 4-fold symmetry containing 18 π electrons. H₂TMPyP has the typical porphyrin type absorption spectrum due to $\pi \rightarrow \pi^*$ type transitions. The weaker bands in the 500nm - 600nm region are called the *Q* bands and strong absorption bands in the 370nm - 450nm region are called *B* bands or *Soret* bands.

The structure and the absorption spectra of H₂TMPyP are similar to those of porphyrin (Figure 3-1-1(b)), thus the assignments given for the electronic structure of porphyrin are accepted as valid for H₂TMPyP.

(a) .

(b)

Figure 3-1-1: Structure of H_2TMPyP (a) and structure of porphyrin (b).

The following is a simple model of the electronic structure of porphyrin molecules [94,95]. Qualitatively, this model can be used to assign the absorption bands of H₂TMPyP.

The model treats porphyrin as a free electron wire ring with 18 electrons. The molecular orbital is constructed by form $e^{i\mu\phi}$ with energy of $\hbar^2\mu^2/2mR^2$, where $\hbar$ is Planck's constant divided by 2π , m is the electron mass, and R is the radius of the ring, μ is the quantum number of the angular momentum [95]. Orbitals with $\mu = 0, \pm 1, \pm 2, \pm 3, \pm 4$ are occupied in the ground state. The lowest-energy excited states were constructed as transitions from $\mu = \pm 4 \rightarrow \mu = \pm 5$. There are four transitions with total angular moments $\Delta L_z = \pm 1$ and ± 9 ; each transition give rise to one state with $S = 0$ (singlet) and three states with $S = 1$ (triplet), where S is the spin angular momentum of the electrons. Altogether, there are 16 lowest-energy excited states. From the effect of electron interaction in the 16 low-energy excited states, it can be deduced that the lowest-energy excited state should be a triplet state, and the singlet state with $\Delta L_z = \pm 1$ should have higher energy than those with $\Delta L_z = \pm 9$. Since the ground state is a closed shell with $L_z = 0$, the selection rule $\Delta L_z = \pm 1$ predicts that the higher energy singlet is allowed and the lower-energy singlet is forbidden. Thus, the Soret absorption band is identified with the $\Delta L_z = \pm 1$ excited singlet state and absorption band Q with the $\Delta L_z = \pm 9$ excited singlet state. The large difference in angular momentum for Q and Soret bands was verified by Zeeman and MCD studies [96].

H_2TMPyP and its metal derivatives are able to bind to DNA molecules [91]. This type of compound has the potential for therapeutic use. It is observed that certain porphyrins can serve as photo sensitizers for destruction of tumour cells and a number of porphyrins have been shown to be effective in the treatment of malignant tumours [92,93,132-134].

3.1.2 H_2TMPyP /DNA Complexes

There are three degrees of intimacy in the DNA-drug interactions: (i) covalent binding, (ii) intercalation and (iii) nonintercalative groove binding. The first one represents a strong chemical interaction, the remaining two significantly weaker physical associations [166].

Intercalation of H_2TMPyP into DNA was first discovered by Fiel et al. in 1979 [91]. Since that time the binding of H_2TMPyP and its metal derivatives to DNA had been studied by a variety of techniques, examples are shown below:

Viscometry [91,97,108,109,113,114,125],

Electrophoresis [97,102,114,119,131],

Absorption [91,97,98,99,113,116,121,130],

Anisotropic Absorption [105],

Circular Dichroism [91,97,99,118,122],

Flow Dichroism (Linear Dichroism) [100,112,117,128],
Thermodynamic and Kinetic Studies [99,101,115-117],
Fluorescence [73,102,104,125],
Nuclear Magnetic Resonance (NMR) [97,98,108,109,112,124,129],
Resonance Raman Scattering (RR) [98,103,106,110,111,123,126,127],
Electron Spin Resonance (ESR) [107],
Melting Studies [91],
X-ray Crystallography [120].

The major conclusions obtained from a variety of these measurements indicate that the binding between H₂TMPyP molecules and DNA molecules is a non-covalent binding and there are two binding modes for H₂TMPyP/DNA complexes. The intercalated complex is formed between H₂TMPyP and G-C base pairs of DNA; the externally groove-bound complex is formed between H₂TMPyP and A-T base pairs of DNA. Intercalation results from a π - π overlap between the porphyrin core and the purine/pyrimidine ring of the DNA. External groove binding occurs when H₂TMPyP fits into the major/minor groove and interacts with the nucleic acids electrostatically. A columbic interaction between the PO₂ group of DNA and N⁺-CO₃ group of the H₂TMPyP reinforces both types of binding [97,99].

The principal criterion used to identify intercalative binding of drug to DNA is the ability to unwind super-coiled DNA. Unwinding DNA means the angle between the

adjacent base pairs of DNA is changed. In a normal situation, all base pairs of DNA are perpendicular to the axis of DNA and the angle between the adjacent base pairs is 36° .

Intercalation of the drug molecules into DNA requires adjacent base pairs to move apart $\sim 7\text{\AA}$. This gives rise to a displacement of the axis of DNA. With a helical axis displacement of $1.0\text{\AA}$, and an effective angular unwinding of 10° [135]. Unwinding can be detected by viscometry and electrophoresis techniques. The unwinding angle for H_2TMPyP is 19° determined from viscometry measurements [113].

Intercalating drugs also can increase the length of DNA. The relative increase in length, L/L_0 , is related to a corresponding increase in the relative viscosity given by:

$$\frac{L}{L_0} = \left(\frac{\eta}{\eta_0} \right)^{1/3} \quad (3-1-1)$$

where L is the length of the DNA molecule in the presence of bound ligands, L_0 is the length of free DNA, and η and η_0 are the corresponding viscosities.

H_2TMPyP /DNA complexes have been shown to give rise to a rapid increase of relative length (L/L_0) with increasing concentration of H_2TMPyP . The maximum relative length is ~ 1.3 at a binding ratio of approximately one H_2TMPyP molecule to four base pairs of DNA [131].

The results of electrophoresis titration for H_2TMPyP /DNA and ethidium bromide/DNA indicate that both H_2TMPyP and ethidium bromide can unwind DNA. Ethidium bromide is a well-known intercalator, this result suggests that H_2TMPyP may also be an intercalator [119]. On the other hand, H_2TMPyP is positively charged, and DNA is negatively charged, then if H_2TMPyP binds to DNA, this binding should reduce the electrophoretic mobility of the DNA. The experimental results indeed show that H_2TMPyP affects the migration rate of DNA [102,119].

The binding sequence specificity can be determined by DNase I footprinting methodology [131]. Such an analysis has revealed that the outside-binding 5- and 6-coordinate metalloporphyrins bind in the minor groove of the A-T rich regions of DNA; while the 4-coordinate structure appears to bind to both A-T and G-C sequences of the DNA [99].

Spectroscopic measurements are used in the study (i) to identify binding modes of H_2TMPyP with DNA and (ii) to detect the structure of H_2TMPyP /DNA complexes.

Absorption

Absorption spectra have been used to monitor the interaction of H_2TMPyP with DNA, the first results being reported in 1979 [91]. It was found that the intensity of the Soret band of H_2TMPyP decreased with increasing concentrations of DNA in the

H₂TMPyP/DNA solution and the Soret band was red-shifted [91].

H₂TMPyP intercalation with DNA is due to π - π overlap of the two molecules; external binding occurs when H₂TMPyP fits into the major/minor groove and interacts with the nucleic acids electrostatically. The Soret band of H₂TMPyP originates from a $\pi \rightarrow \pi^*$ type transition, which is consistent with the fact that the influences of intercalated binding on the Soret band are much more important than those of external binding. By comparing the absorption spectrum of H₂TMPyP with calf thymus DNA, poly(dG-dC), and poly(dA-dT) [99], it was concluded that the substantial red-shift and hypochromicity of the Soret band of H₂TMPyP is characteristic of the intercalation binding mode. Smaller changes in the absorption spectra are indicative of other binding modes.

The influences of ionic strength on the binding of H₂TMPyP to calf thymus DNA and poly(dG-dC) were studied by optical titration. The results of these experiments indicated that the binding ability of H₂TMPyP does decrease with increasing [Na⁺], and the H₂TMPyP molecules relocate from the G-C rich regions to the A-T rich regions [121]. The experimental results obtained from circular dichroism spectra [91,97,113,135] stated that at low ionic strength, H₂TMPyP binds to DNA by intercalation with G-C specificity and that the fraction of H₂TMPyP bound by intercalation decreases as the ionic strength increases and shifts to outside binding at A-T sites.

Anisotropic absorption spectroscopy is used to determine the geometric binding structure of H_2TMPyP /DNA complexes [105]. The theoretical aspects of anisotropic absorption are treated in chapter 2 (Section 2.3) of this thesis. By fitting the experimental data with the developed theories, the relative orientation of the intercalated H_2TMPyP molecules and the externally bounded H_2TMPyP molecules can be obtained. This work will be described in chapter 4 - chapter 6.

Circular Dichroism (CD)

A pure H_2TMPyP buffer solution does not display circular dichroism. When nucleic acids are added to the H_2TMPyP solution, circular dichroism can be detected in the region of the Soret band. Because DNA has no absorption in the visible region, this signal is due to the induced circular dichroism of H_2TMPyP .

Circular dichroism spectra of H_2TMPyP with DNA, H_2TMPyP with poly(dA-dT), H_2TMPyP with poly(dG-dC) have been reported in many studies [91,99,122]. The circular dichroism spectrum of H_2TMPyP with DNA consists of a positive component and a negative component. Only a positive component was found for H_2TMPyP with poly(dA-dT). With poly(dG-dC), circular dichroism spectra displayed a negative component with relatively large ellipticities. The negative component in the Soret band was assigned as characteristic of H_2TMPyP intercalated binding to DNA with G-C base pairs. The positive component was found to be characteristic of external binding to DNA

with A-T base pairs. Circular dichroism spectra are widely used as a spectroscopic characteristic to identify binding modes of porphyrins to DNA.

Fluorescence

Fluorescence spectroscopy is a suitable technique to investigate the porphyrins/DNA systems because of its high sensitivity. Kelly et al. first measured fluorescence spectra of H_2TMPyP with DNA, H_2TMPyP with poly(dA-dT), H_2TMPyP with poly(dG-dC) in 1984 [102].

A pure H_2TMPyP buffer solution has a broad fluorescence band between 600nm and 800nm (centred at about 675nm). When DNA was added to the H_2TMPyP solutions, the fluorescence band split into two peaks (centred at about 650nm and 715nm respectively) and the intensity of the fluorescence was enhanced. For solutions of H_2TMPyP /poly(dA-dT) the intensity of the fluorescence was markedly enhanced; but for H_2TMPyP /poly(dG-dC) solutions the intensity was reduced.

From time-resolved fluorescence measurements [73], it was found that fluorescence decay time of intercalated bound H_2TMPyP was different from that of externally bound H_2TMPyP . The shape of the fluorescence bands was also different. This result gives another spectroscopic characteristic through which the binding modes can be identified. This work and the related experimental results will be described later.

(chapter 4 - chapter 6) in more detail.

Nuclear Magnetic Resonance (NMR)

Nuclear magnetic resonance spectroscopy has been used to evaluate binding between porphyrins and DNA by many workers [98,108,109,112,124,129].

The intercalator H₂TMPyP was found to induce a broad downfield peak in the ³¹P NMR spectrum of DNA and a slight upfield shift to the main peak. The magnitude of this effect increased with DNA which had a higher G-C content. The effects of H₂TMPyP on the ³¹P NMR spectra of poly(dG-dC) were more striking. For the ¹H NMR spectra of DNA, both A-T and G-C imino hydrogen resonances decreased and shifted upfield when H₂TMPyP was added.

