PHOTOCHEMICAL PROPERTIES OF PHOTOALLERGENS AND THEIR DEPENDENCE ON HUMAN SERUM ALBUMIN

By

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Abstract

Photoallergic compounds cause an allergy when applied to the skin and the skin is then exposed to light. Photoallergy is connected with the ability of the allergen to bind to the skin. This thesis describes much basic photochemistry of several photoallergens and has explained any differences in that photochemistry when on surfaces and in the presence of H.S.A.

Overall it has shown there is not one photochemical property which can be held responsible for photoallergy but that it is probably due to a combination of properties. The ground state absorption spectra were all below 400 nm and the fluorescence energies and phosphorescence energies were similar for all the compounds. Although radicals were found in 6 of the 8 compounds and the other two were suspected to form radicals it is still not known whether these are the radicals causing photoallergy, the quantum yield required to cause photoallergy and how the photochemical binding of the compounds to H.S.A. effects the photoallergy mechanism. There maybe minor radicals which have not been observed in this thesis which cause the allergic response. It is strongly suspected from these results however that the mechanism is not the same for all compounds. It may be possible that some photoallergens cause photoallergy via a singlet oxygen mechanism whereas others proceed via a radical mechanism.

Fentichlor and bithionol have been shown to randomly dark bind to multiple sites on the H.S.A. Although it is clear that the compounds must bind with the skin, it is not clear if this binding must be dark binding or photochemical binding.

Keywords : Photoallergen, Photochemistry, Laser flash photolysis, Human serum albumin, Dark binding, Radical.

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1.Introduction

1.1. Photochemical principles

Photochemistry is the study of the chemical changes which occur due to the interaction of visible or Ultra Violet light with a molecule. Light is a form of electromagnetic radiation and can be described using both the wave theory of electromagnetic radiation and particle theory. Electromagnetic radiation consists of an oscillating electric field and an oscillating magnetic field operating in planes perpendicular to each other and to the direction of propagation. The wavelength and the velocity of light are related by equation 1.1.[1,2]

$$c = \nu\lambda \qquad (1.1)$$

Where, λ = wavelength, the distance travelled during a complete cycle, (Length).

ν = frequency, which is the number of cycles per second, ($Time^{-1}$).

c = the speed of light in a vacuum, ($Length\ Time^{-1}$).

1.1.1. Regions of the electromagnetic spectrum

The electromagnetic spectrum was classically thought to consist only of the region which could be seen unaided by the eye. The spectrum is now known to consist of all the frequencies from $3x10^6$ Hz to $3x10^{20}$ Hz. The electromagnetic spectrum can be split up into definite regions as listed below.

1) Radiofrequency region:- $3x10^6$ - $3x10^{10}$ Hz, this is the region studied in Nuclear magnetic resonance spectroscopy.
2) Microwave region:- $3x10^{10}$ - $3\ x10^{12}$ Hz, this is the region of rotational spectroscopy and is the region studied in electron spin resonance spectroscopy.
3) Infra red region:- $3x10^{12}$ - $3x10^{14}$ Hz, this is the region of molecular vibration spectra.
4) Visible and Ultra Violet (UV) region:- $3x10^{14}$ - $3x10^{16}$ Hz, this is the region of electronic spectroscopy due to outer electrons. This can detected by the eye or by the use of a photomultiplier.
5) X-ray region:- $3x10^{16}$ - $3x10^{18}$ Hz, this is the region of energy changes due to the inner electrons of an atom or a molecule.

6) γ-ray region:- $3x10^{18}$ - $3x10^{20}$ Hz, this is the region of energy changes due to the rearrangement of the nuclear particles within an atom.

The region most often studied by photochemists is the UV/Visible region where the electronic transitions occur.[3]

1.1.2. Quantisation of energy

In 1900 Max Planck proposed the idea of distinct energy levels. This showed the energy of the molecule could only be changed by a jump in energy between these levels shown in figure 1.1. The electromagnetic radiation can therefore only be emitted, or absorbed in discrete quanta of radiation called photons.

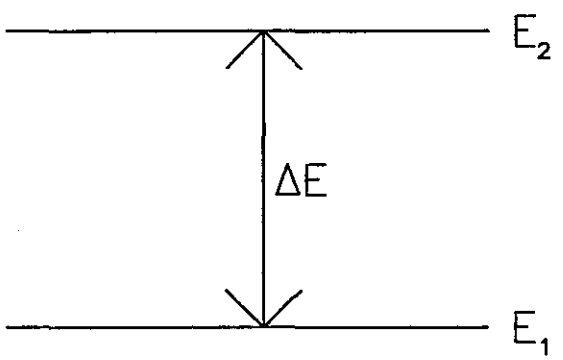


Figure 1.1. - Two energy states of a system of a molecule.

Grottus and Draper formulated a law of photochemistry which stated that only the light absorbed by a molecule could produce photochemical change in the molecule. Stark and Einstein later said that if a species absorbs radiation then one particle is excited for each quantum of radiation absorbed.[1] The energy of excitation is the same as the energy difference between the different energy levels which is given by the Planck relation in equation 1.2.

$$\Delta E = Nh\nu \qquad (1.2)$$

Where, h = Planck's constant, $6.63x10^{-34}$, (Energy Time).

N = Avagadro's number, $6.02x10^{23}$, (mol^{-1}).

ΔE = Energy difference between two different energy levels, (KiloJoules).

This equation refers to the primary photochemical process of a species. These processes may induce chain reactions consuming many further molecules of the starting material without the need for more light.[2]

1.1.2.1. Types of transition

The Highest Occupied Molecular Orbitals (HOMO) are usually bonding, π and σ, or nonbonding, n. The absorption of radiation may cause promotion of an electron into the Lowest Unoccupied Molecular Orbital (LUMO) which is usually an antibonding π^* or σ^* orbital

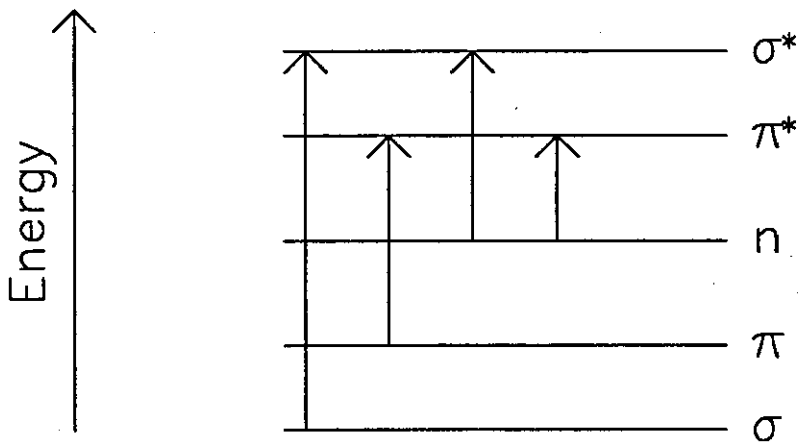


Figure 1.2. - Energy level diagram showing the most common electronic transitions.

1.1.3. Electronic structure of atoms

Schrodinger's wave equation gives three results which define the electronic state of a atom. These solutions are called the quantum numbers and consist of n, the principal quantum number, l, the Azimuthal quantum number and m_l the magnetic quantum number. The principal quantum number gives the position of the electron relative to the nucleus and has integer values greater than zero. The Azimuthal quantum number is related to the electron orbital angular momentum and takes values 0,1,2,....n-1. The magnetic quantum number, m_l takes values l, l-1, l-2,....-l. [1]

There is a fourth quantum number which is found from Dirac's equation called the magnetic spin quantum number represented by m_s which takes values of $+^1/_2$ and $-^1/_2$. These values represent the two spin states of the electrons, usually considered as the spin up and spin down configurations. In 1924 Wolfgang Pauli said that no two

electrons in an atom should possess the same set of quantum numbers n, l, m_l and m_s. This restricts the number of electrons to two per orbital, these electrons are spin paired.

1.1.4. Molecular orbitals (MO)

The electronic structure of molecules is best understood using Molecular orbitals. Molecular orbitals are formed as linear combinations of the valence shell atomic orbitals.

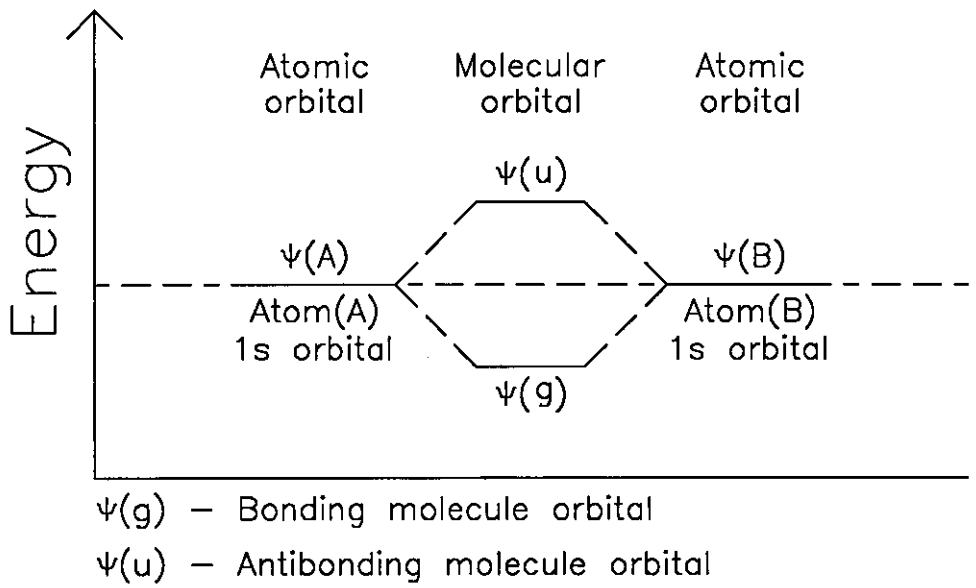


Figure 1.3. - Energy levels of s-s atomic and molecular orbitals.

One MO is a bonding orbital and the other is an antibonding orbital. The bonding MO is more stable than the initial Atomic Orbitals (AO) and the antibonding MO are of higher energy than the initial AO and therefore less stable. The electrons from the AO's occupy the lowest energy bonding molecular orbitals before the antibonding MO therefore gaining stability compared to the atomic orbitals. However Hund's rule stated that electrons must occupy different orbitals when energetically possible and 2 electrons occupying degenerate orbitals will have parallel spins in the low energy state. Three types of molecular orbitals may be distinguished, σ and π bonding, σ^* and π^* antibonding and nonbonding orbitals, n. The quantum numbers for molecular orbitals are calculated from the vector sum of the atomic quantum numbers of the electrons in the valence shell.

The total spin angular momentum, S, for the molecule is used to calculate the spin multiplicity of the molecule by the equation 1.3.

$$\text{Spin multiplicity} = 2S+1 \qquad (1.3)$$

For example if the excited state has 2 unpaired electrons with the same spin, S = 1, the excited state is a triplet state and if they have a different spin, S = 0, the excited state is a singlet state. The singlet and triplet states have different physical and chemical properties. The triplet state has a lower energy than the singlet state due to the repulsive nature of the spin-spin interaction between electrons of the same spin.[4]

1.1.4.1. Selection rules for transitions

There are several main selection rules for electronic transitions which decide whether the transition is allowed or forbidden.