The NMR spectral investigations of the interaction of porphyrins with synthetic oligomers led to the identification of 5'CG3' as a predominant sequence for intercalation [124,136]. This conclusion is consistent with the results of footprinting tests [131], resonance Raman scattering measurements [98] and theoretical studies [120,137]. The NMR measurements indicate that additional binding sites are available, one of them being 5'GC3'. However most of the investigations indicate that 5'CG3' is a primary sequence for intercalation.

Resonance Raman Scattering (RR)

Resonance Raman scattering occurs when the energy of the exciting radiation falls within an electric absorpior band. It is treated as a special case of Raman scattering. Resonance Raman scattering is dependent on the specific vibrational and rotational quantum numbers of the selected excited state. Because of the resonance Raman effect, certain vibrational band intensities can be enhanced up to a factor of 10^3 .

Resonance Raman spectroscopy has been used to study the structure of H_2TMPyP /DNA complexes. Through the use of resonance Raman spectroscopy it is possible to resonance-enhance chromophore (H_2TMPyP and DNA) vibrations individually by tuning the laser wavelength to the electronic transition of each chromophore. Three resonance Raman scattering bands of H_2TMPyP appeared to be sensitive to structure. These bands which occur near 1360cm^{-1} , 1460cm^{-1} and 1570cm^{-1} were assigned to $\nu(C_\alpha - N)$, $\nu(C_\alpha - C_\beta)$, $\nu(C_\beta - C_\beta)$ transitions respectively. From the changes of these sensitive Raman bands, the information about binding structure can be obtained [111]. Another resonance Raman scattering measurements indicated that a Raman band near 1095cm^{-1} ($\nu(C_\beta - H)$) red shifted and got boarder when DNA was added to H_2TMPyP [103]. Resonance Raman excitation profiles have also been used to identify tautomeric structure of H_2TMPyP /DNA complexes [103].

3.2 THE STRUCTURE AND PROPERTIES OF MX/DNA COMPLEXES

3.2.1 Mitoxantrone: MX

Mitoxantrone, 1,4-Dihydroxy-5,8-bis[2-[(2-hydroxyethyl) amino] ethyl] amino]-9,10- anthracenedione, is an anthraquinone. Mitoxantrone was first synthesized by Zee-Cheng and Cheng [138] and independently by C.Murdock and associates [139].

Mitoxantrone is one of the anthraquinone compounds which has shown a wide spectrum of antitumour activity in experimental systems [141,142]. Mitoxantrone has been found to be effective in the treatment in vivo of numerous murine tumour models [140]. It has been used to treat breast cancer [143-145] and malignant lymphomas [146-148].

There are three peaks in the absorption spectrum of Mitoxantrone solution (ethanol) in the visible region, ~525nm, ~610nm and ~660nm. The peak positions are solvent dependent. Mitoxantrone dissolves sparingly and is only slightly soluble in methanol [35].

The structure of Mitoxantrone (abbreviated below as MX) is shown in Figure 3-2-1. MX has the essential structural characteristics of other compounds which intercalate

with DNA, namely it is a planar, electron-rich compound. Various approaches have been used to elucidate the binding mechanism between MX and DNA [73,140,149-156].

Figure 3-2-1: Structure of MX.

3.2.2 MX/DNA Complexes

Because of a planar aromatic chromophore of MX (Figure 3-2-1), it is commonly considered that MX intercalates with DNA. The binding of MX to DNA is enhanced by the presence of a side chain bearing a basic amino group which could bind electrostatically to the phosphate groups of DNA.

Experiments to determine the unwinding angle of DNA by MX were performed by **Viscosity assay**. The results of these experiments indicated an unwinding angle of 17.5°. This value agrees with the value obtained independently by **Topoisomerase assay** [149]. **Melting point** measurements have shown that the melting point of DNA is increased by ~16°C when MX was added [151]. A number of investigations have established that MX inhibits RNA and DNA synthesis [152,156]. These results support the concept that MX is intercalated with DNA.

Spectroscopic measurement have also been used to detect the binding mode of MX to DNA.

Absorption [149,150,154,155]

The absorption spectra of MX/DNA with a low ratio of [MX]:[phosphate] were shown to have a red-shift and a lower amplitude when compared with the spectra of the

pure MX buffer solution in the low concentration [154].

The binding constant for interaction of MX with calf thymus DNA and the influences of ionic strength changing were determined by absorption measurements [149].

Fluorescence [73,150,151,155]

MX has been found to fluoresce with excitation maxima at 610nm and 660nm and an emission maximum at 685nm. The intensity of the fluorescence was altered by the pH value and the fluorescence was red-shifted by adding DNA [155].

The fluorescence polarization has been used to determine the extent of inhibition of the binding of acridine orange to DNA. Calculations of the fluorescence polarization were made using the relationship:

$$P = \frac{(I_{vv}/C_F) - I_{hv}}{(I_{vv}/C_F) + I_{hv}} \quad (3-2-1)$$

where I_{vv} and I_{hv} are the fluorescence intensities in the vertical and horizontal orientations respectively and C_F is a correction factor, I_{vh}/I_{hh} . Excitation and emission polarizer position are indicated by the order of subscripts. The fluorescence polarization of MX is enhanced by DNA, which confirms the binding of MX to DNA.

Time-resolved fluorescence measurements show that the fluorescence decay time of free MX is ~ 160 picosecond and the decay time changes to ~ 240 picosecond when DNA was added to it [73]. These experiments and their results will be discussed in this thesis (chapter 4 - chapter 6).

Electron Microscopy [140,153,156]

Electron Microscopy is used to determine the interaction of MX with DNA. The information which can be obtained from the electron microscopy measurements is (i) the length of DNA before and after MX intercalates to it and (ii) the intercalation sequences specificity.

Electron microscopy studies have confirmed, by the characteristic extension in length of different DNAs, the intercalation of MX with DNA [153]. The electron microscopy measurements suggest that there may be two binding modes of MX with DNA, (i) the binding mode of intercalated binding and specificity at G-C base pair [149], and (ii) an additional external binding [153,156].

4. EXPERIMENTAL SECTION

This chapter describes the experimental procedures and equipment used in this work.

Section 4.1 outlines the picosecond laser system used to measure time-resolved anisotropic fluorescence and isotropic fluorescence. Section 4.1.1 describes the components of the system. The principles of the main procedures in the picosecond system are described in Section 4.1.2: generation of picosecond light pulses; Section 4.1.3: pulse-width measurement and Section 4.1.4: detection techniques. Section 4.1.5 outlines the method used for calibration.

Section 4.2 introduces the nanosecond laser system, with which the anisotropic absorption measurements were performed. Section 4.2.1 introduces the elements of the system and Section 4.2.2 describes the operation of the system.

Section 4.3 describes the methods used for data analysis. Section 4.4 describes the sample handling procedures. Section 4.5 describes other equipment used in this work.

4.1 PICOSECOND LASER SYSTEM

4.1.1 Components of the Picosecond System

Fig 4-1-1 gives a schematic diagram of the picosecond laser system. The major components are as follows:

(i) A Nd:YAG laser (Quantronix Model 116 head with InfraAction Corp. Model ML-50D accost-optic mode-locker) which produces ~ 100 picosecond light pulses at 1060nm and is operated at a repetition rate of ~ 100 MHz.

(ii) A second harmonic generation (SHG) crystal which is used to double the frequency of the Nd:YAG laser pulses. After travelling through the crystal, light pulses with the wavelength of 530nm are selected as a pump source for a dye laser. The average power of the laser pulses (at 530nm) is about 800mW.

(iii) A Dye laser (Coherent model 590 with extended length of the cavity) which is synchronously pumped by the frequency doubled mode-locked Nd:YAG laser and is tunable between 560nm and 680nm (Rh6G or DCM dye). The dye laser generates stable 5-6 picosecond pulses with average power of 250mW at 630nm.

(iv) A low-voltage travelling-wave electro-optic modulator, **EOM**, (Lasermetrics Model 3130F) which is used to reduce the repetition rate of the dye laser pulses.

In a time-resolved measurement, it is usually necessary to reduce the repetition rate of the laser pulses. This reduction in repetition rate allows a longer "dead time" space between two adjacent laser pulses such that (i) the response time of the system can be matched and (ii) the decay time can be measured more easily.

The repetition rate of the laser pulses produced by the dye laser is $\sim 100\text{MHz}$. The EOM can reduce the repetition rate to $100 \div ((N+1) \times 128)\text{MHz}$ (N can be set to any integer which ≥ 2 and ≤ 10). The repetition rate in this work is 270KHz ($N = 2$).

(v) An **Auto-correlator** is used to measure the width of the laser pulses.

(vi) A **Fresnel Rhomb** is used to change the polarization direction of the linear polarized dye laser beam. An optical detection system usually has a light sensitivity which changes with the polarization direction. Using a Fresnel Rhomb, this disadvantage can be avoided by fixing the polarization direction of the emission light (using a polarizer: **P**) and changing the polarization direction of the excited light beam by the Fresnel Rhomb.

(vii) A Jarrel-Ash double monochromator, **DMC**, operating in the subtractive mode is used to select the excited fluorescence. The subtractive configuration of the monochromator components is used because this configuration can eliminate time broadening of short light pulses induced by single monochromator.

(viii) A Hamamatsu R1564U microchannel plate photomultiplier tube (**MCP-PMT**) cooled to -20°C is used as a single photon detector. Single photoelectron pulses from the photomultiplier are amplified by two broad band amplifiers (Mini Circuits ZHL1042J) and the output fed into a constant fraction discriminator, **CFD**, (Tennelec model TC454) producing the Stop pulses for the time-to-amplitude converter (**TAC**). The Start pulses of TAC are obtained from a fast photodiode (**Ph.**) which monitors the extracted laser pulses.

(ix) A 4k multichannel analyzer PC board (**MCA**) is used to accumulate the time-to-amplitude converter (**TAC**) output. A multi-tasking graphics software for PC/AT microcomputers is used to manage the digit data.

Figure 4-1-1: Experimental set-up of the picosecond system.

4.1.2 Generation of Picosecond Light Pulses

A laser is typically constructed with a pair of mirrors which enclose a gain medium. The range of the frequencies over which a laser oscillates is determined by the gain bandwidth and the loss in the cavity. The precise frequencies which oscillate within the allowed range are determined by the shape of the resonator.

If the length of a resonator is assumed to be L and the other two resonator dimensions are much longer than L , the resonant frequencies are given by

$$\nu = n \cdot \frac{c}{2L} \quad (4-1-1)$$

where c is light speed; n is an integer. The frequency difference of two adjacent modes ($\Delta n = \pm 1$) is given by either

$$\Delta \nu = \frac{c}{2L}$$

or

$$\Delta \omega = \frac{\pi c}{L} \quad (4-1-2)$$

The oscillation modes which show the dependencies given in equations 4-1-1 and 4-1-2 are called longitudinal modes. The discussion in this section only considers the longitudinal modes.

The number of modes, N , is limited by the bandwidth, $\Delta\omega_{osc}$, over which the laser gain exceeds the loss of the resonator.

$$N = \frac{\Delta\omega_{osc}}{\Delta\omega} = \frac{\Delta\nu_{osc}}{\Delta\nu} \quad (4-1-3)$$

If one considers the case of $2N+1$ longitudinal modes in a laser system, the electric field can be written as

$$E(t) = \sum_{n=-N}^N \alpha_n(\omega_0 + n\Delta\omega) e^{i[(\omega_0 + n\Delta\omega) \cdot t + \phi_n(\omega_0 + n\Delta\omega)]} \quad (4-1-4)$$

where α_n is the amplitude of the n th mode; ω_0 is the frequency of the central mode; $\Delta\omega$ is the frequency difference between two consecutive modes (equation 4-1-2); and ϕ_n is the phase of the n th mode.

When free oscillation occurs, the phases of the modes are randomly distributed in the range from $-\pi$ to π and the temporal pattern of the intensity, $I(t)$ ($= E(t)E^*(t)$), are similar to thermal noise.

Mode-locking forces the modes to maintain a fixed phase and frequency relationship. In this case, $I(t)$ is a well defined periodic function of time. If one assumes that the $2N+1$ modes (equation 4-1-4) oscillate with the same amplitude α_0 and the phase of the modes is fixed as $\phi_n - \phi_{n-1} = \phi_c$ where ϕ_c is a constant, equation 4-1-4 can be written as

$$E(t) = \sum_{n=-N}^N \alpha_0 e^{i[(\omega_0 + n\Delta\omega) \cdot t + n\phi_c]} = A(t) e^{i\omega_0 t} \quad (4-1-5)$$

Here $A(t)$ is given by

$$A(t) = \sum_{n=-N}^N \alpha_0 e^{in(\Delta\omega t + \phi_c)} \quad (4-1-6)$$

Equation 4-1-5 shows that $E(t)$ can be represented in terms of a sinusoidal carrier wave with a centre-mode frequency ω_0 whose amplitude $A(t)$ is time modulated. And $A(t)$ can be represented by

$$A(t) = \alpha_0 \frac{\sin \frac{1}{2} (2N+1) (\Delta\omega t + \phi_c)}{\sin \frac{1}{2} (\Delta\omega t + \phi_c)} \quad (4-1-7)$$

Equation 4-1-7 shows that the width of the output pulses, t_p , determined by the time spacing between a maximum of $A(t)$ and its first zero point, is approximately equal to

the reciprocal of the total mode-locked bandwidth, $(2N+1)\Delta\nu = \Delta\nu_{\text{tot}}$, and that the time period (separation of two consecutive pulses), τ , determined by the time between two consecutive maxima of the $A(t)$ is equal to $2L/c$. Equation 4-1-7 represents the amplitude of the electric field of a perfect mode-locked laser in the time domain.

Various mode-locking methods have been used to produce picosecond light pulses since 1965 [157]. Generally speaking, mode-locking methods can be divided into two categories (i) active mode-locking, in which the loss or gain of the laser pulses is modulated by an external driving source, and (ii) passive mode-locking, usually achieved by a saturable absorber.

The processes involved in mode-locking can be understood by considering its behaviour in the time domain. If a pulse is assumed to pass through the modulator at a time t_{min} of minimum loss, it will be returned to the modulator after a time $2L/c$, and the frequency of time-varying loss is adjusted to be $c/2L$, so that the loss of the pulse is again at minimum. On the other hand, if a pulse is assumed to initially pass through the modulator at a time, slightly before t_{min} , the leading edge of the pulse will suffer more attenuation than the trailing edge of the pulse. Thus after passing through the modulator, the peak of the pulse will be changed in such a way, that during the next passage the peak will arrive at a time closer to t_{min} . Finally after the passage of sufficient time, all pulses will arrive at the modulator at time t_{min} and the laser is considered to be "mode-locked".

2

MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS
STANDARD REFERENCE MATERIAL 1010a
(ANSI and ISO TEST CHART No. 2)

An acoust-optic mode locker (active amplitude-modulation mode-locking) is used with the Nd:YAG laser in this system. The mode-locking is accomplished by inserting a standing wave acoust-optic modulator into the laser cavity near one of the mirrors. The acoustic resonant frequency is 50.232MHz and the modulator produces a sinusoidal time-varying loss at the frequency of 50.232×2 MHz. The length of the Nd:YAG laser cavity, L , is adjusted to satisfy the relationship, $c/2L = 50.232 \times 2$ MHz. The width of laser pulse produced by the mode-locked Nd:YAG laser is ~ 100 picosecond.

The cavity length of the dye laser is adjusted to be equal to that of the Nd:YAG laser and the dye laser is synchronously pumped by the frequency doubled Nd:YAG laser. According to equation 4-1-7, the width of a laser pulses is approximately equal to the reciprocal of the bandwidth of the laser. Because the bandwidth of the dye laser is much broader than that of Nd:YAG laser, the width of the light pulses is compressed by the dye laser. The width of the light pulses produced by the dye laser is 5-6 picosecond.

4.1.3 Pulse-width Measurement

In a pulse-width measurement of ultrashort light pulses, satisfactory time resolution can be achieved either by a streak-camera system or by a nonlinear correlation method. The streak-camera system can be used to directly demonstrate picosecond or subpicosecond time resolution. The nonlinear correlation method is an indirect pulse

width measurement. The technique used in this system is the nonlinear correlation method.

For a nonlinear correlation measurement, the important point is to understand the relationship between a signal $I(t)$ and its correlation function $G(\tau)$. The second order autocorrelation function of intensity is given in normalized form by [158]

$$G^{(2)}(\tau) = \frac{\langle I(t) I(t+\tau) \rangle}{\langle I^2(t) \rangle} \quad (4-1-8)$$

where the brackets indicate an average over a sufficiently long interval of time. If $I(t)$ is a single isolated pulse, $G^2(\tau)$ vanishes for large relative delay τ and its half-width provides a measurement of the duration of $I(t)$.

This technique is illustrated diagrammatically in Figure 4-1-2. The optical pulse to be measured is divided into two beams which travel different paths before being recombined in a nonlinear crystal. The signal $S(\tau)$ is detected by the detector. The relationship between $G^{(2)}(\tau)$ and $S(\tau)$ is given by

$$S(\tau) = W \cdot (1 + 2G^{(2)}(\tau)) \quad (4-1-9)$$

where W is a parameter independent of τ .

Figure 4-1-2: Diagram for nonlinear correlation technique.

From equation 4-1-8, it is clear that (i) $G^{(2)}(0) = 1$ and (ii) $G^{(2)}(\tau) = 0$ when $\tau > \Delta t$ or $\tau < -\Delta t$ (Δt is the width of the laser pulse). Substitution these relations in equation 4-1-9 gives

$$\frac{S(0)}{S(\tau > \Delta t)} = 3 \quad (4-1-10)$$

Thus equation 4-1-10 shows that for a detected correlation function of $s(\tau)$, the ratio of its peak to its background is 3. And that half-width of its peak is equal to the width of the laser pulse. The trick in this technique is changing a time resolution measurement to a length resolution measurement. The time scale τ is determined by the displacement of a pyramid (pyramid 2). The signal S measured by the detector is a function of the displacement of pyramid 2, and displacement of 0.15mm corresponds to 1 picosecond.

4.1.4 Time-correlated Single Photon Counting [159]

In an ideal time-correlated experiment each photon emitted by a sample is able to be timed and recorded. The time dependent number of photons represents the time dependent emission intensity. In fact the response time of the detection system and the recording system makes it necessary to time only the first photon in a given time interval. This is called the time-correlated single photon counting technique. The major principles underlying this technique are given here [160,161].

During one excitation cycle an average number of photons, n_{pi} , impinge on the photocathode of the photomultiplier at the i th time interval. These photons eject an average number n_{ei} of photoelectrons from the photocathode of the photomultiplier

$$n_{ei} = qn_{pi} \quad (4-1-11)$$

where q is the quantum efficiency of the photocathode.

If $n_{ei} \ll 1$, after a large number of excitation cycles (N_{exc}), the number of anode pulses in the i th time interval is N_{ai}

$$N_{ai} \approx N_{exc} (n_{ei} + n_{ei}^2) \approx N_{exc} n_{ei} = N_{exc} qn_{pi} \quad (4-1-12)$$

Therefore the number of anode pulses at the i th time interval, N_{ai} , is proportional to the detected photons at the i th time interval. This is the case of an ideal time-correlation.

If only the first photon in a given time interval is detected, after N_{exc} cycle, N_{di} anode pulses are detected in the i th time interval. N_{di} is related to N_{ai} by equation [162]

$$N_{ai} = \frac{N_{di}}{1 - \frac{1}{N_{exc}} \sum_{j=1}^{i-1} N_{dj}} \quad (4-1-13)$$

Since

$$\sum_j N_{dj} \leq N_D$$

where N_D is the total anode pulses to be detected. If $N_D \ll N_{exc}$, it follows that $N_{ai} = N_{di}$. Thus a time-correlated single photon counting technique can be used satisfactorily under such conditions.

The choice of the value of N_D/N_{exc} depends on the level of distortion that can be experimentally accepted. Most studies keep N_D/N_{exc} in the region of 0.02 or lower. The value of N_D/N_{exc} in this work is in the region < 0.01 .

Figure 4-1-3 shows a diagram of the single photon counting detection system used here. Overall the system dynamics are as follows: a **Start** signal obtained from a fast photodiode (Ph.) monitoring the extracted laser pulses is sent to the **Start** input of the time-to-amplitude converter (TAC). In the meantime, the double monochromator (DMC) selects the emission light in the designated region of wavelengths. The selected photon pulse is detected by the photomultiplier (MCP-PMT). The single photoelectron pulse induced by the photon pulse is amplified by two broad band amplifiers and is fed into the constant fraction discriminator (CFD) producing the **Stop** pulses. The **Stop** pulses are sent to the **Stop** input of the TAC. The multichannel analyzer PC board (MCA) accumulates the time-to-amplitude converter (TAC) output. The output is digitized and

the analyzer directs this output into the corresponding channel depending on the time interval between Start and Stop. The data obtained is a curve of the time dependent photon counts at a designated wavelength.

Figure 4-1-3: Diagram of the single photon counting detection system.

The time response of the entire system is 42 picosecond. Figure 4-1-4 shows the response of the system to a 6 picosecond light pulse.

Figure 4-1-4: Time response of the detection system to a 6 picosecond laser pulse.

The detection system can also be used in a manner similar to that of a boxcar. In this case the Start and Stop times are fixed, and the double monochromator is adjusted to detect the whole spectrum of a fluorescence. The multichannel analyzer (MCA) inputs the signals to different channels depending on the wavelength of the signals. The data obtained in this way represents the fluorescence spectrum in a designated time region.

One of the most important uses of the above method is to isolate the spectrum with the longest decay time from a multi decay time process. A time region in which the longest decay time component can be detected and the other decay time components can be neglected, the spectrum taken in this time region represents a processes with only one decay time.

4.1.5 Calibration

One of the most accurately known fluorescence life-times is that of Rhodamine B. When excitation is at 568nm and detection at 630nm, the life-time of fluorescence of Rhodamine B (optic density, O.D., from 0.05 to 0.1) is 2.468 nanosecond [83].

The picosecond system is thus calibrated by measuring the fluorescence life-time of Rhodamine B (RB). The O.D. of the RB solution is 0.1 diluted by spectra grade methanol. With an excitation wavelength of 570nm and detection wavelength of 630nm, the fluorescence life-time measured is 2.21 nanosecond. This indicates that the error of the picosecond measuring system is less than 0.3 nanosecond.