1) $\Delta S = 0$ for allowed transitions that means there should be no change in the spin during the transition.
2) $\Delta L = 0, \pm 1$ for diatomic and linear molecules, where $\Delta l = \pm 1$ for the electron.
3) There should be good spatial overlap between the orbitals of the molecule involved in the transition.[1]

There are transitions which are allowed even though there appears to be a change in spin during the transition, this is due to spin orbit coupling. This is where the spin and orbital angular momentum are combined, blurring the distinction between the singlet and triplet states, so causing each state to take a little of the other's character. This effect is more significant with heavier atoms

1.1.4.2. Franck Condon principle

The electronic transitions occur rapidly compared to nuclear motion, therefore the nuclei remain essentially frozen in their ground state configuration during the transition and vice versa. The excited state is likely to have different structural properties to the ground state, therefore a change in the nuclear configuration must occur after the transition has taken place. The Franck Condon principle states that electronic transitions are most favourable where the nuclear geometries of the initial and final

states are similar. The most probable transition takes place from the position of highest Franck Condon probability, represented as the centre of the vibrational level on the potential energy diagram. In a molecule where the nuclear geometry of the ground state and excited state are similar, the most probable transition is from v"=0 to v'=0 levels as seen in figure 1.4a. If the molecule has a very different equilibrium geometry in the excited state to the ground state, the most probable transition would be from v"=0 to v'>0 levels, possibly due to excitation into the antibonding state. In complex molecules there will be rotational quanta coupled to the vibrational spectra and the excitation would be to several different vibrational levels so giving broad absorption spectra.[1]

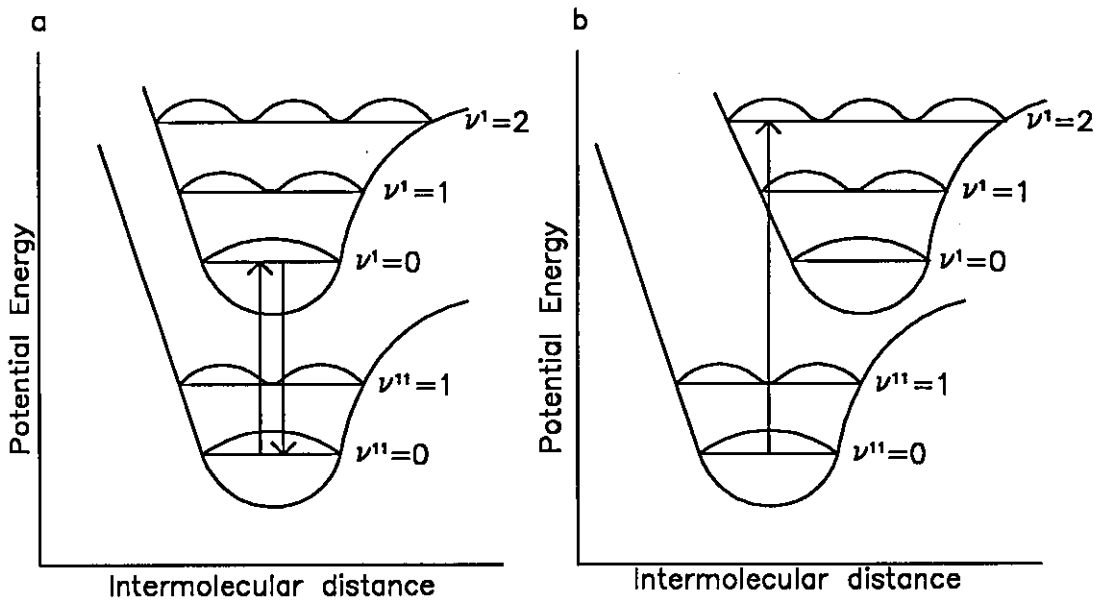


Figure 1.4. - Franck Condon transitions for a) similar ground state and excited state geometry, b) different ground state and excited state geometry.

1.1.5. Absorption of radiation

When a photon passes close to a molecule there is an interaction between the electric field from the molecule and the electric field of the radiation. This causes the photon to be absorbed by the molecule so changing the electronic structure. In terms of molecular orbitals this means a change in the occupation patterns within the orbitals, which is the same set of orbitals in the excited state as in the ground state. This is a one electron excitation. The energy of the photon may not exactly match the energy difference between lowest vibrational levels of the ground and excited electronic states

of the absorbing molecules. This is because the initially produced electronic state is an upper vibrational state and will later lose energy and relax back down to the lowest vibrational state. The electrons in the ground state of a diamagnetic are spin paired and therefore singlet. On excitation the spin of the electrons can be can preserved to produce an excited singlet state or inverted to produce an excited triplet state. The absorption of a monochromatic beam of light by a molecule at a given frequency is related to its concentration and the pathlength of the solution through the Beer Lambert law (1.4).

$$\log_{10} \frac{I_0}{I} = \varepsilon c l \qquad (1.4)$$

where, I_0 = Incident light intensity.

I = Transmitted light intensity.

c = Concentration, (mol Length^{-3}).

l = Pathlength of the light through the solution, (Length).

ε = The decadic molar absorption coefficient which characterises the probability of an interaction for the molecule, (mol^{-1} Length^2).

The function $\log_{10} I_0/I$ is called the absorbance or the optical density of the solution. [4,5]

1.1.6. Deactivation of electronically excited molecules

After absorption of electromagnetic radiation the electronically excited molecule is energetically unstable with respect to its ground state. The molecule must either lose its excitation energy, rearrange or fragment for the molecule to return to a more stable conformation.

There are three methods for a molecule to lose its excitation energy

1) Radiative processes- This is the emission of electromagnetic radiation by excited molecules as they return to the ground state.
2) Non radiative processes- This is where the population of one excited quantum state is transferred into another without any accompanying emission.

3) Quenching process- This is the bi or termolecular quenching of an excited molecule by the transfer of energy from the excited molecule to the molecules which collide with it.

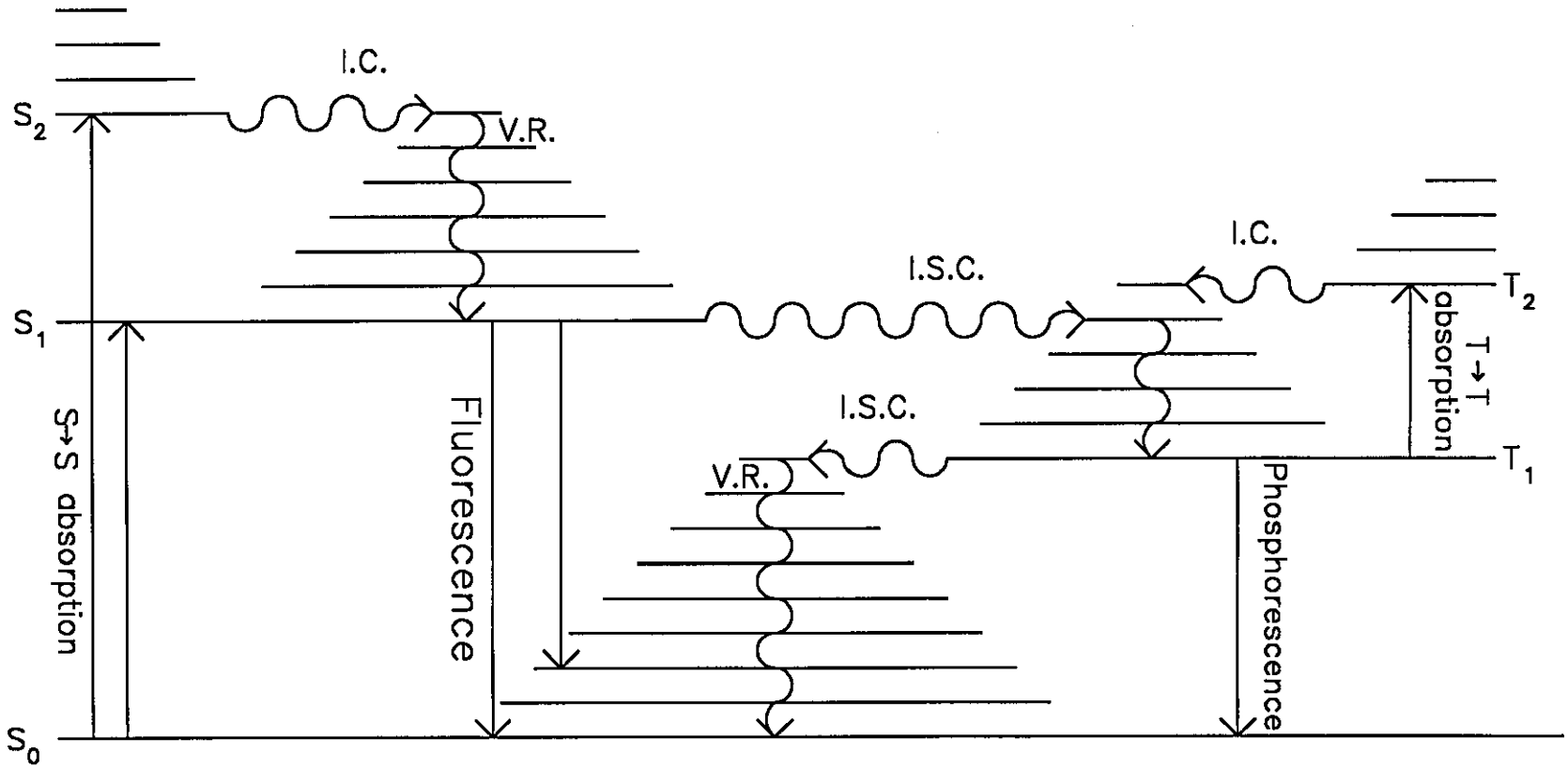
1.1.6.1. Radiative transitions

There are two different types of spontaneous radiative transition fluorescence and phosphorescence.

Fluorescence is the emission from an excited state of the same spin multiplicity as the lower state in the transition. There are two types of fluorescence

1) Prompt fluorescence, where excitation to the excited singlet state is followed by the immediate emission of radiation to the ground state. This almost always occurs from the lowest vibrational excited singlet state, as other processes such as internal conversion compete favourably to lower the vibrational level. This means that the fluorescence is independent of the exciting wavelength. This transition is strongly spin allowed, and therefore is a relatively fast transition, with typical time scales of 10^{-10} s to 10^{-6} s. It is characterised by first order exponential kinetics. Fluorescence is similar to absorption in that it essentially occurs before changes in the nuclear configuration have time to take place. Emission occurs from $\nu' = 0$ level, therefore emission intensity according to Franck Condon is greatest from 0-0 transition but it can also go to higher vibrational levels. If the vibrational level spacings are similar in both the excited and ground states the emission spectrum will be a mirror image of the absorption spectrum.