4.2 NANOSECOND LASER SYSTEM

4.2.1 Components of the Nanosecond System

A schematic of the experimental apparatus used in the nanosecond laser system is shown in Figure 4-2-1. The major components are:

(i) A pulsed excimer laser (Lumonics model TE-320-2) which produces ~ 8 nanosecond pulses of 35mJ at a repetition rate of 25Hz is used as the pump source for two dye lasers. The wavelength of the excimer laser is 308nm.

(ii) Two dye lasers (dye laser 1: Lumonics model TE-320-2; dye laser 2: Lumonics model EPD 330) pumped by the excimer laser are used as the pump light source and the probe light source. The wavelengths of the two dye lasers can be tuned from the UV to the Visible region. The width of the light pulses is ~ 5 nanosecond.

(iii) A double monochromator, DMC, (Jobin-Yvon, HG2) equipped with two holographically ruled gratings is used to select the signals. There are four sets of slits which are independently controlled by stepper motors and by re-transmitting potentiometers. The slits can have a width of up to 2mm with $1\mu\text{m}$ resolution. The mechanical range of the monochromator is 400nm to 780nm and the optimum visible

range is 440nm to 740nm.

(iv) The light emerging from the double monochromator is detected by a photomultiplier, **PMT**, (RCA 31034) with a ~ 2 nanosecond response.

(v) The output of the photomultiplier is fed into a **boxcar** (Stanford Research Associates model SR 250, SR 235, SR 245 and SR 275) and the data is processed by the **boxcar**.

(vi) The output by the boxcar is recorded by a **recorder** or fed into a **PC**.

Figure 4-2-1: Experimental set-up of the nanosecond system.

4.2.2 Performance of the Nanosecond System

The excimer laser beam is split to two beams by M1, one beam is used to pump dye laser 1 and the other beam is used to pump dye laser 2. The radiation wavelengths of the dye laser 1 and dye laser 2 can be tuned to that required by the desired experiment. These two laser beams (usually with two colours) arrive at the same spot of a dichroic mirror M7 which transmits one beam (from dye laser 1) and reflects the other one (from dye laser 2). The polarization direction of the two laser beams are adjusted by two $\lambda/4$ plates and two polarizers (P1 and P2). After passing through and / or reflecting from M7, these two laser beams propagate co-linearly and are focused into a sample.

The light generated by the pump and probe beams which transmits through the sample is fed to the double monochromator which selects the probe light only. The light emerging from the double monochromator is detected by a photomultiplier and fed to the boxcar. The data is processed by the boxcar. The output from the boxcar is accumulated by the recorder or the PC.

The signals obtained by the two beam absorption experiments are categorized into two type of data, I(on) and I(off).

I(ofi) is the transmission intensity of the probe beam when the pump beam is off,

i.e. $I(\text{off})$ is the transmission intensity equivalent to that from a normal absorption experiment.

$I(\text{on})$ is the transmission intensity of the probe beam when the pump beam is on. This means that the pump beam and the probe beam must arrive at the sample at the same time. An optical delay line (not shown in Figure 4-2-1) is used to control the time at which the pump beam arrives at the sample.

The time control through the use of the optical delay line is obtained by: (i) measuring $I(\text{off})$ and recording the intensity of $I(\text{off})$; (ii) making the polarization directions of the pump beam and the probe beam either parallel or perpendicular as required; (iii) measuring $I(\text{on})$ with different optical delays to find a position which corresponding to the maximum of $\Delta I (= |I(\text{on}) - I(\text{off})|)$ and fixing the delay at this position.

In a two beam absorption measurement it is also necessary to keep the detected light (probe beam light) in a fixed polarized direction to avoid distortion due to changes in the monochromator sensitivity with polarization induced by the gratings. This is achieved by changing the polarization direction of the pump beam and keeping the probe beam polarization direction constant. The intensity of the pump beam is adjusted by a group of optical attenuation plates (not shown in Figure 4-2-1) to keep its intensity constant for different polarization directions.

4.3 DATA ANALYSIS [163]

4.3.1 Convolution

The ideal experimental conditions for a time-resolved measurement are (i) the excitation laser pulse is infinitely narrow ($\sim \delta$ function) and (ii) the response of the detection system is infinitely fast (response time $\ll$ required time resolution of decay curve). A decay curve obtained by a time-resolved fluorescence measurement is a true decay curve only under the ideal experimental conditions.

The data, $I(t)$, obtained in an experiment which does not satisfy the ideal experimental conditions, is not the true decay curve. If $G(t)$ is used to represent the true decay curve, the relationship between $I(t)$ and $G(t)$ can be written

$$I(t) = P(t) \otimes G(t) = \int_0^t P(t') G(t-t') dt' \quad (4-3-1)$$

Here $P(t)$, the instrument response function, is a convolution of $E(t)$ and $H(t)$

$$P(t) = E(t) \otimes H(t) \quad (4-3-2)$$

where $E(t)$ represents the excitation function which is not a δ function; and $H(t)$ represents the δ pulse response of the detection system. Equation 4-3-1 represents the

true decay curve distorted by convolution with both the pump pulse and the detector response.

Equation 4-3-1 can be solved for $G(t)$ (true decay curve) in the case that $I(t)$ and $P(t)$ are measured under the same conditions. $I(t)$ is the experimental data; $P(t)$ represents the instrument response to the excitation pulses. In this work, the instrument response function $P(t)$ is measured under the conditions used for the related time-resolved fluorescence measurements. The true decay curve $G(t)$ can be obtained from $I(t)$ and $P(t)$ by a deconvolution technique.

4.3.2 Deconvolution

The deconvolution technique used in this work is a standard non-linear least-squares iterative deconvolution method [30].

The basic concept of least-squares curve fitting is that the quantity χ^2 is minimized. Here χ^2 is defined by

$$\chi^2 = \sum_{i=1}^n W_i [y(t_i) - Y(t_i)]^2 \quad (4-3-3)$$

where W_i is the weighting factor for the i th data point; n is the total number of points; $y(t_i)$ is the experimental point; $Y(t_i)$ is the point from the calculated fitting function.

The plot of the residuals, $r(t)$, and the Serial Variance Ratio (SVR) are used to judge the goodness of the fitting in the deconvolution technique.

The residuals are determined by

$$r(t_i) = \{W_i [y(t_i) - Y(t_i)]\}^{1/2} \quad (4-3-4)$$

where W_i , $y(t_i)$ and $Y(t_i)$ represent the same parameters as equation 4-3-3.

SVR is related to the von Neumann Ratio, VNR. The ratio of the mean-square successive difference to the variance of a series was proposed by von Neumann (1944) as a statistic for testing the independence of successive observations in an ordered series for which the underlying distribution is normal. In large samples taken from a random series, the distribution of the ratio tends to be 2. SVR is related to VNR by

$$SVR = VNR \times \frac{N}{N-1} \quad (4-3-5)$$

where N is the number of observations. When $N \gg 1$, $SVR \approx VNR$.

For this study, the data produced by the deconvolution technique has been considered satisfactory only where SVR is larger than 1.5.

4.4 SAMPLE HANDLING

The DNA samples were gifts from Dr. Y.Yevdokimov (Institute of Molecular Biology, Moscow) [164] and were used without further purification. The DNA, "low-molecular mass chicken blood erythrocytes DNA" (Reanal, Hungary), was obtained by ultrasonic depolymerization of initial high-molecular mass DNA (4, 50S, 22KHz, ultrasonic generator UZDN-2T, USSR). The molecular mass of the DNA is $(5-7) \times 10^4$, estimated by a 3.5% polyethylene gel electrophoresis. Figure 4-4-1 shows the absorption spectrum of the DNA sample, the ratio of O.D.(260.0nm) / O.D.(280.0nm) is about 2.0. Here, O.D. is the optical density of the sample, i.e. the absorbency of the sample.

Tetrakis (4-N-methyl-pyridyl) porphyrin, H_2TMPyP , was purchased from Aldrich Chemical Company, Inc. as the tosylate salts and was used without further purification.

Mitoxantron hydrochloride (MX) was purchased from Cyanamid Canada, Inc and was used without further purification.

A **Phosphate buffer** (0.01M phosphate; 0.3M NaCl; $pH = 7.0 \pm 0.1$) was used as solvent to dilute the drugs and the DNA solutions. The buffers were prepared from analytical grade reagents (K_2HPO_4 , KH_2PO_4 and NaCl) by dissolving them in ultra-pure

deionized water (18.2M Ω). The pH value of the buffer was measured by a Fisher Accumet pH Meter (Model 825 MP).

The concentration of the drugs in the DNA solution was expressed in terms of the r(c) number, where r(c) represents the molarity ratio of base pairs of DNA to H₂TMPyP or MX molecules.

The molarity of the base pairs of the DNA was determined by (i) the O.D of the DNA samples at 260nm and (ii) the molar extinction coefficient of the sample, ϵ . According to Beer's law [80], if the length of the sample is 1cm, the molarity of the base pair, [b.p.], is

$$[b.p.] = \frac{O.D.}{\epsilon} \quad (4-4-1)$$

The molar extinction coefficient of the different kinds of DNA at 260nm varies, depending on the ratio of A-T base pair to G-C base pair. The ratio of these two kinds of base pairs of the DNA was not measured in this study. The generally accepted value of 6600 cm⁻¹M⁻¹ as the molar extinction coefficient for B form DNA has been used throughout.

Beer's law is valid only for dilute solutions where the O.D. < 1.0. The O.D. of the DNA samples used for the experiments reported here was larger than 1.0, the

concentration of the DNA was kept at 150.0 $\mu\text{g/mL}$. The molarity of the base pairs of the sample DNA was determined from separate samples with lower concentrations, 50.0 $\mu\text{g/mL}$. The O.D. of the lower concentration sample is 0.91 and the molarity of the base pair is $1.38 \times 10^{-4}\text{M}$ (for extinction coefficient = $6600 \text{ cm}^{-1}\text{M}^{-1}$). The molarity of the higher concentration sample (150.0 $\mu\text{g/mL}$) derived from the low concentration sample was $4.15 \times 10^{-4}\text{M}$.

Fresh samples were prepared before each measurement.

Figure 4-4-1: Absorption spectrum of the DNA sample: $\text{O.D. (260.0nm)} / \text{O.D. (280.0nm)} = 2.0$.

4.5 OTHER RELEVANT EXPERIMENTAL EQUIPMENT

Circular dichroism spectra and absorption spectra were taken for quality control. Circular dichroism spectra were measured at Dr. B.R. Hollebone's laboratory (Carleton University, Chemistry Department) by a home-made instrument. Absorption spectra were taken by a Lambda 4B UV/VIS Spectrophotometer (C688-0001). The fluorescence spectra were also taken by an Ar⁺ laser (COHERENT RADIATION LABORATORIES Model 52 Ion laser).

5. RESULTS AND DISCUSSION

5.1 H₂TMPyP/DNA COMPLEXES

5.1.1 Identification

Absorption

When DNA is added to H₂TMPyP, the Soret absorption band decreases and red-shifts [91]. These spectral changes have been used to monitor the interaction of H₂TMPyP with DNA for over 20 years.

Figure 5-1-1 shows the absorption spectra of H₂TMPyP with and without DNA in the visible range (350nm - 700nm). The concentration of H₂TMPyP was 7.4×10^{-6} M for the both situations. For the H₂TMPyP/DNA complexes, the concentration of DNA was 150.0 μ g/mL and the r(c) number was 56.0. The definition of r(c) is given in Section 4.4. Hypochromicity and red-shift of the Soret band of H₂TMPyP were seen in these measurements (Figure 5-1-1).

Figure 5-1-1: Absorption spectra of H_2TMPyP (dashed line) and H_2TMPyP/DNA complexes (solid line).