2) Delayed fluorescence is a longer lived emission from excited states of the same multiplicity and can occur by two mechanisms. The first mechanism occurs due to the formation of the singlet excited state from two excited triplet states. This is called p-type delayed fluorescence and depends on the accumulation of a high triplet state concentration. The other type of delayed fluorescence is called e-type delayed fluorescence. This is where thermally activated reverse intersystem crossing occurs from vibrationally excited levels of the T_1 state.[2]



I.S.C. – Intersystem crossing
I.C. – Internal conversion
V.R. – Vibrational relaxation

Figure 1.5 – A Jablonski diagram showing the different transitions which a molecule may undergo.

Phosphorescence occurs when the spin multiplicity of the excited state differs from that of the lower state. This emission is usually seen from the lowest excited triplet state to the singlet ground state. Phosphorescence is spin forbidden ($\Delta S = 1$) but occurs due to spin orbit coupling which makes it weakly allowed. This means that the transitions are longer lived with typical lifetimes of 10^{-3}s - 10s . The phosphorescence emission is always at higher wavelengths than the fluorescence emission. It is very susceptible to quenching collisions with impurities in the solvent and in very pure solvents bimolecular depopulation, due to its long radiative lifetime.[6] This means that experiments are usually performed in rigid organic glasses to reduce the diffusion of the quencher and at low temperatures to reduce the rate of quenching.[7,8]

Heavy atom effect

In cases where the fluorescence is strong the phosphorescence will be lower, it is therefore necessary to increase the phosphorescence by increasing the intersystem crossing. In light atoms there can be some interaction between spin angular momentum and orbital angular momenta of an electron. This means transitions between states of different spin multiplicity's can occur with a small probability. This interaction is known as spin orbital coupling. When a molecule has heavy atoms present strong interactions occur between the spin angular momentum and the orbit angular momentum of the electron . This is called the internal heavy atom effect. The external heavy atom effect occurs when a heavy atom is added to the molecule to form a weak transient complex. This means that the triplet state acquires some singlet character and the singlet state acquires some triplet character. This condition favours a high probability of intersystem crossing from the lowest excited singlet state to the lowest excited triplet state in a molecule.[9]

1.1.6.2. Non radiative transitions

This is the conversion of one molecular quantum state to another without the emission of radiation. This is most probable in molecules with a small energy gap between the different states.

There are two types of non radiative transitions:-

1) Internal conversions - This is the transfer of the population between electronic states of the same spin multiplicity. The rate of internal conversion is dependent on the separation between the two states, the larger the energy gap the slower the transition and vice versa due to the Franck Condon principle.

2) Inter system crossing- This is due to the transfer of the population between electronic states of different multiplicity, which is allowed due to spin orbit coupling. Inter system crossing often occurs from the S_1 to T_1 states as the triplet state is at lower energy than the singlet state. This produces a T_1 state with excess vibrational energy which must be lost before phosphorescence can occur. T_1 - S_1 intersystem crossing can occur where the energy difference between T_1 and S_1 is very small, which is the origin of e type delayed fluorescence.

1.1.6.3. Vibrational relaxation

Some electronically excited states produced following intersystem crossing or internal conversion have a large vibrational energy. In solution or in the gas phase at high pressure, collisions between the excited molecules and solvent molecules rapidly remove the excess vibrational excitation, converting it into heat, vibrational, rotational and translational excitation or if the collisions are infrequent, vibrational de-excitation may be achieved by emission of I.R. radiation with a frequency which equals the energy gaps between vibrational levels.[10]

1.1.6.4. Quantum Yields

The quantum yield of a photochemical reaction is defined as

$$\phi_B = \frac{No.\ of\ molecules\ under\ going\ a\ process\ per\ unit\ volume\ per\ unit\ time}{No.\ of\ quanta\ of\ light\ absorbed\ by\ molecules\ per\ unit\ volume\ per\ unit\ time} \qquad (1.5)$$

This also applies to the quantum yields of fluorescence , phosphorescence, product formation etc. The quantum yields should be measured in the same solvent or it should be corrected for the difference in the refractive indices of the two solvents. The sum of

the quantum yields for all primary processes, including deactivation, going from one energy level to another is unity. Quantum yields greater than 1 may be seen, for example where the photochemical process initiates a radical chain reaction.[1]

1.1.7. Energy Transfer

This is the transfer of energy from the singlet or triplet state of one molecule to excite another molecule into the excited singlet or triplet state. This reaction is shown for two molecules A and D in equation 1.6.

$$D^* + A \rightarrow D + A^* \qquad (1.6)$$

For this reaction to occur the energy level of A* must be lower than D*. There are 3 types of energy transfer which are classed as either radiative or non radiative energy transfer.

1.1.7.1. Radiative

This is where the emission from D* is reabsorbed by A

$$D^* \rightarrow D + h\nu \qquad A + h\nu \rightarrow A^* \qquad (1.7)$$

This requires the emission spectrum of D* to overlap the absorption spectrum of A. The efficiency of the process is dependent on the extent of the overlap.

1.1.7.2. Non- radiative

This involves a specific interaction of D* and A. There are two types of non radiative energy transfer

1) Coulombic - This is due to long range dipole-dipole interactions causing perturbations of the electronic structures of the energy donor and acceptor. The dipole perturbations of the donor molecule will cause the acceptor molecule to oscillate leading to the excitation of an electron into the lowest unoccupied molecular orbital of the acceptor with a corresponding deactivation of the excited electron in the donor molecule.

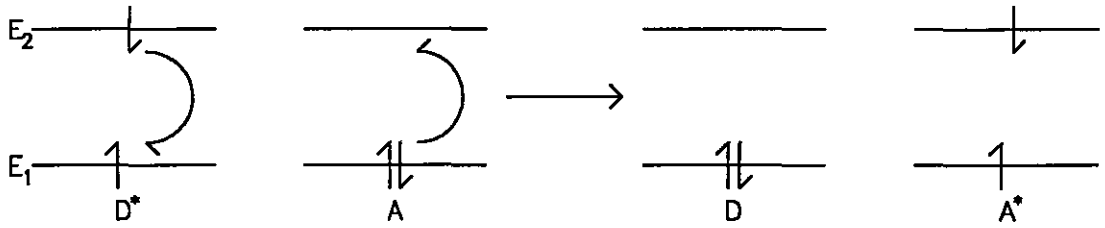


Figure 1.6. - Energy level diagram showing the electron movement in long range Coulombic energy transfer.

2) Electron-exchange energy transfer - This is where the excited electron from the donor molecule transfers into the lowest unoccupied molecular orbital of the acceptor molecule. Simultaneously an electron from the highest occupied molecular orbital of the acceptor molecule is transferred to the corresponding orbital of the donor molecule. This requires close contact between the excited state donor and the ground state acceptor molecules so their electron orbitals overlap. The energy transfer is more efficient when the distance between the donor and acceptor electronic energy levels is small.

The most common example of this is triplet - triplet energy transfer which occurs via electron exchange due to the close approach of the donor and acceptor molecules. This process can not occur via coulombic energy transfer due to the spin forbidden nature of the process.

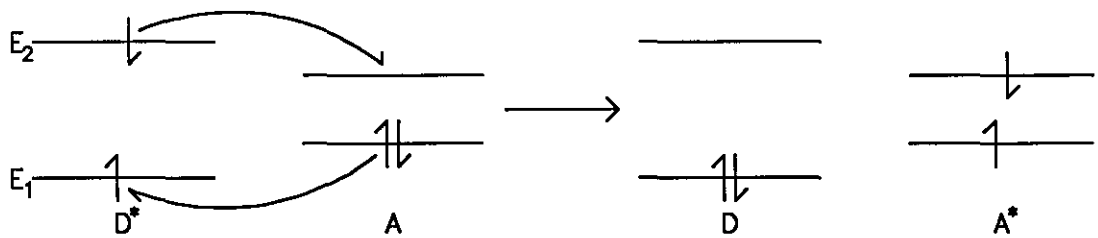


Figure 1.7. - Energy level diagram showing electron exchange energy transfer.

1.2. Flash Photolysis

In 1949 Porter and Norrish developed a technique called flash photolysis. This was where an extremely high intensity light was passed into an absorbing system forming a high concentration of intermediates. These intermediates could then be studied using spectroscopic techniques such as the absorption of light, allowing the

mechanisms of reactions to be followed. This technique allowed the identification of free radicals and triplet species and elucidation of the primary photochemical processes of different compounds. The initial sources used for this work were white light sources such as gas discharge lamps which had a flash duration of 10^{-6} s. In 1970 Porter and others started using Q switched lasers as the excitation source with flash lengths of 10^{-9} s allowing shorter lived intermediates to be studied.

1.3. LASER

LASER is an acronym for Light Amplification by the Stimulated Emission of Radiation.

1.3.1. Stimulated emission

A sample can emit energy spontaneously or as the result of a stimulus acting on the molecule, this is called stimulated emission. In a normal situation the total rate of absorption will be balanced by the rates of both spontaneous and stimulated emission as shown in figure 1.8.

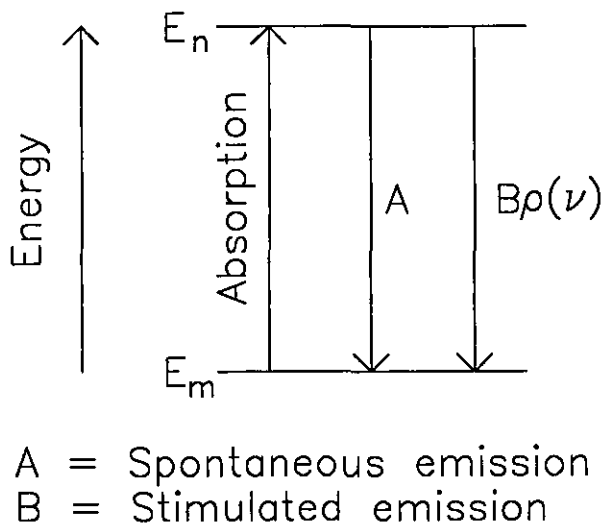


Figure 1.8. - Energy level diagram showing the different types of emission.

The relationship between absorption and the two types of emission is shown by equation 1.8.

$$B_{mn}N_m\rho_\nu = A_{nm}N_n + B_{nm}N_n\rho_\nu \qquad (1.8)$$

Where, N_m,N_n are the populations of two energy state E_m, E_n.

A and B are the Einstein coefficients for spontaneous and stimulated emission respectively.

ρ_ν = density of radiation of energy, $h\nu$

For stimulated emission to occur the energy of the light directed into the system must have a photon energy that exactly matches the gap between the excited state and the lower energy state. The light emitted will be of the same frequency as the supplied radiation. The stimulated emission will occur in the direction of the stimulating beam, unlike spontaneous emission which is random. This is then amplified in intensity. The radiation emitted by stimulated emission has three important qualities -

1) The beam will be highly collimated with low divergence properties.

2) The radiation will be coherent i.e. the radiation is in phase with the stimulating emission in both time and space.