For the H₂TMPyP/DNA complexes, the Soret band was measured for r(c) numbers from 1.8 to 224.0 (concentration of H₂TMPyP from 2.3×10^{-4} M to 1.85×10^{-6} M). The measurements show that:

(i) when the r(c) number is lower than 4.5, the concentration of H₂TMPyP is too high and the Soret band is saturated, thus it is impossible to observe hypochromicity and red-shift of the Soret band;

(ii) when the r(c) number is between 11.2 to 224.0, the Soret band of H₂TMPyP (centred at 421.9nm without DNA) red-shifts and hypochromicity is observed.

Circular Dichroism

H₂TMPyP does not display circular dichroism in the absence of DNA. When DNA is added to H₂TMPyP, circular dichroism can be detected in the Soret band. The negative component in the Soret band is caused by intercalated binding of H₂TMPyP molecules and the positive component is caused by externally binding of H₂TMPyP molecules [91,99,122].

The circular dichroism spectrum of a H₂TMPyP/DNA sample is shown in Figure 5-1-2. The r(c) number of the sample is 11.2. A positive component around 423.0nm and a negative component around 437.0nm were observed. A circular dichroism

measurement was also taken for another sample which had an $r(c)$ number 56.0. In this case, the intensities of the signal were very weak (< 0.1), but both the positive signal and the negative signal could be detected.

Figure 5-1-2: Circular dichroism spectrum of H₂TMPyP/DNA complexes ($r(c)$: 11.2).

These absorption spectra and the circular dichroism spectra show H₂TMPyP bound to DNA in H₂TMPyP/DNA complexes. The circular dichroism spectra also indicate that two types of binding modes (the intercalated binding mode and the external binding mode) exist in the complexes.

5.1.2 Fluorescence Measurements

Fluorescence Spectrum

Fluorescence spectra of H₂TMPyP and H₂TMPyP/DNA complexes are shown in Figure 5-1-3. The spectra were produced by excitation from an Ar⁺ laser (476.5nm) with emission detection from 600nm to 800nm. DNA in the buffer solution does not fluoresce in this region, thus the fluorescence spectra shown in Figure 5-1-3 are due to H₂TMPyP.

Without DNA, H₂TMPyP shows a broad fluorescence band between 600nm and 800nm, centred at about 675nm (curve I in Figure 5-1-3). With DNA, this fluorescence band splits into two peaks centred at about 650nm and 715nm respectively (curve II and curve III in Figure 5-1-3). The concentration of the H₂TMPyP was the same for curve I and curve II (3.7×10^{-5} M). The r(c) number for curve II was 11.2 and the r(c) number for curve III was 56.0. With DNA, fluorescence of H₂TMPyP was markedly enhanced and its spectral shape was changed when compared with fluorescence without DNA.

Fluorescence spectra were measured for H₂TMPyP/DNA complexes with different concentrations. The two peaks in the region between 600nm and 800nm were observed for samples which had r(c) numbers between 1.8 to 224.0.

Figure 5-1-3: Fluorescence spectra of H₂TMPyP (dashed line) and H₂TMPyP/DNA complexes (solid line).

Time-resolved Fluorescence Spectrum

Time-resolved fluorescence spectra were taken with the picosecond system shown in Figure 4-1-1. The isotropic time-resolved fluorescence intensity was measured at the the "magic angle".

Time-resolved fluorescence spectra of H₂TMPyP without DNA were measured at three wavelengths: 656nm, 667nm and 716nm. The emissions were mono-exponential and the decay time was 4.8 ± 0.5 nanosecond, 4.8 ± 0.5 nanosecond and 4.9 ± 0.5 nanosecond at 656nm, 667nm and 716nm respectively. Figure 5-1-4 shows the decay curves in a logarithmic form, excitation was at 566nm and probe was at 656nm (a), 667nm (b) and 716nm (c). These results indicate that fluorescence of H₂TMPyP without DNA has a decay time of ~ 5.0 nanosecond.

Figure 5-1-4: Fluorescence decay of H_2TMPyP in a buffer solution: excitation at 566nm, probe at 656nm (a), 667nm (b) and 716nm (c).

Time-resolved fluorescence spectra of H₂TMPyP/DNA were measured for samples with an $r(c)$ number from 1.8 to 224.0. The excitation was at 572nm and detection was at 662nm.

With an $r(c)$ number > 112 , the signal was too weak to be detected. With $r(c)$ numbers > 5.4 , the fluorescence spectrum were bi-exponential, exhibiting two decay times of ~ 2 nanosecond and ~ 10 nanosecond. When $r(c)$ was ≤ 5.4 , the fluorescence spectra were tri-exponential with three decay times, ~ 1.2 nanosecond, ~ 4.0 nanosecond and ~ 10.5 nanosecond.

Table 5-1 gives the experimental results in detail. Table 5-1 also gives the relative contribution to the spectrum of the individual specific decay time components. The data shown in Table 5-1 were obtained by the deconvolution method introduced in Section 4.3.

Figure 5-1-5 shows a bi-exponential decay curve (in linear and logarithmic form) which was measured for a sample with $r(c) = 11.2$. Figure 5-1-6 shows a tri-exponential curve (in linear and logarithmic form) which was measured for a sample with $r(c) = 5.4$.

Table 5-1: Radiative Decay Time and Relative Contribution to the Fluorescence
Intensity of H₂TMPyP/DNA Complexes

H ₂ TMPyP (M)	r(c)	Decay Time (ns)	Contribution (%)
3.7×10^{-6}	112.0	9.1	84
		1.5	16
9.25×10^{-6}	45.0	10.2	81
		1.7	18
3.7×10^{-5}	11.2	10.0	79
		1.9	21
7.7×10^{-5}	5.4	10.6	56
		1.2	11
		4.1	33

The concentration of DNA is 150.0 $\mu\text{g/mL}$.

Estimated error: in decay time ± 0.5 ns; in contribution 10%.

Figure 5-1-5: Fluorescence decay of H₂TMPyP/DNA complexes (r(c) : 11.2) in a buffer solution: excitation at 572nm, probe at 662nm.

Figure S-1-6: Fluorescence decay of H_2TMPyP/DNA complexes ($r(c) : 5.4$) in a buffer solution: excitation at 572nm, probe at 662nm.

Separation of Fluorescence Components Based on Different Decay Times

It has been shown in Section 4.1.4 that by taking a fluorescence spectrum at a designed time region one can isolate the component with the longest decay time from a multi-exponential spectrum. Measurements in a selected time region were taken for a sample with $\tau(c) = 45.0$ (Figure 5-1-7). The time-resolved measurements show that the fluorescence of the sample was made up of two components with decay times of 10.0 nanosecond and 2.0 nanosecond (Table 5-1).

The spectrum shown in Figure 5-1-7(a) was taken in a time window from 0 to 6 nanosecond after excitation. The emission of the 2.0 nanosecond component and the 10.0 nanosecond component almost equally contributed to the spectrum intensity. The ratio of the two peaks in the spectrum is 1.7. The spectrum shown in Figure 5-1-7(b) was taken in a time window from 2 to 8 nanosecond after excitation, the spectrum is primarily due to the 10.0 nanosecond component. The ratio of the two peaks in the spectrum is 1.35. The spectrum in Figure 5-1-7(c) was taken in a time window from 9 to 15 nanosecond after excitation. During this time, the emission of the 2.0 nanosecond component was too weak to contribute to the overall spectrum. The spectrum is a mono-exponential spectrum with the decay time of 10.0 nanosecond. The ratio of the two peaks in the spectrum is 1.1.

Figure 5-1-7 (a): Time dependent fluorescence spectrum of H_2TMPyP/DNA complexes
($r(c) : 45.0$): excitation was at 572nm, detection was after the excitation
from 0 - 6 nanosecond.

Figure 5-1-7 (b): Time dependent fluorescence spectrum of H₂TMPyP/DNA complexes
($r(c) : 45.0$): excitation was at 572nm, detection was after the excitation
from 2 - 8 nanosecond.

Figure 5-1-7 (c): Time dependent fluorescence spectrum of H_2TMPyP/DNA complexes
($r(c) : 45.0$): excitation was at 572nm, detection was after the excitation
from 9 - 15 nanosecond.

Therefore through a selected delayed time fluorescence measurement technique, a mono-exponential spectrum with 10.0 nanosecond decay was isolated from the bi-exponential spectrum generally exhibited by the H_2TMPyP/DNA solution.

A mono-exponential spectrum with a decay time of 2.0 nanosecond was experimentally derived by the following procedures:

(i) Time-resolved fluorescence intensities were measured at between 620nm to 760nm with the interval between neighbouring measurements of ~ 10 nm. Throughout these measurements, the intensity of the excitation laser was maintained constant.

(ii) The two decay time components for the all individual time-resolved spectrum were separated by: first determining the 10.0 nanosecond decay function by computer simulation, and second, determining the 2.0 nanosecond component by subtracting the 10.0 nanosecond component from the bi-exponential function.

(iii) The intensities of the different decay time components at time = 0 were determined from the results of step (ii).

(iv) Based on the intensities (at time = 0) of the different decay time components at different wavelengths, the fluorescence spectra were plotted using a single decay time.

Figure 5-1-8 shows the fluorescence spectra with a single decay time obtained by this method. The $r(c)$ number of the sample was 56.0. The original spectra were bi-exponential with two decay times, 2.0 nanosecond and 10.0 nanosecond. The 10.0 nanosecond decay time spectrum obtained by the delayed "window" (Figure 5-1-7(c)) agrees with the spectrum acquired by the time-resolved fluorescence measurements within the experimental error. This result indicates that the two separated spectra in Figure 5-1-8 represent the 10.0 nanosecond decay component and the 2.0 nanosecond decay component respectively.

Both the 2.0 nanosecond spectrum and the 10.0 nanosecond spectrum have two peaks in the region of 600nm to 800nm. The ratio of the two peaks is close to 1.1 in the 10.0 nanosecond decay spectrum. The intensity of the peak around 650nm is much higher than the other one (at 715nm) in the 2.0 nanosecond decay spectrum, in this case the peak : peak ratio is > 2 .

Figure 5-1-9 shows the results obtained by the same procedure for a sample with the $r(c)$ number of 11.2 . Qualitatively, the fluorescence spectra in Figure 5-1-9 image the fluorescence spectra in Figure 5-1-8.

Figure 5-1-8: Separation of fluorescence components based on different decay time for H_2TMPyP/DNA complexes ($r(c) : 56.0$).

Figure 5-1-9: Separation of fluorescence components based on different decay time for H_2TMPyP/DNA complexes (r(c) : 11.2).

Measurements of the fluorescence spectra of H_2TMPyP , without DNA, show only one decay time, 5.0 nanosecond, and a broad peak in the region of 600nm to 800nm. Similar measurements of the fluorescence of H_2TMPyP , with DNA, showed the emission to be indicative of two or three decay times (Table 5-1) and the broad peak at 600 - 800nm is split to produce two maxima. Combining these results with the results from the absorption measurements and the circular dichroism measurements, suggests that H_2TMPyP molecules exist as three forms in H_2TMPyP /DNA complexes, i.e. (i) free H_2TMPyP , (ii) intercalated bound H_2TMPyP and (iii) externally bound H_2TMPyP . The fluorescence spectra corresponding to the three components have different decay times and emission contours.