3) The radiation will be monochromatic i.e. one frequency, which will be the same as the frequency of the stimulating emission.[3]

1.3.2. Population inversion

Stimulated emission is unlikely to happen naturally as there are few molecules in the excited state due to a bias towards the ground state as described by the Boltzmann law. Therefore the chance of the photon encountering an excited molecule is low. It is therefore necessary to achieve a population inversion to produce a laser. This can be achieved using an intense excitation source such as a flash lamp or another laser. This process of exciting is called pumping and the excitation source is called the pump. The

medium in which the population inversion occurs is called the active medium which can be gas, liquid or solid and depends on the type of output required. Each substance has its own set of energy levels so the frequency of the light emitted depends on which levels are excited. The active medium in the laser needs to be one where the rate of spontaneous emission is slow. The population inversion is both hard to achieve and to maintain. In Neodymium : Yttrium Aluminium Garnet (Nd:YAG) lasers this is achieved by using a four level laser system. The laser transition involves the Nd ions in the host lattice Yttrium aluminium garnet.

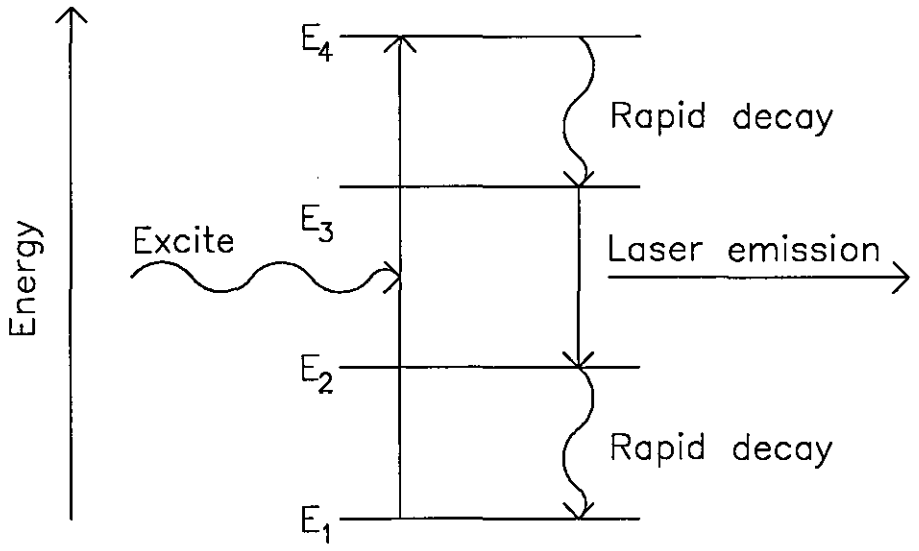


Figure 1.9. - A diagram showing the energy levels in a 4-level laser.

The population inversion is maintained in the laser in figure 1.9 as the E_2 state decays rapidly to the ground state and the population of the ground state is not important.

1.3.3. Amplification of the stimulated emission

A single pass of photons through a medium does not usually produce enough stimulated emission for a useful laser, therefore the intensity needs to be amplified by passing the beam through the active medium several times. This is achieved by placing mirrors at each end of the laser medium, so the light emitted perpendicular to the mirrors is trapped and will bounce back and forth indefinitely. Any photons which are spontaneously emitted in random directions will not be reflected and therefore will pass out of the medium. These mirrors are called the resonators and are usually planar and perfectly parallel. The use of confocal concave mirrors allows slightly off axis beams to

be reflected as well as the perpendicular beams. The beam is emitted by making one of the mirrors partially transmitting. The total amplification can be enhanced by increasing the path length over which the light beam interacts with the medium or by increasing the number of times the light beam passes through the medium. The region where the beam is amplified is called the optical cavity.

1.2.4. Frequency conversion

The fundamental wavelength from a Nd:YAG laser is at 1064 nm and for most photochemical studies it is necessary to change this to a more useful wavelength. This is achieved using frequency doubling and mixing crystals

This process give an output which is twice the frequency of the original i.e. half the wavelength at 532 nm. The crystals used to frequency double to 532 nm are either KDP (KH_2PO_4) or Lithium Niobate (Li_3NBO_4). The third and fourth harmonics at 355 nm and 266 nm can also be achieved by this process..

1.2.5. Pulsing techniques

The laser output can be a continuous wave but it is often desirable to have a pulsed laser beam. This effect can be achieved by a process known as Q-switching. Q-switching is where the rate of energy loss from the laser cavity is suddenly reduced. The idea of Q switching is that a large population inversion is achieved before a high intensity beam is emitted in a shutter type action. With the Q-switch the Q factor is first decreased then increased, where the Q-factor is defined as

$$Q = \frac{2\pi \; x \; energy \; stored \; in \; the \; cavity}{energy \; loss \; per \; optical \; cycle} \qquad (1.9)$$

The shutter is based on an electro-optical principle using a Pockel cell. A Pockel cell is a cell of crystalline material (usually potassium dihydrogen phosphate) which shows a proportional change in the refractive index on applying an electric field, i.e. the Pockels effect. With the potential difference applied there is a 90° rotation of the polarisation plane of the light in the Pockel cell, causing a shutter effect. This allows a high population inversion to be achieved in the laser material. When the voltage is removed the polarisation returns to normal and a high intensity beam can be emitted. This

usually on a nanosecond scale.[9,11] Picosecond pulses can be achieved using a technique called mode locking. This is where all the light waves in the laser cavity are constrained to show a phase relationship i.e. all the same phase at 1 time. This causes intervals where maxima of the waves will coincide in time so the light output will be a series of evenly spaced short pulses.

1.3 Diffuse reflectance

The reflection of light from a surface consists of two components

1) Specular reflection - a mirror like reflection which takes place at the surface of the sample.

2) Diffuse reflection - this is due to light penetrating into the interior of the sample, where some of the light is absorbed. This light is then scattered many times at the boundaries of the particles making up the sample, before returning to the surface. This reflection mainly occurs from matt or dull surfaces.[12]

The proportion of specular and diffuse reflection depends on the nature of the reflecting material.[13]

1.31. Kubelka Munk theory

Kubelka and Munk[14,15] devised a theory to connect the diffuse reflectance with the concentration of absorbers in optically thick solid samples. This theory makes the following assumptions

1) The sample contains randomly distributed, uniformly absorbing and scattering particles, which are a lot smaller than a layer thickness.

2) The sample is irradiated with monochromatic light.

3) The layer has an infinitely lateral extension, i.e. no edge effects.

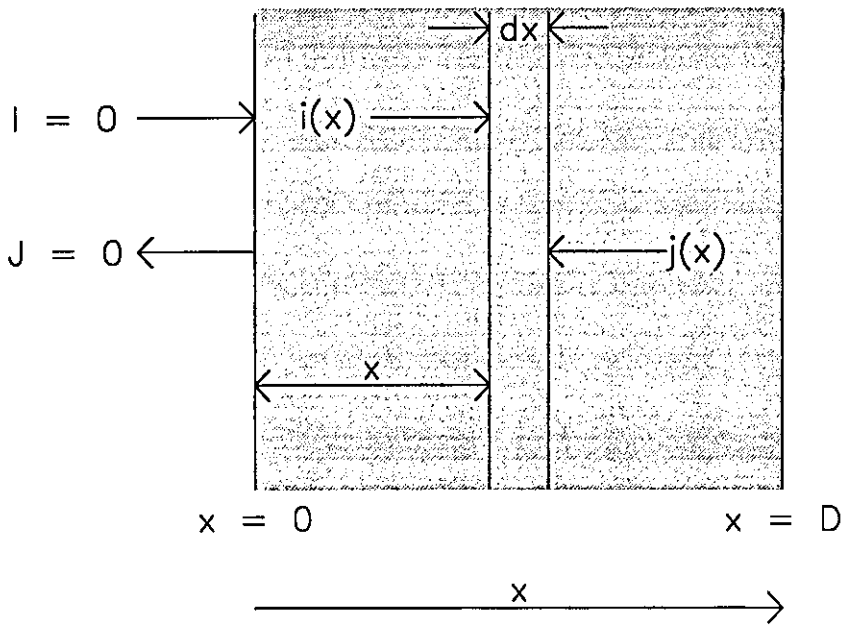


Figure 1.10: The absorption and scattering of light through a layer of particles

I(0) is the light entering the sample and J(0) is the light reflected from the sample. The decrease in the intensity of I in the direction +x will fall off exponentially with the depth of penetration into the sample.

The light entering the sample can be expressed using equation 1.10

$$dI(x) = \{-I(x)(K+S)dx\}+\{J(x)Sdx\} \qquad (1.10)$$

Where K = Absorption coefficient

S = Scattering coefficient

The generated flux has the opposite sign due to the direction of the reflected light being regarded as travelling in the -x direction, therefore,

$$dJ(x) = \{J(x)(K+S)dx\}-\{I(x)Sdx\} \qquad (1.11)$$

The reflectance (R) of the sample can be calculated using equation 1.12.

$$R = \frac{J(0)}{I(0)} \qquad (1.12)$$

If the sample is an ideal diffuser then the radiation has the same intensity in all directions. The above equations can be solved for the entire thickness of an infinitely thick sample, where the transmittance will be zero and the reflectance will be equal to R_∞.

$$J(x) = RI(x) = RI_0 \exp\left\{-\left(\frac{(K^2 + 2KS)^{\frac{1}{2}}}{S}\right)Sx\right\} \qquad (1.13)$$

Therefore, from the equations 1.13 for an infinitely thick sample, the Kubelka Munk equation is stated in 1.14.

$$F(R_\infty) = \frac{(1 - R_\infty)^2}{2R_\infty} = \frac{K}{S} \qquad (1.14)$$

Where $F(R_\infty)$ = remission function, which is directly proportional to the concentration of the absorber[16]

As K can also be defined using equation 1.15.

$$K = \omega\varepsilon C \qquad (1.15)$$

where ε = extinction coefficient of absorbing species

C = concentration of absorbing species

ω = geometry factor which allows for the angular distribution of diffusely reflected light.

The value for ω for an ideal diffuser is 2, therefore the remission function can be defined according to equation 1.16.

$$F(R_\infty) = \frac{2\varepsilon C}{S} \qquad (1.16)$$

$F(R_\infty)$ is dependent of wavelength, therefore F(R) against wavelength will give the ground state diffuse reflectance spectrum.[17] The ground state reflectance spectrum is usually measured against a total reflector (white) where K=0 and R is taken to be 1 with actual values being usually 0.99 or 0.98. The reflectance will be found from the ratio of the sample reflectance against the standard reflectance as shown in equation 1.17.

$$\frac{R_{\infty SAMPLE}}{R_{\infty STANDARD}} = r_\infty \qquad (1.17)$$

If the value of $R_{\infty STANDARD} = 1$ then the remission function can be written as shown in equation 1.18.

$$F(R) = \frac{(1-r_\infty)^2}{2r_\infty} = \frac{K}{S} \qquad (1.18)$$

The remission function is directly proportional to the concentration giving a relationship for diffuse reflectance which is comparable to the Beer Lambert law for solutions. Strongly absorbing samples will show a deviation from the Kubelka Munk theory because there is incomplete diffusion in scattering processes.