By comparing time-resolved fluorescence measurements of H_2TMPyP without DNA to those of H_2TMPyP with DNA, it can be seen that the 2.0 nanosecond decay fluorescence and the 10.0 nanosecond decay fluorescence in H_2TMPyP /DNA complexes are from the bound H_2TMPyP and the 4.0 nanosecond component originates from the free H_2TMPyP molecules or unbound H_2TMPyP molecules.

The free H_2TMPyP (with decay time of ~ 4 nanosecond) was not detected in samples with $r(c)$ number > 5.4 , but this does not mean there is no free H_2TMPyP in those samples. The fluorescence emitted by the free H_2TMPyP is probably too weak to be measured. Because of the effects of DNA, the decay time of the free H_2TMPyP is shorter than that without DNA by ~ 1 nanosecond.

Relationship between Decay Time and Binding Mode

It has been found that there are two decay times (2.0 nanosecond and 10.0 nanosecond) corresponding to the two forms of H_2TMPyP molecules, intercalated bound H_2TMPyP and externally bound H_2TMPyP . The next step is to determine the relationship between the decay time and the binding mode.

The fluorescence contributed by the intercalated bound H_2TMPyP can be obtained by an experiment [104] which is briefly introduced below.

The concept of the experiment is based on the fact that intercalated binding is specific for double stranded DNA. If the concentration of H_2TMPyP is very low, only one binding mode, the intercalated binding mode, can be detected. By increasing the concentration of H_2TMPyP , another binding mode, the externally binding mode, appears. These effects were observed by circular dichroism spectroscopy and visible absorption spectroscopy [91,113,122,118].

The fluorescence of H_2TMPyP /DNA complexes with different concentrations of H_2TMPyP were measured in the experiment. The concentration of H_2TMPyP varied from $3.3 \times 10^{-8}M$ to $4.16 \times 10^{-6}M$ ($r(c)$ from 12800.0 to 100.0). From the dependence of the fluorescence cross section on the concentration of H_2TMPyP , it was determined that when the $r(c)$ number is ≥ 6400.0 (concentration of $H_2TMPyP \leq 6.5 \times 10^{-8}M$),

H₂TMPyP molecules occupy only one specific site of DNA that site giving rise to intercalation [104].

The fluorescence spectrum of a sample with an $r(c)$ value of 6400.0 is shown in Figure 5-1-10. Only the intercalated H₂TMPyP contributes to the spectrum because of the low concentration. A comparison of this spectrum with the 2.0 nanosecond decay spectrum and the 10.0 nanosecond decay spectrum (Figure 5-1-8) indicates that the 10.0 nanosecond spectrum corresponds to the intercalated bound H₂TMPyP.

Figure 5-1-10: Fluorescence spectrum of H₂TMPyP/DNA complexes ($r(c) : 6400.0$).

Selective excitation fluorescence was used to verify these conclusions. The measurements were made in the nanosecond system but the laser system was different from that shown in Figure 4-2-1. Figure 5-1-11 shows the experimental set-up used specifically for the selective excitation fluorescence measurements.

A dye laser (Lumonics model EPD 330) is pumped by a pulsed excimer laser (Lumonics model TE-320-2). The output of the dye laser is frequency doubled by a BBO crystal (Second Harmonic Generation crystal). The frequency doubled pulses, with a duration of 5 nanosecond, can be tuned from 230nm to 330nm. This UV light is used as the excitation in the measurements. The fluorescence is measured by the detection system described in Section 4.2.

Figure 5-1-11: Experimental set-up for the selective excitation fluorescence measurements.

Figure 5-1-12 shows the results when the excitation wavelength was at 260nm. The solid line represents the results observed for H₂TMPyP without DNA, and the dashed line for H₂TMPyP/DNA complexes. The concentration of H₂TMPyP was 5.9×10^{-6} M for the two samples and the r(c) was 70.0 for the complexes.

Energy transfer DNA to bound drugs has been detected by fluorescence spectroscopy [89]. Recognizing the strong absorption of DNA in the UV region and the differences of the two spectra (Figure 5-1-12), show that energy transfer has taken place from DNA to H₂TMPyP.

Figure 5-1-13 shows the results of a similar fluorescence excitation experiment in which a sample with an r(c) value of 70.0 is excited at different UV wavelengths. The solid line represents fluorescence when the excitation is at 287nm; the dashed line when the excitation is at 260nm. From the ratio of the two peaks in the spectrum, it is found that (i) the externally bound H₂TMPyP are more effectively excited with excitation at 287nm; and (ii) the two types of bound H₂TMPyP are equally excited when the excitation is at 260nm.

It is known that the absorption band of base pairs of DNA is around 260nm. When the excitation is 260nm, both G-C base pairs and A-T base pairs are strongly excited. However the maximum of absorption for G-C pairs is at a slightly lower wavelength than 260nm and the maximum of A-T pairs at wavelength slightly higher than

260nm [89]. Thus, when the excitation is 287nm, A-T base pairs are more strongly excited than G-C base pairs.

The conclusions obtained from previous measurements [97,98] indicate that the intercalated H_2TMPyP with DNA is formed between H_2TMPyP and G-C base pairs of DNA and the externally bound H_2TMPyP with DNA is formed between H_2TMPyP and A-T base pairs of DNA.

The experimental results obtained from the selective excitation measurements show that when A-T base pairs are excited more efficiently than G-C base pairs, then energy transfer from DNA to externally bound H_2TMPyP is more efficient. These results mean that the externally bounded H_2TMPyP is more sensitive to A-T base pairs, which is in agreement with the previous conclusions.

Figure 5-1-12: Fluorescence spectra of H_2TMPyP (solid line) and H_2TMPyP/DNA complexes (dashed line), excitation at 260nm.

Figure 5-1-13: Fluorescence spectra of H_2TMPyP/DNA complexes, excitation at 260nm (dashed line) and 287nm (solid line).

Effects of Temperature

Time-resolved fluorescence were taken at different temperatures. The temperature of the sample was controlled by a water bath (HAAKE F3 2×T6,3 /DIN 61662). The results obtained by deconvolution from the experimental data are shown in Table 5-2.

Table 5-2: Temperature Dependent Contribution to Fluorescence by Intercalated and Externally Bounded H₂TMPyP of H₂TMPyP/DNA Complexes.

H ₂ TMPyP/DNA r(c)	Contribution (%)		Total Counts	Temperature °C
	Intercalated	External		
112.0	84	16	221669	20
	92	8	233238	50
45.0	81	18	343233	20
	90	9	386319	50
11.2	79	21	595303	20
	93	7	870680	50

Estimated error: in contribution 10%.

When the temperature rises, (i) the contribution by the intercalated bound H_2TMPyP increases; and (ii) the contribution by the externally bound H_2TMPyP decreases. The events can be explained by the mechanism proposed for drug binding to DNA.

Drug intercalation into DNA requires adjacent base pairs to move apart, thus allowing a drug molecule to enter the gap and sit between the two base pairs. When temperature is increased, the frequency of thermal motion of the base pairs increases giving rise to a higher probability for the conditions required for drug binding to occur.

External binding of a drug to DNA is primarily due to electrostatic interactions between the drug and the phosphate groups of DNA. Temperature does not significantly increase the binding affinity of such external binding. However increasing the temperature increases the probability of the de-binding processes, i.e. the bound drugs leave the DNA and become free drugs.

If these processes are present, then when temperature is increased, (i) the concentration of externally bound drugs decreases; and (ii) the free drugs and the intercalated bound drugs are dependent on the competition of the relevant binding and de-binding processes.

The present measurements show that when the temperature is increased from 20°C

to 50°, the concentration of intercalated bound H₂TMPyP is increased while that of the externally bound H₂TMPyP is decreased (Table 5-2). The fluorescence contribution by the free H₂TMPyP was too weak to be detected.

Anisotropic Fluorescence

Figure 5-1-14 shows an anisotropic fluorescence spectrum of H₂TMPyP. The concentration of the sample was $3.7 \times 10^{-5}\text{M}$, the excitation at 566nm and detection at 660nm. In Figure 5-1-14, $I_{\parallel}(t)$ represents the direction of fluorescence polarization parallel to the polarization direction of the excitation laser pulses; $I_{\perp}(t)$ represents the direction perpendicular to the polarization direction of the laser pulses; $r(t)$ is given by equation 2-4-1.

Figure 5-1-14: Time resolved anisotropic fluorescence spectra of H_2TMPyP in a buffer solution.

An assignment [36] has been proposed for absorption and fluorescence spectra of *tetraphenylporphyrin* (TPP). This assignment gives (i) The position of $Q_x(0,0)$ and $Q_x(1,0)$ at 647nm and 591nm; (ii) the position of $Q_y(0,0)$ and $Q_y(1,0)$ at 549nm and 515nm; (iii) the maxima in the fluorescence spectrum at 652nm and 719nm assigned to $Q(0,0)$ and $Q(0,1)$.

The structure of TPP (Figure 5-1-15) is similar to H_2TMPyP . Also the absorption and fluorescence spectra of TPP occur in the same spectral regions as that of H_2TMPyP . Therefore based on these similarities, it is assumed that the assignment for TPP is also valid for H_2TMPyP and it then is used to analyze the anisotropic fluorescence spectrum in this work.

Figure 5-1-15: Structure of tetraphenylporphyrin (TPP)

The anisotropic fluorescence spectra shown in Figure 5-1-14 were obtained with an excitation at 566nm and detection at 660nm. Translating the assignments for TPP to these spectra of H_2TMPyP indicates: (i) the fluorescence was excited with overlapping absorption bands composed of transitions between $Q_x(1,0) \leftarrow S_0$ and $Q_y(0,0) \leftarrow S_0$; and (ii) the emission of the fluorescence was $Q_x(0,0) \rightarrow S_0$.

The no motion anisotropy value ($r(0)$) is dependent on an angle between the absorption transition moment and the emission transition moment (equation 2-4-4). Thus, the absorption by the $Q_x(1,0)$ transition specifies that the angle is 0° and $r(0) = 0.4$; the absorption by $Q_y(0,0)$ specifies that the angle is 90° and $r(0) = -0.2$. Because of the overlapping of the absorption bands, $Q_x(1,0)$ and $Q_y(0,0)$, it is estimated that the value of $r(0)$ may be between -0.2 to 0.4. The measurements show that $r(0) = 0.1$ (Figure 5-1-15), this indicates that the two absorption bands make almost equal contribution to the fluorescence.

The rotational relaxation time of H_2TMPyP in a buffer solution is 300 ± 75 picosecond which corresponds to the diffusion of H_2TMPyP molecules in the buffer solution.

Anisotropic fluorescence spectra were measured for H_2TMPyP /DNA complexes with $r(c)$ value from 1.8 to 45.0. The results are shown in Table 5-3.

Table 5-3: No Motion Anisotropy Value of H₂TMPyP/DNA Complexes

H ₂ TMPyP r(c)	No Motion Anisotropy Value: r(0)	Temperature °C
1.8	0.10	20
	0.10	50
4.5	0.12	20
	0.12	50
5.4	0.11	20
	0.10	50
11.2	0.08	20
	0.10	50
45.0	0.08	20
	0.10	50
112.0	0.10	20
	0.11	50

The no motion anisotropy value, $r(0)$, is from 0.08 to 0.12, and this can be due to the overlap of the absorption bands.

The rotational relaxation function for H_2TMPyP/DNA complexes is complicated. In a solution with drug/DNA complexes, there are numerous processes which can contribute to the rotational relaxation processes [61, 62, 64, 65]. The relaxation decay times include (i) wobbling of the drug in the intercalation site at a region of 50 - 100 picosecond; (ii) torsional motion of DNA at times from 1 to 50 nanosecond; (iii) bending of DNA helix in a region of a microsecond. If fluorescence of the free drug can be detected, the diffusion relaxation of the free drugs will contribute to the relaxation function as well.

Not all of these decay times can be detected in an anisotropic fluorescence measurement. It is limited by the fluorescence life-time of the drug molecules and by the time resolution of the detection system.

For the rotational relaxation processes of $r(c) < 5.4$, two relaxation decay times were detected: (i) 300 picosecond and (ii) > 10 nanosecond (Figure 5-1-16). A comparison with the results obtained by H_2TMPyP without DNA (Figure 5-1-14), suggests that the 300 picosecond decay originates from the free H_2TMPyP . The other measured decay, > 10 nanosecond, originates from the torsional motion of DNA.

For $r(c) > 5.4$, there is only one measured decay time, > 10 nanosecond (Figure 5-1-17) which also originates from the torsional motion of DNA.

The fluorescence life-time of bound H_2TMPyP is 10.0 nanosecond and 2.0 nanoseconds. This is not sufficiently long to determine the accurate decay time of the torsional motion of DNA. It is also not possible to determine the decay time due to bending of DNA. Wobbling of H_2TMPyP could not be detected by the measurements, because the signal was too weak. The diffusion decay time of the free H_2TMPyP was detected at the higher drug concentration (Figure 5-1-16), the results agree with the isotropic time-resolved measurements (Table 5-1).

Figure 5-1-16: Time resolved anisotropic fluorescence spectra of H_2TMPyP/DNA complexes ($r(c) : 3.7$).

Figure 5-1-17: Time resolved anisotropic fluorescence spectra of H₂TMPyP/DNA complexes ($r(c) : 37.0$).

5.1.3 Anisotropic Absorption

Anisotropic absorption measurements of H_2TMPyP/DNA complexes are performed by the nanosecond system (Figure 4-2-1). By fitting the experimental data with a simulated curve (equation 2-3-14), one can get the angle between the pump transition moment and the probe transition moment.

The circular dichroism spectrum of H_2TMPyP/DNA complexes (Figure 5-1-2) shows a positive component around 423nm and a negative component around 437nm. This indicates that the Soret band of the externally bounded H_2TMPyP molecules is around 423nm (the positive signal in the circular dichroism spectrum) and Soret band of the intercalated bound H_2TMPyP molecules is around 437nm (the negative signal in the circular dichroism spectrum).

It is known that in free-base porphyrins two inner hydrogens bound to opposite nitrogen may jump from one pair of nitrogen to another. For symmetrical porphyrins, NH-tautomers are not distinguishable in structure and have identical electronic spectra [90]. The measurements of resonance Raman excitation profiles [103] show that that (i) Soret band (423nm) of the externally bound H_2TMPyP is composed of two bands (419nm and 427nm) and (ii) Soret band (437nm) of the intercalated bound H_2TMPyP is composed of two bands (434nm and 441nm). These results suggest that when H_2TMPyP is bound to DNA, (i) NH-tautomers of H_2TMPyP do not remain symmetrical; (ii) Soret band is

deferent for the two NH-tautomers, one is B_x and another one is B_y .

In the anisotropic absorption measurements, the four Soret bands (419nm, 427nm, 434nm, 441nm) are selected as the probe transition moments.

The Q_y band of H_2TMPyP is selected as the pump transition moment. The Q_y band is around 520nm in H_2TMPyP /DNA complexes which is contributed by the intercalated bound H_2TMPyP and the externally bound H_2TMPyP . The pump is tuned from 510nm to 530nm.

When the pump is at 519nm, the experimental results show that (i) the angle between the pump transition moment and the probe transition moment is 0° for the probe at 419nm, and (ii) the angle is 90° for the probe at 427nm (Figure 5-1-18). Because 419nm and 427nm correspond to the Soret band of externally bound H_2TMPyP molecules, these results indicate that (i) 519nm corresponds to the Q_y band of the externally bound H_2TMPyP molecules, (ii) 419nm is the B_y band of the externally bound H_2TMPyP molecules and (iii) 427nm is the B_x band of the externally bound H_2TMPyP molecules.

When the pump is at 525nm and the probe is at 434nm, the angle between the pump transition moment and the probe transition moment is 90° (Figure 5-1-19). This result indicates that (i) 525nm corresponds to the Q_y band of the intercalated H_2TMPyP

molecules, and (ii) 434nm corresponds to the B_x band of the intercalated H_2TMPyP molecules. This result implies that 441nm corresponds to the B_y band of the intercalated H_2TMPyP molecules.

Table 5-4 gives the assignments for those transition moments.

Figure 5-1-18: Anisotropic absorption measurements of H_2TMPyP/DNA complexes (pump at 519nm; probe at 419nm and 427nm), solid line represents theoretical curve.

Figure 5-1-19: Anisotropic absorption measurements of H₂TMPyP/DNA complexes (pump at 525nm; probe at 434nm), solid line represents theoretical curve.

Table 5-4: Assignments for Part of Bands of H₂TMPyP/DNA Complexes

Wavelength (nm)	Band	Component
419	B _y	Externally Bound H ₂ TMPyP
427	B _x	Externally Bound H ₂ TMPyP
434	B _x	Intercalated H ₂ TMPyP
441	B _y	Intercalated H ₂ TMPyP
519	Q _y	Externally Bound H ₂ TMPyP
525	Q _y	Intercalated H ₂ TMPyP

Figure 5-1-20 shows the results when the pump is at 519nm and the probe is at 434nm and 441nm. The results show that (i) the angle between Q_y of the externally bound H_2TMPyP molecules and B_y of the intercalated H_2TMPyP molecules is 60° , and (ii) the angle between Q_y of the externally bound H_2TMPyP molecules and B_x of the intercalated H_2TMPyP molecules is 40° .

Figure 5-1-21 shows the results when the pump is at 525nm and the probe is at 419nm and 427nm. The results indicate that (i) the angle between Q_y of the intercalated H_2TMPyP molecules and B_y of the externally bound H_2TMPyP molecules is 60° , and (ii) the angle between Q_y of the intercalated H_2TMPyP molecules and B_x of the externally bound H_2TMPyP molecules is 50° .

The relative orientation between the intercalated H_2TMPyP molecules and the externally bound H_2TMPyP molecules is shown in Table 5-5. If one assumes that the plane of the intercalated H_2TMPyP molecules is perpendicular to the helical axis of DNA, the simulated results show that the angle between the normal direction of the externally bound H_2TMPyP and the helical axis of DNA is $50^\circ \pm 10^\circ$.

Figure 5-1-20: Anisotropic absorption measurements of $\text{H}_2\text{TMPyP/DNA}$ complexes (pump at 519nm; probe at 434nm and 441nm), solid line represents theoretical curve.

Figure 5-1-21: Anisotropic absorption measurements of H_2TMPyP/DNA complexes (pump at 525nm; probe at 419nm and 427nm), solid line represents theoretical curve.

Table 5-5: Relative Orientation of H₂TMPyP Molecules of H₂TMPyP/DNA Complexes

	X	Y	Z
X'	90°	50° *	140°
Y'	40° *	60° *, 60° *	66°
Z'	120°	126°	50°

XYZ represent intercalated H₂TMPyP molecules; X'Y'Z' represent externally bound H₂TMPyP molecules.

The values with * are obtained from the measurements (Figure 5-1-21 and 5-1-22).

The values without * are obtained from calculation according normalized condition and orthogonal condition.

Estimated error: $\pm 5^\circ$ (the value obtained from the measurements) and 10° (the value obtained from calculation).

5.2 MX/DNA COMPLEXES

Absorption Spectrum

Figure 5-2-1 shows the measured absorption spectra of MX and MX/DNA complexes. The concentration of MX was $1.38 \times 10^{-5} \text{M}$ for the two samples; the concentration of DNA was $150.0 \mu\text{g/mL}$ ($r(c) = 30.0$) for the complexes. The absorption spectrum of MX has a peak at 596.4nm and a shoulder around 650nm. When DNA was added to the MX solutions, the peak shifted to 620nm and became slightly weaker; the shoulder separated as another peak with a higher intensity and shifted to 672nm. Similar results have been reported by other workers [154].

Fluorescence Spectrum

Figure 5-2-2 shows the fluorescence spectra for the same samples of MX and MX/DNA complexes used in the absorption measurements. The excitation was at 590.2nm, the detection was from 600nm to 800nm. The fluorescence of MX became about 3 times as strong and red-shifted 17nm when DNA was added to the solution. The contour of the emission was similar to that measured without DNA. The red-shift of the fluorescence has also been reported by other workers [155].

Figure 5-2-1: Absorption spectra of MX (dashed line) and MX/DNA complexes (solid line).

Figure 5-2-2: Fluorescence spectra of MX and MX/DNA complexes.

It has been shown that (i) MX is strongly intercalated bound to DNA [149,151,152,156]; (ii) no fluorescence was detected for DNA in a buffer solution using the above experimental conditions; (iii) fluorescence of MX increases by a factor of 3 when DNA is added. These results suggest that the fluorescence spectra of MX/DNA complexes primarily originates from an intercalated bound MX.

Time-resolved Fluorescence Spectrum

The isotropic time-resolved fluorescence measurements were performed on MX and MX/DNA complexes. The samples were the same as those used for the absorption measurements. The excitation was at 590.2nm, the detection was at 671.4nm.

The fluorescence decay of MX and MX/DNA are mono-exponential. The fluorescence life-time of MX in the buffer solution is determined to be 160 ± 50 picosecond (Figure 5-2-3). The fluorescence life-time of MX in MX/DNA complexes is 240 ± 50 picosecond corresponding to the intercalated bound MX (Figure 5-2-4), because the source of the fluorescence spectra of MX/DNA is primarily contributed by the intercalated bound MX. With intercalation to DNA, the life-time of the MX fluorescence became longer. A similar effect was observed in H_2TMPyP where the lifetime increased from 5.0 nanosecond to 10.0 nanosecond.

Figure 5-2-3: Fluorescence decay of MX in a buffer solution.

Figure 5-2-4: Fluorescence decay of MX/DNA complexes in a buffer solution.

The fluorescence spectra of MX/DNA complexes detected at different time windows are shown in Figure 5-2-5. Comparing the two spectra in Figure 5-2-5, it can be concluded that (i) only the one component, namely intercalated bound MX, was detected in the measurements; (ii) no contribution by the free MX to the fluorescence could be detected.

Figure 5-2-5: Time dependent fluorescence spectra of MX/DNA complexes ($r(c) : 30.0$):
excitation was at 590.2nm, detection were after the excitation 0 to 2
nanosecond and 480 picosecond to 2.5 nanosecond.

Anisotropic Fluorescence Spectrum

Figures 5-2-6 and 5-2-7 show the anisotropic fluorescence spectra of MX and MX/DNA complexes. The samples were the same as the earlier measurements. The excitation was at 590.2nm, the detection was at 671.4nm.

For MX without DNA (Figure 5-2-6), the measured rotational decay time is 300 ± 50 picosecond which corresponds to the diffusion of the MX in the buffer solution. The no motion anisotropy value is 0.4. Therefore the absorption transition moment (absorption at 590.2nm) is parallel to the emission transition moment (emission at 671.4nm).