1.3.2. Diffuse Reflectance Laser Flash Photolysis (D.R.L.F.P.)

In the early 1980's it was discovered that opaque and highly scattering systems could be studied by observing the changes in the diffusely reflected analysing light following laser excitation.[16] An apparatus was developed to allow measurements to be made on highly scattering powders[18] and microcrystalline materials[19] which is now available for both nanosecond and picosecond measurements.[20] D.R.L.F.P. allows the photoexcitation of a homogeneously distributed ground state to give a transient or permanent change in the absorption of the compound. This change occurs just below the irradiated surface. For DRLFP the parameter measured is $\Delta R(t)$ which is the change in diffusely reflected light, at the analysing wavelength, at time t after the laser flash, as shown in equation 1.19.

$$\Delta R_t = \frac{R_0 - R_t}{R_0} = \frac{\Delta J_t}{J_0} \qquad (1.19)$$

where R_0 = sample reflectance before excitation,

R_t = reflectance at time t after the laser pulse,

ΔJ_t = Change in diffusely reflected light intensity at time t following laser pulse,

J_0 = intensity of diffusely reflected light before laser excitation.[21]

In order to analyse the kinetic data from diffuse reflectance laser flash photolysis the concentration of the excited state as a function of the penetration depth must be known. This is dependant on many parameters including the concentration of the ground state molecules before excitation, the molar extinction coefficient at the laser excitation wavelength, the scattering coefficient of support material, the reflectance, the pulse intensity and the quantum yield of conversion.

There are 2 limiting cases for the concentration profiles of excited states as a function of depth into the sample:

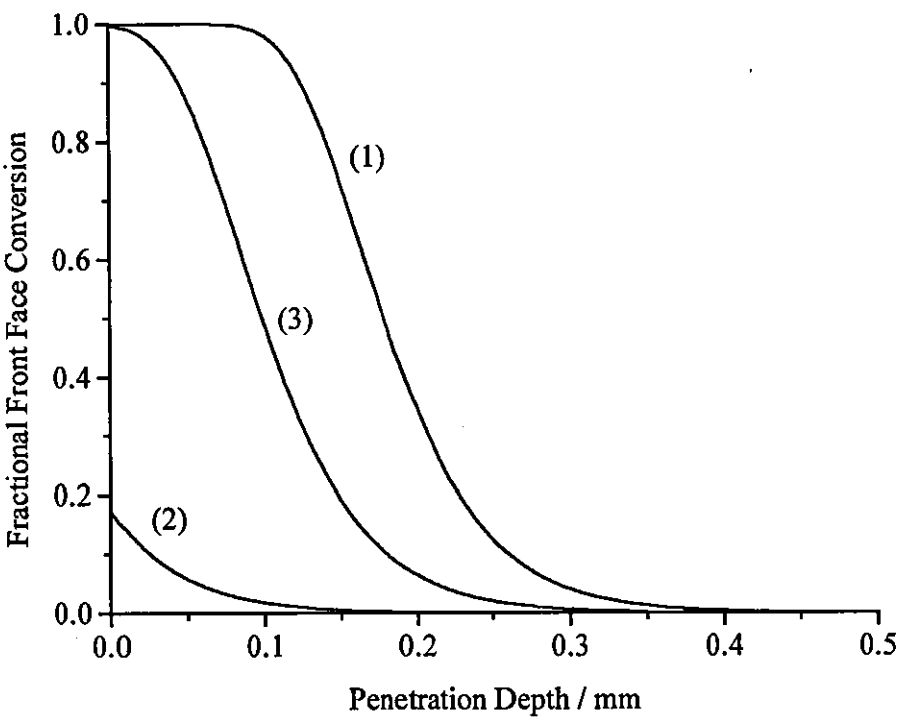


Figure 1.11. - A diagram showing the limiting cases for concentration in diffuse reflectance laser flash photolysis.

1) This is a homogeneous profile obtained with large laser energy and/or a lower concentration of ground state absorbers, with a low extinction coefficient at the laser excitation wavelengths. This gives total conversion from the ground state to the excited state to a certain depth into the sample, called a plug of the excited state. On reaching a certain depth the concentration of the excited state will fall off sharply. The analysing light does not reach as far into the sample as the plug, therefore the sample is homogeneous in the presence of the analysing light. The Kubelka Munk equation therefore applies, providing the sample is infinitely thick and only the transient species absorbs.[15,22]

2) In this profile the transient concentration falls off exponentially as a function of the penetration depth. This is for samples with a low ground state to excited state conversion, which mostly occurs when there is a low laser intensity and a high ground state extinction coefficient. This results in the laser pulse losing intensity rapidly on entering the sample. For this concentration profile the Kubelka Munk equation is not valid, as the concentration of the absorber is not randomly distributed as a function of penetration depth into the sample. It is found from equation 1.13 that $\Delta R(t) \propto C_T$ where $\Delta R(t) < 0.1$. For $\Delta R(t)$ values of up to 0.3 deviation from linearity is small, becoming more significant as $\Delta R(t)$ approaches one. The decay function for concentrations below 0.1 is defined using equation 1.20.

$$f(t) = \frac{\Delta R_T(t)}{\Delta R_T(0)} = \frac{C_T(t)}{C_T(0)} \qquad (1.20)$$

3) There are intermediate cases where concentration profiles show behaviour of both the plug and exponential models. This is where there is a total conversion of the ground state at the surface but this falls off rapidly deeper into the sample in an exponential manner. In this case neither of the previous analytical methods can be used to analyse the kinetic data. The transient concentration can however be calculated via computer simulation, using several parameters such as the laser intensity, concentration, the scattering coefficient, the quantum yield of conversion, and the extinction coefficient of the ground state at the laser excitation wavelength.

1.4. Singlet oxygen

The ground state of molecular oxygen contains two unpaired p electrons of the same energy spin, i.e. it is paramagnetic in the ground state. This conformation produces the maximum multiplicity and therefore the lowest energy state according to Hund's rule.

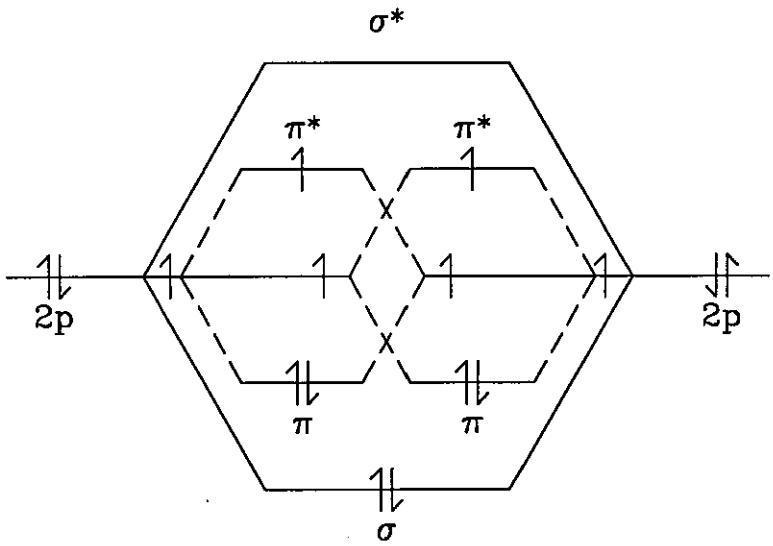


Figure 1.12. - Molecular orbital diagram for oxygen in the ground state.

The ground state of oxygen is a triplet and is given the group symbol $^3\Sigma_g^-$. There are two different types of transition, usually seen on the excitation of molecular oxygen, to the two low lying singlet excited states.[23] The $^1\Delta_g$ state is 400 times longer lived than the $^1\Sigma_g^+$ state and the $^1\Sigma_g^+$ state decays to the $^1\Delta_g$ state with unit efficiency very quickly therefore, the singlet oxygen observed is due to the $^1\Delta_g$ state. These transitions give an emission at 1269 nm and 742 nm for $^1\Delta_g$ and $^1\Sigma_g^+$ states respectively.[24] Due to the forbidden nature of intersystem crossing, these transitions are rarely seen by direct excitation, but are readily seen by sensitisation from both the triplet and singlet excited states of other molecules.

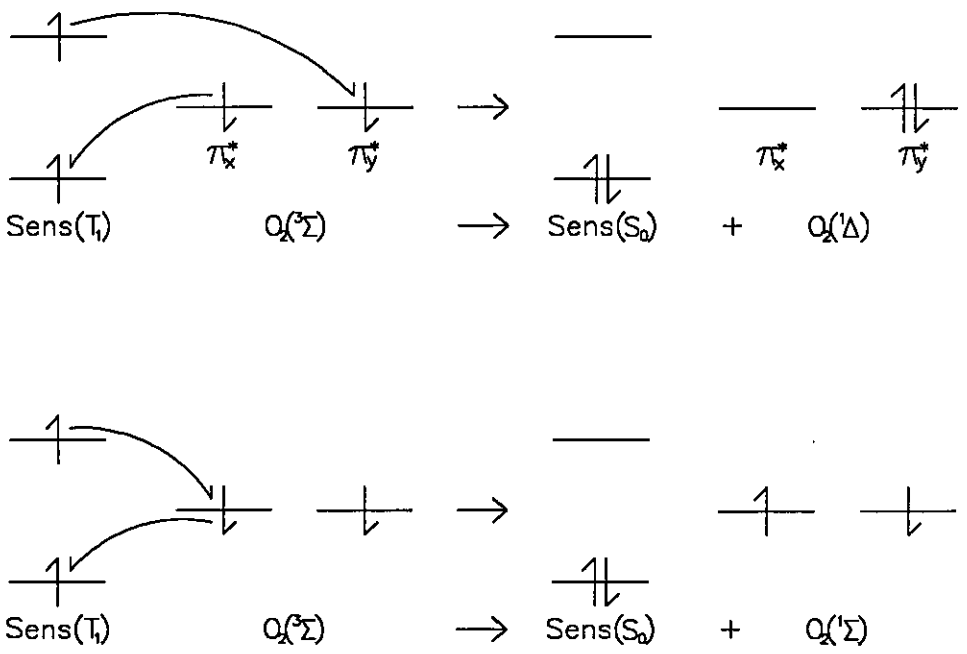


Figure 1.13. - Sensitisation of ground state molecular oxygen to the lowest lying singlet excited state.

This sensitisation proceeds via an electron exchange energy transfer mechanism, as described in section 1.1.7.2., so quenching the excited state of the molecule involved. The triplet excited states of the molecule when quenched by oxygen, may or may not produce singlet oxygen as shown in equations 1.21 and 1.22.

$$^3M^* + O_2\left(^3\Sigma_g^-\right) \rightarrow {}^1M + O_2\left(^1\Delta_g\right) + heat \qquad (1.21)$$

$$^3M^* + O_2\left(^3\Sigma_g^-\right) \rightarrow {}^1M + O_2\left(^3\Sigma_g^-\right) + heat \qquad (1.22)$$

The quenching of a sample by oxygen is quite often used to identify the triplet state. The singlet state of a molecule can be quenched by molecular oxygen, due to intersystem crossing into the excited triplet state. This lowers the energy of the molecule, and may or may not produce singlet oxygen.

$$^1M^* + {}^3O_2\left(^3\Sigma_g^-\right) \rightarrow {}^3M^* + {}^1O_2\left(^1\Delta_g\right) \qquad (1.24)$$

$$^1M^* + {}^3O_2\left(^3\Sigma_g^-\right) \rightarrow {}^3M^* + {}^3O_2\left(^3\Sigma_g^-\right) \qquad (1.25)$$

If the oxygen quenches both the singlet and triplet states the quantum yield of singlet oxygen could have a value of two. The radiative lifetime of singlet oxygen from measurement of the integrated absorption coefficient is 45 minutes. However in solution the lifetime is considerably lower, due to the interaction of the $^1\Delta_g$ state with the solvent.[25,26] The lifetime is shortened the most in solvents such as hydrocarbons, water and alcohols. The singlet oxygen therefore deactivates by electronic vibrational energy transfer. The amount of singlet oxygen can be measured by several different techniques including photothermal methods, chemical methods and near I.R. methods.[27] The near I.R. method uses a germanium photodiode, to detect the 1270 nm phosphorescent emission due to the excited singlet state of oxygen returning to the ground state.

$$^1O_2 \xrightarrow{k_{rad}} {}^3O_2 + h\nu_{(1270nm)} \qquad (1.26)$$

The lifetime of the singlet oxygen may be reduced from that expected in the solvent, this may be due to energy transfer to the ground state of the molecule, if the energy of the triplet state of the molecule is lower than the excited state of the singlet oxygen.

$$^1O_2\left(^1\Delta_g\right) + {}^1M \rightarrow {}^3O_2\left(^3\Sigma_g^-\right) + {}^3M^* \qquad (1.27)$$

High laser energy or high sensitiser concentration can also give lifetimes which are lower than expected. This is due to multiphoton processes producing species which can quench the singlet oxygen.[28]

Chapter 2
Literature Survey

2.1. Photoallergy

In the 1960's there was an outbreak of photodermatitus that was found to be caused by salicylanilides in soaps. The main cause was found to be tetrachlorosalicylanilide (T_4CS).[29] The photodermatitus was discovered to be due to a photoallergic reaction. After T_4CS was identified as a photoallergen other compounds in soaps cleansers, sunscreens, antifungal agents and preservatives were also found to be photoallergens.[30-36] All these products are either frequently used or have a long contact time with the skin. This led to the introduction of the photopatch tests to test various chemicals for a photoresponse, which showed that many compounds were phototoxic, but relatively few were photoallergens.[37]

Photoallergy is defined as the acquired altered capacity of the skin to react to light energy, alone or in the presence of an exogenous photosensitizer.[33,34,38-40] Typically phototoxic compounds affect a large number of people and they only require one application to cause a reaction, but as soon as the substance is removed the toxicity will stop.[41] Photoallergy however, typically requires several exposures but only effects a small fraction of the population. Photoallergy can cause either a transient light reaction or a persistent light reaction. The transient light reaction is where removal of the allergen will stop the allergy.[42] A persistent light reaction is where once exposed a person remains sensitive for a long time afterwards even if there is no further contact with the photoallergen.[43] The substance or its effects can remain in the skin for over 6 months and sometimes for years after application. The reaction must therefore occur in the dermis, as the epidermis is totally replaced every month. There are several possible explanations for the persistent light reaction, the first is that although the initial photosensitizer has been removed there could be a cross reaction with other photosensitizers.[43] Another explanation is that since many of the photoallergens are not soluble in water they could precipitate out in the dermis. This would also account for the fact that photoallergy is usually restricted to the site of application.[32]

There are several factors which affect photoallergy

1) the concentration of the photoallergen and its capacity to penetrate the skin,

2) the quantity of radiation supplied and its capacity to penetrate the skin,

3) the immunological state of the person

4) temperature, humidity, pH, concentration of enzymes and solubility at the site.

Photoallergy occurs when light energy of certain wavelengths, between 290nm and 800nm,[44] present in sunlight, are absorbed by the photosensitizer and chemically alter it. There are two major photochemical pathways by which these compounds could cause photoallergy.

Type 1 - radical formation

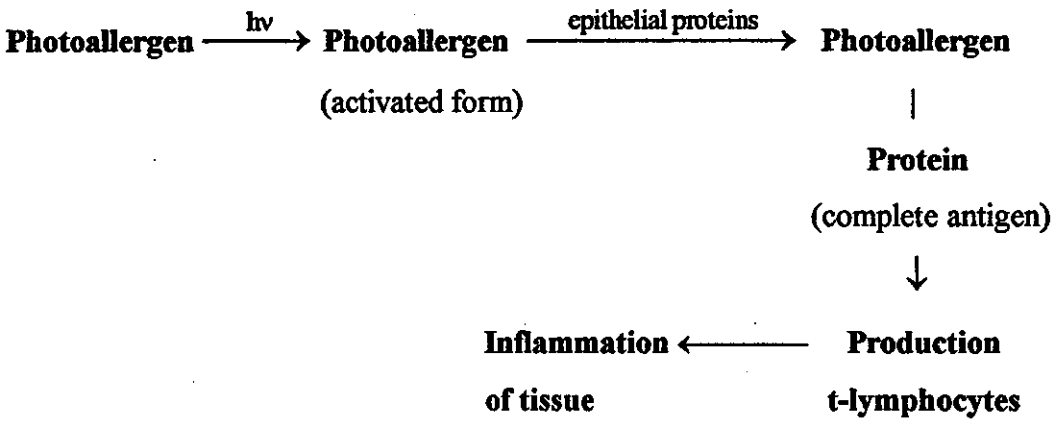
Type 2 - singlet oxygen mechanism

It has been found from *invivo* tests that some photoallergens are dependent on oxygen being present, whereas others proceed whether oxygen is present or not.[45]

It has been shown that photoallergy is connected to the ability of a compound to photochemically bind to proteins in the skin. Photochemical binding is the binding of a protein and a photoallergen in the presence of UV light of the appropriate wavelength, to form covalent conjugates. It is thought to be free radicals produced on UV irradiation which react photochemically with the proteins. Not all proteins are capable of being the carrier protein, the protein which is present for binding in the skin is human serum albumin (HSA). HSA has a molecular weight of 66439 and is an acidic and very soluble stable protein.[46,47] From previous studies it has been shown that T_4CS must non-covalently bind to a protein before photochemically binding to it. This is due to the lifetime of the reactive species formed being short therefore T_4CS needs to be in close proximity to the protein to bind photochemically. The molar ratio of T_4CS bound to HSA was found to be1:1 photoallergen:protein. In addition it was found that there was two major binding sites for T_4CS on HSA, positioned between residues 182-298 close to the tryptophan molecule.[48-52]

The way bithionol and fentichlor bind to proteins is different to the way T_4CS binds because they react non specifically with H.S.A. Bithionol however shows more specificity when binding to proteins than fentichlor. Although bithionol and fentichlor

can bind non covalently to H.S.A. it does not appear to be essential for this to happen before photochemical binding can occur. This suggests that the active species of fentichlor and bithionol have longer lifetimes than the active species of T_4CS, therefore they have longer to react with the protein photochemically and it is not important that any be bound to it prior to irradiation. There is also a connection between radical lifetime and specificity of binding, the longer the lifetime of the radical the less specific the binding. When irradiated with H.S.A. 8 molecules of fentichlor were found to bind to 1 molecule of H.S.A., and the fentichlor was found to be distributed fairly evenly through the entire H.S.A. sequence. Bithionol was found to bind photochemically to H.S.A. in a molar ratio of 6:1 photoallergen to protein. The molar ratio for photochemical binding of 6-methylcoumarin with protein was found to be 1:1.[53-55] Another possible mechanism is where the protein carrier is altered, which could then accept altered or unaltered chemicals so acting as a complete antigen.



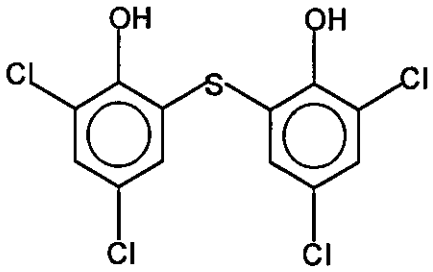
Scheme 1 : A proposed mechanism of photoallergy.[39,40]

2.2. Photoallergens studied.

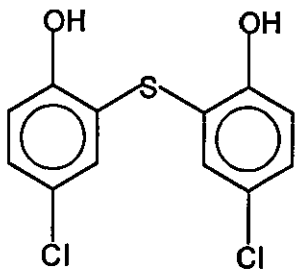
The photoallergens investigated in this thesis were 6-methylcoumarin, fentichlor, bithionol, TBS, chlorpromazine, promethazine, ketoprofen and omadine. T_4CS although being one of the strongest photoallergens has been investigated in detail by another Loughborough student[56], and therefore was not studied. The strength of the photoallergens is unknown as the difference between photoallergy and phototoxicity is

hard to distinguish in photopatch tests, this results in photoallergens being ranked differently by different research groups.[57]

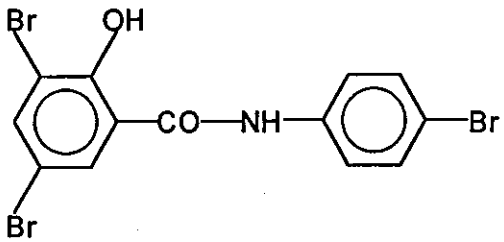
2.2.1. Fentichlor bithionol and Tribromosalicylanilide (TBS)



BITHIONOL



FENTICHLOR



TRIBROMOSALICYLANILIDE
(TBS)

Bithionol (Bis(2-hydroxy-3,5-dichlorophenyl)sulfide) was used in antiseptic creams.[46] Fentichlor (Bis(2-hydroxy-5-chlorophenyl)sulfide) was used as a hair cream and as a antifungal agent.[58] TBS was widely used as an antibacterial agent. TBS, bithionol, fentichlor and T_4CS are all structurally similar with a substitution on the 2-position of the phenol and a halogen on the 4 position.[36]

2.2.3.1. Photochemistry of bithionol and fentichlor

The photochemistry of these compounds has not been studied in detail before. Most of the studies on these compounds has concerned the radicals produced by steady state irradiation, using techniques such as electron spin resonance spectroscopy (E.S.R.). The absorption spectra of bithionol and fentichlor have both been shown to change with pH. The absorption peaks have λ_{max} at 330 nm for bithionol and 324 nm for fentichlor in alkaline solutions which move to lower wavelengths as the pH becomes more acidic.[59-61] The irradiation of fentichlor and bithionol at wavelengths > 300 nm

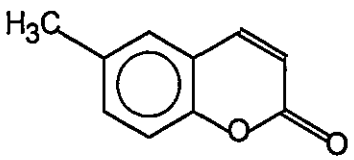
leads to dehalogenation demonstrating the formation of aryl radicals and free halogen ions. This is a process seen in many polyhalogenated phenols[59,62-64] including hexachlorophene which is structurally related to bithionol and fentichlor.[65] It is well known that compounds which have substituents in the 2 position can donate electrons into the benzene ring, this helps to stabilize the radicals formed on UV irradiation.[66] Aryl radicals are highly reactive chemicals which can abstract a hydrogen atom from any suitable donor.[59] Fentichlor and bithionol form a benzoquinone type radical by the loss of 1 chlorine. The bithionol then loses the second chlorine to form a fentichlor type molecule and will then follow the fentichlor mechanism.[67,68] From irradiation in air the bithionol has been said to give different products to those seen in degassed solution and to produce hydroxyl radicals and to consume oxygen upon irradiation.[69] This suggests the difference in the products is due to oxidation by molecular oxygen in the air.[59] However it has since been found that the same products are seen in both aerated and degassed solution[60,70] and the substituents come from the solvent and not the air. This results in different products being seen in different solvents. In aqueous solvents the radical would abstract a hydroxyl group from the water to produce a semiquinone type radical.[60,71] The pH was found to decrease the amount of semiquinone radical seen from pH's 10.7- 8.5.