For the MX/DNA complexes (Figure 5-2-7), a short decay time, 200 ± 50 picosecond was measured. Two kinds of components may contribute to this decay time, wobbling of the bound MX and the free MX. Because the fluorescence due to the free MX could not be detected in the experiments (Figure 5-2-4), the 200 picosecond decay corresponds to wobbling of the intercalated bound MX. Because the fluorescence lifetime of intercalated bound MX (240 picosecond) is too short, it is not possible to measure torsional motion of DNA and bending of DNA helix by MX/DNA complexes in this system. Through a comparison at the results shown in 5-2-6 and 5-2-7, it is found that, after the initial fast decay, (i) the signal of the rotational relaxation function of MX vanishes directly (becomes 0); but (ii) the relaxation function of MX/DNA does not go

to 0, the signal (after 1 nanosecond and > 0) is at least in part due to the motion of DNA.

Figure 5-2-6: Time resolved anisotropic fluorescence spectra of MX.

Figure 5-2-7: Time resolved anisotropic fluorescence spectra of MX/DNA.

6. SUMMARY AND CONCLUSION

Summary

A theory has been developed which images a new experimental method used to measure time-resolved anisotropic spectroscopy. Using this theory, the angle between any two transition moments in a molecular system can be determined by the following experimental procedures:

(i) tuning the pump and the probe lasers to the wavelengths related to those two transition moments;

(ii) measuring the transmission of the probe light when the pump light is on and the angle between the polarization directions of the pump beam and the probe beam changes from 0° to 90° ;

(iii) measuring the transmission of the probe light when the pump light is off under the same experimental conditions as (ii) and

(iv) fitting the experimental data to the formula developed through the new theoretical approach.

theoretical approach.

The influence of (i) the rotation of the molecules, (ii) the life-time of the molecules, (iii) probe light being absorbed by two transition moments and (iv) the strong pump intensities can also be determined by applying this theory to the experimental data.

Conclusion

Compared with the previous experimental methods [1,2], the new experimental method is more sensitive and covers a wider range of conditions for the determination of the structure of a molecular system. The previous methods can only be used to determine the angle between two transition moments at a weak pump intensity and even then the only parts of the angles between two transition moments can be determined. The new method covers all angles between any two transition moments and can be used with strong pump intensities, thus increasing the measurement sensitivities.

The proposed experimental method can be used to measure the rotational relaxation function of a molecular system. Two separate dye lasers are used as the pump source and the probe source; this experimental situation limits the time resolution, because it is difficult to reduce the jitter between the two dye lasers to a value below 8 picosecond [34].

Fluorescence measurements on two different drug/DNA complexes, H₂TMPyP/DNA and MX/DNA, show that when the drugs are bound to DNA, their fluorescence decay time and the fluorescence spectral shape are changed. It is found that large life-time differences exist for drugs intercalated and externally bound to DNA.

The fluorescence decay time of H₂TMPyP molecules in a buffer solution is determined to be 5.0 ± 0.5 nanosecond. For H₂TMPyP/DNA complexes in a buffer solution, (i) the fluorescence decay time of the intercalated H₂TMPyP molecules is found to be 10.0 ± 0.5 nanosecond, (ii) the fluorescence decay time of the externally bound H₂TMPyP molecules is found to be 2.0 ± 0.5 nanosecond and (iii) the fluorescence decay time of the free H₂TMPyP molecules is found to be 4.0 ± 0.5 nanosecond.

The fluorescence decay time of MX molecules in a buffer solution is 160 ± 50 picosecond. For MX/DNA complexes in a buffer solution, the fluorescence decay time of intercalated MX molecules is 240 ± 50 picosecond.

These measurements indicate that fluorescence spectroscopy can be used to study the binding mechanism of a drug to DNA and the fluorescence decay time of the drug molecules can be used as a spectroscopic characteristic by which the binding modes can be identified.

The establishment and / or acceptance of a spectroscopic characteristic for a

drug/DNA complex by fluorescence spectroscopy requires validation through comparison with other techniques. After the spectroscopic characteristic is built up, fluorescence spectroscopy could be used to identify the binding modes of a drug to DNA directly.

Using the fluorescence decay time as a spectroscopic characteristic, the measurements show that when the temperature rises (from 20°C to 50°C), the intercalated bound H₂TMPyP increases and the externally bound H₂TMPyP decreases for the H₂TMPyP/DNA complexes with an $r(c)$ number from 5.4 to 112.0.

Comparing with the other techniques, the advantages of fluorescence spectroscopy are:

(i) Fluorescence spectroscopy is more sensitive. In fluorescence spectra measurements, sufficient signal can be obtained from the samples with a concentration of H₂TMPyP $\approx 10^{-8}$ M; for time-resolved fluorescence measurements the limit of the concentration of H₂TMPyP is $\approx 10^{-6}$ M.

(ii) The operation of fluorescence spectroscopy is easier, faster and not destructive. It is also a routine well known in many laboratories and institutes.

Anisotropic absorption measurements of H₂TMPyP/DNA complexes show the relative orientation of the intercalated H₂TMPyP molecules and the externally bound

H₂TMPyP molecules (Table 5-5). If one assumes that the plane of the intercalated H₂TMPyP molecules is perpendicular to the helical axis of DNA, the results indicate that the angle between the normal direction of the externally bound H₂TMPyP molecules and the helical axis of DNA is $50^\circ \pm 10^\circ$.

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A resolution test chart featuring various patterns of vertical and horizontal lines. The patterns are arranged in a grid-like fashion. Each pattern is accompanied by a numerical value indicating its resolution. The values include 1.0, 1.1, 1.25, 1.4, 1.6, 1.8, 2.0, 2.2, 2.5, 2.8, 3.2, 3.6, 4.0, and 5.0. The patterns consist of groups of lines that become increasingly dense and difficult to distinguish as the numerical value increases.

MICROCOPY RESOLUTION TEST CHART
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STANDARD REFERENCE MATERIAL 1010a
(ANSI and ISO TEST CHART No. 2)

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APPENDIX 1

Program LIDE [26] is used to calculate $g(\alpha^u, \gamma, a)$ which is represented by equation 2-3-12.

```

C      JUNE 4, 1991
C      PROGRAM LIDE
C      EXTERNAL FUNC, Y1, Y2, Z1, Z2
C      DIMENSION GAMA(10), RAK(10), ALPH(10)
C      DATA RAK/0., 10., 20., 30., 40., 54.7, 60., 70.,
*          80., 90./
C      REAL LN, PI
C
C      FUNC, Y1, Y2, Z1, Z2 are used to do integration.
C      GAMA (GAM) is the angle between the pump and the probe.
C      ALPH (ALP) is the angle between the two transition moments
C      RANK (RAK) is variable used to do calculation.
C      RESULT is integration result.
C      PI is constant of 3.1415...
C
C      PI=3.1415926
C      DO 10 I=1,10
C          ALPH(I)=RAK(I)/180.*PI
C          ALP=ALPH(I)
C          WRITE(2,*) 'ALPH='
C          WRITE(2,*) RAK(I)
C      DO 20 J=1,10
C          GAMA(J)=RAK(J)/180.*PI
C          GAM=GAMA(J)
C          STA=0.0
C          END=1.0
C          CALL QUAD3D(STA, END, GAM, ALP, RESULT)
C
C      RESULT is integration result.
C
C          RESULT=3./8./PI*RESULT
C          WRITE(2,*) 'GAMA          RESULT'
C          WRITE(2,*) RAK(J),RESULT
C
C      20  CONTINUE
C      10  CONTINUE
C      END
C
C      SUBROUTINE QUAD3D(X1, X2, C, D, SS)
C      C:GAMA, D:ALPH, SS:RESULT
C      X1:START, X2.END for integration of X
C      EXTERNAL H
C      CALL QGAUSX(H, X1, X2, C, D, SS)

```

```

RETURN
END

```

```

C
C

```

```

FUNCTION F(ZZ, CC, DD)
called by QGAUSZ, calls FUNC.

```

```

C
C

```

```

EXTERNAL FUNC
COMMON /XYZ/ X,Y,Z
Z=ZZ
C=CC
D=DD
F=FUNC(X, Y, Z, C, D)
RETURN
END

```

```

C
C

```

```

FUNCTION G(YY, CC, DD)
called by QGAUSY, calls QGAUSZ.

```

```

C
C

```

```

EXTERNAL Z1, Z2, F
COMMON /XYZ/ X, Y, Z
Y=YY
C=CC
D=DD
CALL QGAUSZ (F, Z1(X, Y), Z2(X, Y), C, D, SS)
G=SS
RETURN
END

```

```

C
C

```

```

FUNCTION H(XX, CC, DD)
called by QGAUSX, calls QGAUSY.

```

```

C
C

```

```

EXTERNAL Y1, Y2, G
COMMON /XYZ/ X, Y, Z
X=XX
C=CC
D=DD
CALL QGAUSY(G, Y1(X), Y2(X), C, D, SS)
H=SS
RETURN
END

```

```

C

```

```

FUNCTION FUNC(X, Y, Z , C, D)
C
C
The 3-dimensional function to be integrated.

FUNC=(2.0*(COS(C)**2)*(COS(Z)**2)*(SIN(Z))+
*
(SIN(C)**2)*(SIN(Z)**3))/((SIN(D)**2)*
*
(SIN(Y)**2)*(SIN(Z)**2)-(1./2.)*(SIN(D*2.))
*
*(SIN(Z*2.))*(SIN(Y))+1.0+(COS(D)**2)
*
*(COS(Z)**2))

C
C

FUNCTION Y1(X)
      Y1=0.1
END

C
C

FUNCTION Y2(X)
      Y2=2.0*PI
END

C
C

FUNCTION Z1(X, Y)
      Z1=0.0
END

C
C

FUNCTION Z2(X, Y)
      Z2=PI
END

C
C

SUBROUTINE QGAUSX(FUNC, A, B, C, D, SS)
DIMENSION X(5), W(5)
DATA X/.1488743389, .4333953941, .6794095682,
*
      .8650633666, .9739065285/
DATA W/.2955242247, .2692667193, .2190863625,
*
      .1494513491, .0666713443/
XA=0.5*(B+A)
XR=0.5*(B-A)
SS=0
DO 11 J=1,5
      DX=XR*X(J)
      SS=SS+W(J)*(FUNC((XM+DX), C, D) +
*
      FUNC((XM-DX), C, D))

```

```

11      CONTINUE
        SS=XR*SS
        RETURN
        END

C
C
      SUBROUTINE QGAUSY(FUNC, A, B, C, D, SS)
      DIMENSION X(5), W(5)
      DATA X/.1488743389, .4333953941, .6794095682,
*           .8650633666, .9739065285/
      DATA W/.2955242247, .2692667193, .2190863625,
*           .1494513491, .0666713443/
      XA=0.5*(B+A)
      XR=0.5*(B-A)
      SS=0
      DO 12 J=1,5
          DX=XR*X(J)
          SS=SS+W(J)*(FUNC((XM+DX), C, D) +
*           FUNC((XM-DX), C, D))
12      CONTINUE
      SS=XR*SS
      RETURN
      END

C
C
      SUBROUTINE QGAUSZ(FUNC, A, B, C, D, SS)
      DIMENSION X(5), W(5)
      DATA X/.1488743389, .4333953941, .6794095682,
*           .8650633666, .9739065285/
      DATA W/.2955242247, .2692667193, .2190863625,
*           .1494513491, .0666713443/
      XA=0.5*(B+A)
      XR=0.5*(B-A)
      SS=0
      DO 13 J=1,5
          DX=XR*X(J)
          SS=SS+W(J)*(FUNC((XM+DX), C, D) +
*           FUNC((XM-DX), C, D))
13      CONTINUE
      SS=XR*SS
      RETURN
      END

```

END

1 8-0 7-9 4

FIN