Fentichlor and bithionol are both made up of two 4-chlorophenols therefore the photochemistry is expected to be very similar to the photochemistry of 4-chlorophenol which has been studied.[72-79] Upon irradiation 4-chlorophenol is also found to give aryl radicals by dehalogenation in older reports with E.S.R. or in more recent reports using laser flash photolysis, a carbene species by loss of HCl.[72] In the reports where radicals were said to be formed they were assigned as benzoquinone radicals which then went onto form hydroquinone with more irradiation[73-77] which is a well known photoproduct of p-benzoquinone photolysis.[78] These products were not affected by oxygen however when applying the carbene mechanism these products were only seen in the presence of molecular oxygen.[72,79] The distribution of the products was found to change with pH.[80]

2.2.3.2. Photochemistry of TBS.

From previous studies it has been shown that T_4CS dechlorinates in alkaline solution initially from the 3 chloro position to give 3',4',5-tri CSA with the chlorines being replaced by hydrogen atoms.[81] The loss of the chlorine gives an aryl radical which is a very reactive species.[63,82] The anilide ring was found not to be involved in the photochemical reaction except to stabilize the radical. This was found to be the same for TBS and most of the work done on T_4CS can be used as a reference for TBS.[83-86] When TBS is irradiated with light above 300 nm the TBS will successively lose 3,5,and 4' bromine atoms.[87] The 3 bromine is lost first then the 5 bromine is lost very quickly afterwards, the 4' bromine will only be lost under intense irradiation and in some solvents not at all.

2.2.2. 6-Methylcoumarin

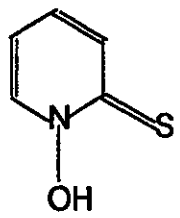


6-METHYLCOUMARIN

6-Methylcoumarin (6-methyl-1,2-benzopyrone) is a synthetic fragrance which is used in many toiletries.[88] It is a proven photoallergen but it shows no phototoxic potential. Coumarin itself however, shows no photoallergic capability and its derivatives only become photoallergic if there is a methoxy or alkoxy group substituted onto the 6 or 7 position of the benzopyran ring.[88,89] The basic photochemistry of 6-methylcoumarin has been studied previously and showed a ground state absorption spectra of 6-methyl coumarin with a peak at 325 nm due to a $\pi \rightarrow \pi^*$ transition. The fluorescence spectra at room temperature showed 1 peak with a λ_{max} at 370 nm and a quantum yield of 0.015. The phosphorescence emission was measured at 77K and showed a peak with a λ_{max} at 465.1 nm, a quantum yield 0.066 and a lifetime of 0.4s.[90] Laser flash photolysis gave a triplet absorption with a maximum at 420 nm which was attributed to a triplet state with a lifetime of 2.3 μs and a quantum yield of 0.04. This transition is thought to be due to a $\pi \rightarrow \pi^*$ transition.[91] The skin sensitization is thought to be due to the $^3(\pi\pi^*)$

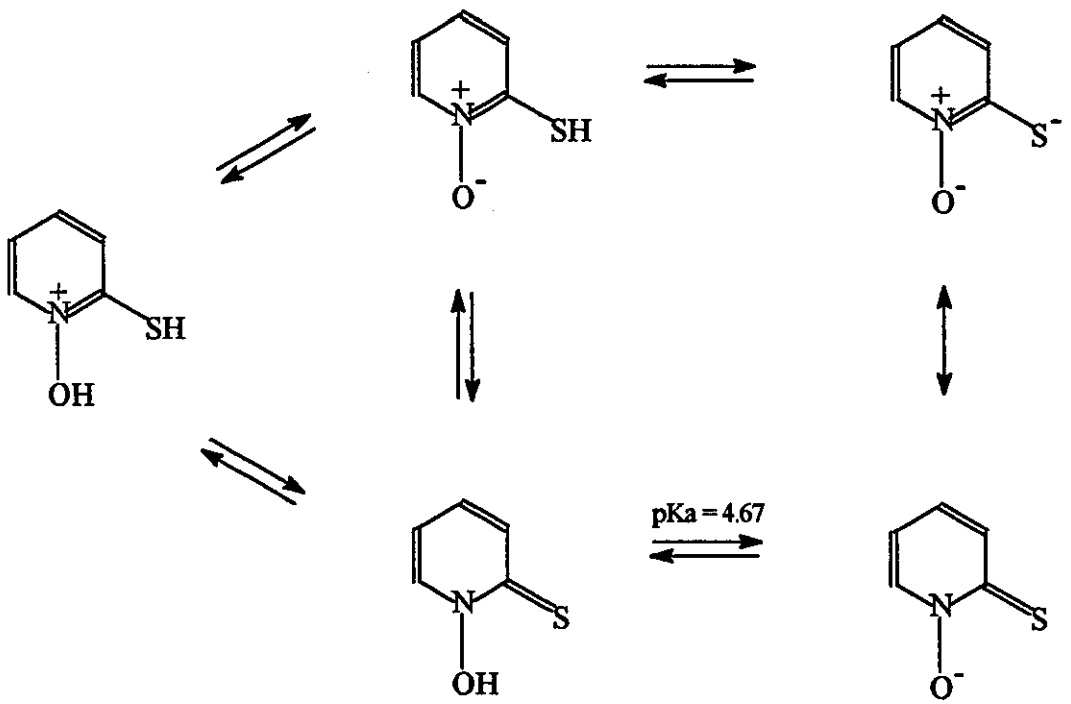
state, which has been shown to be the reactive state for cycloaddition of the 6-methylcoumarin to the DNA pyrimidine bases.

2.2.3. Omadine



OMADINE

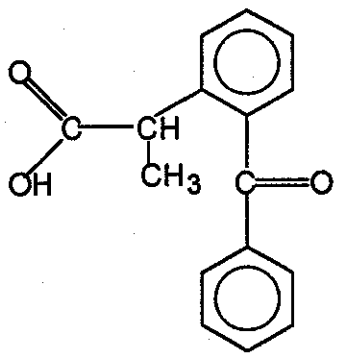
Omadine (1-hydroxypyridine-2-thione) was used in several antibacterial and antifungal formulations and as an active ingredient in several antidandruff shampoos.[30] Omadine exists in 2 tautomeric forms, the thione and thiol forms, each of which may be present as cationic, anionic or neutral species.



Scheme 2: The ionization states of the tautomers of omadine

The ground state absorption spectra however, show that there are only two ionization states seen at pH's between 1 and 10.[92] The thione form was found to be highly preferred to the thiol form for the neutral form of the compound. The equation between the neutral and the anionic species of the thione form has a pK_a of 4.67. Above a pH of 5 the chances of seeing either anion is the same, therefore the ground state absorption of the anion is due to a mixture of the two anions.[93] On UV irradiation there is the production of the thiyl radical[94] with the simultaneous production of hydroxyl radicals which can act as photoreducing agents.[95-97] From flash photolysis at pH 7.4 on ester derivatives of omadine an instantaneous cleavage of the N-O bond occurring during the laser pulse was found, then a peak at 460-490nm was observed which it was suspected was due to the 2-pyridylthiyl radical.[98] At pH 2 the 2-pyridylthiyl radical was still observed but the triplet species, which is very short lived, could also be seen at 660 nm[98] which was also seen with omadine esters.[99]

2.2.4. Ketoprofen

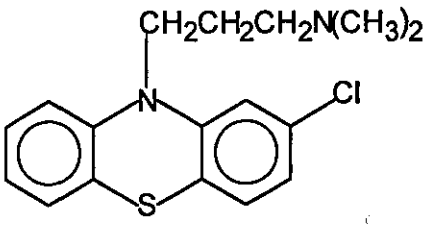


KETOPROFEN

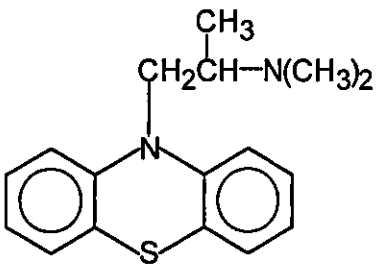
Ketoprofen (2-[3-benzoylphenyl]propionic acid) is a non steroidal anti-inflammatory which is topically used. It was found to be a common but weak photoallergen,[100] which is not surprising due to its propionic acid structure which is phototoxic.[101] Irradiation of ketoprofen in a neutral aqueous solution gives 3-ethylbenzophenone as the major photoproduct. This is formed by the protonation of the benzylic carbanion due to hydrogen abstraction by the benzylic radical. There is also the formation of eight minor products, four formed from the benzylic radicals and the rest formed by the initial hydrogen abstraction by the excited benzophenone chromophore of ketoprofen.[102]

There would be additional products seen in aerobic conditions from those seen in anaerobic conditions due to the addition of molecular oxygen.[103]

2.2.5. Phenothiazines



CHLORPROMAZINE



PROMETHAZINE

Promethazine (2-dimethylaminopropylphenothiazine) is a well known antihistamine[104] and chlorpromazine (2-chloro-N-(3-dimethylaminopropyl)phenothiazine) is a frequently prescribed antipsychotic drug.[105] These substances were first used in 1952 and are still used today. Chlorpromazine is usually taken orally and was found to produce phototoxicity on exposed skin. However the main photoallergy to this compound was found in people who worked on the production of the drug and in the nurses who administered the drug thereby getting it on their skin. Promethazine is usually topically applied and caused a photoallergy at the place of application. These compounds are both phototoxic and photoallergic and can cause either transient or persistent photoallergy.[44]

The photochemistry of both chlorpromazine and promazine has been studied before but no work has been carried out on promethazine. The transient absorption spectra of both chlorpromazine and promazine show bands at 460 nm, 525 nm and 720 nm when excited with a picosecond laser. The 720 nm band was assigned to the hydrated electron which decays totally after 2 μs. The 460 nm band was assigned to the triplet state which lasts about 23 ns for chlorpromazine in a degassed solution. The same result was found for promazine but the triplet had a longer lifetime of 3.1 μs in degassed solutions and 0.4 μs in aerated solutions.[106] After the above two species have decayed the remaining peak at 525 nm is due to the cation radical.[107] Several different mechanisms have been suggested for the formation of the cation radical. Initially the

compound is excited to the singlet excited state, it then has several different routes available. It could form the cation radical directly from the excited singlet state as shown in equation 2.1.

$$^1CPz* \rightarrow CPz^{+\bullet} + e^-_{aq} \qquad (2.1)$$

or the excited singlet state could relax to the triplet state as shown in equation 2.2.

$$^1CPz* \rightarrow {}^3CPz* \qquad (2.2)$$

There are then two possible routes to form the radical cation . The first is by the absorption of another photon of light (equation 2.3) making this a biphotonic process.[108] This process is thought to be dominant at the laser excitation wavelengths above 310 nm. The other route is the spontaneous formation of the radical cation, seen at laser wavelengths below 310 nm due to the chlorpromazine being excited into a higher initial vibrational singlet state.[106]

$$^3CPz* + h\nu \rightarrow CPz^{+\bullet} + e^-_{aq} \qquad (2.3)$$

$$^3CPz* \rightarrow CPz^{+\bullet} + e^-_{aq} \qquad (2.4)$$

Chlorpromazine may also show a peak at 510 nm due to the promazine radical being formed from loss of the chlorine radical from the triplet excited state.[107,109]

$$^3ClP* \rightarrow Cl^{\bullet} + P^{\bullet} \qquad (2.5)$$

Over 50% of chlorpromazine taken orally is metabolized to chlorpromazine sulphoxide during presystemmic absorption. Flash photolysis studies on chlorpromazine sulphoxide also gave the chlorpromazine cation radical in degassed solutions but in aerated solution there was also formation of hydroxyl radicals. In animals where sulphoxides are not formed there was no photoallergy reported.[105] The dechlorination process was found to be very minor for sulphoxides and was therefore suggested not to be major cause of photoallergy for these compounds.[110] Singlet oxygen has been detected in these compounds but would only be an important process if it took place in non aqueous part of body e.g. cell membrane, as very little singlet oxygen was seen in polar solvents.[106]

2.3. Cellulose

Cellulose is a polymer of D-glucose in which individual units are linked by β-glucoside bonds from the anomeric carbon of one unit to the C-4 hydroxy of the next unit. Cellulose is a linear polysaccharide where the isolated form contains an average of 3000 units per chain giving a molecular weight of 500,000. Isolation of cellulose can cause some degradation, therefore in nature cellulose usually contains10000-15000 glucose units, giving a molecular weight of 1.6-2.4 million.[111]

2.3.1. Photochemistry of cellulose

There have been many studies carried out on the photochemistry of compounds adsorbed onto cellulose.[17,112-114]

The ground state diffuse reflectance spectrum of cellulose measured against barium sulfate is shown in figure 2.1.

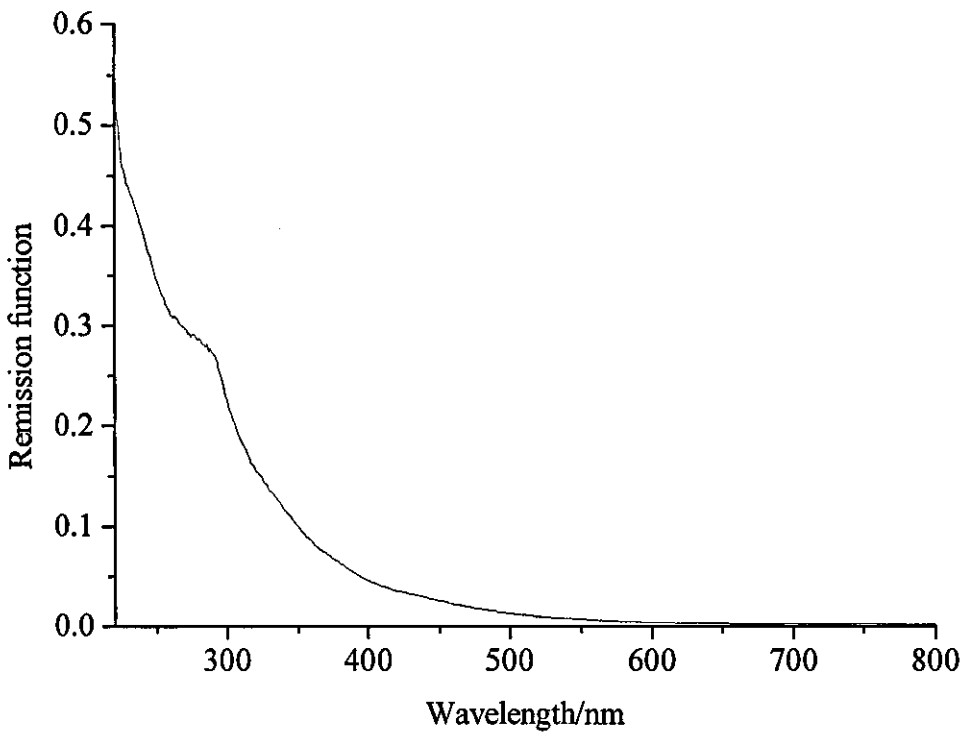


Figure 2.1: Ground state diffuse reflectance spectrum of cellulose.

The cellulose was found to give a weak, structureless, reflectance change from 350 nm -650 nm after laser excitation at 266 nm. However the reflectance change seen from the compounds was a lot greater than seen from cellulose alone. The cellulose weak transient absorption spectra could be due to two possibilities

1. during the formation of cellulose there may be the formation of a few residual carbonyl moieties, which following laser excitation, could form excited states[115]

2. direct photolysis of cellulose at 266 nm leads to formation of free radical species via cleavage of the oxygen bridge between the two linked monomer units.[116]

The lifetimes of excited states of molecules adsorbed onto cellulose are often longer than those seen in solution. This is because the cellulose, through its rigidity and high hydrogen bonding capacity, can protect the excited state of molecules from quenchers, such as molecular oxygen. This effect is enhanced further by thorough drying of the cellulose.[117] The spectra of the compounds are also quite often broader when adsorbed onto cellulose than the spectra seen in solution. This is due to the inhomogeneity of the medium which results in varying adsorption sites.

Chapter 3
Experimental

3. EXPERIMENTAL

3.1 Preparation of samples

3.1.1 Materials

All solvents used were spectroscopy grade (99.9%), purchased from Aldrich. Bithionol, dansylamide, dansylsarcosine and ketoprofen were purchased from Sigma. The H.S.A. used was fraction 5 fatty acid free, purchased from Sigma. The monomer H.S.A. was prepared at Unilever using the method described in section 3.1.3. The 2-acetonaphthone, acetophenone, anthracene, benzophenone, 2-bromobutane, carbon tetrabromide, 4-chlorophenol, chlorpromazine, 1-10 diphenylanthracene, iodine, 6-methylcoumarin, naphthalene, promethazine, potassium iodide and sodium omadine were purchased from Aldrich. The tribromosalicyanilide was used as bought from Kodak. Flowers of sulfur were used as bought from Fisons. The microcrystalline cellulose DS-0 powder was purchased from Fluka. The pH 11.4 aqueous solutions were prepared using deionised water and 0.1M sodium hydroxide to make the solutions alkaline. The pH 7.4 solutions were prepared using pH 7.4, 0.1 M sodium phosphate buffer solution. Fentichlor could not be bought commercially and was therefore prepared using the method described by Dunning, Dunning and Drake[118] outlined in section 3.1.2.

3.1.2 Synthesis of fentichlor

Iodine (0.1 g), flowers of sulfur (0.32 g) and carbon tetrachloride (15 ml) were placed in a round bottomed flask with a side arm delivery tube and a magnetic stirrer in an ice salt bath at -5°C. Chlorine gas (0.7 g) was prepared from concentrated hydrochloric acid and potassium permanganate crystals (>1.58 g) and was bubbled slowly into the reaction mixture. The mixture was allowed to warm up to room temperature before adding 4-chlorophenol (2.57 g) in carbon tetrachloride (15 ml) with stirring. The solution was stirred in the dark for three hours then left to stand in the dark overnight. The resulting white precipitate was filtered off and washed with cold carbon tetrachloride. The product was recrystallised from toluene. The yield of fentichlor was found to be approximately 18%. The melting point was found to be 173°C which was in good agreement with the literature value of 172-175°C.[118]

3.1.3 Preparation of monomer HSA

A sephadex column was prepared by weighing out 25g of G-150 sephadex then adding 0.1M sodium phosphate buffer solution at pH 7.4 and standing the mixture in a boiling water bath for 3 hours to form a swollen gel. The mixture was then poured into a 2.6cm x 100cm column and allowed to pack under gravity. It was then packed at a pump speed of 2.75 for 22.5 hours in a degassed solution of 0.1M sodium phosphate buffer solution at pH 7.4.

4.96mg of Sigma HSA was dissolved in 4.96 ml of 0.1M sodium phosphate buffer solution at pH 7.4. This was loaded onto the column and eluted with 0.01M sodium phosphate buffer solution at pH 7.4, at a pump speed of 2.75, for 15 minutes. The column was then eluted and fractions 45-57 were collected, pooled and then filtered through a 0.22μm minisart filter to reduce microbial contamination. 0.5 ml of this solution was then diluted with 1.5 ml sodium phosphate buffer solution at pH 7.4. The optical density of this solution was found to be 0.677 at 280 nm. The undiluted concentration of the monomer HSA was calculated from equations 3.1 to 3.3.

$$[\text{mHSA}] = OD_{280}/\text{extinction coefficient x path length x dilution factor} \quad (3.1)$$

$$\text{Total protein recovered} = \text{Fraction collected from column x [mHSA]} \quad (3.2)$$

$$\% \text{ Recovery} = \text{protein recovered/protein loaded onto column x } 100 \quad (3.3)$$

The total protein recovered was 340 mg and the % recovery was 69%. The monomer HSA was stored at +4°C in a fridge.[119]

3.1.4. Preparation of solid samples

Solid samples were prepared using cellulose as an adsorbing surface. The cellulose was dried for 3 hours in a vacuum oven at 70°C. Solutions of the compounds (3-100mg) were made up in 20ml ethanol, these were poured onto 3g of the dried cellulose and stirred. The use of ethanol causes swelling of the cellulose which allows penetration of the compound into the submicroscopic pores of the cellulose.[120] The samples were placed in a fume cupboard to allow the solvent to evaporate. They were stirred to ensure a homogeneous distribution of the compounds. The samples were then placed in the vacuum oven at 50°C for 3-4 hours to remove the rest of the solvent.

3.1.5. Degassing of solutions

Triplet and singlet excited states can be quenched by molecular oxygen, therefore it is necessary to degas solutions. There are two ways of achieving this, the first is by saturating the solution with nitrogen, but such solutions do not stay deaerated for long periods of time. A better method for this work was a technique called freeze pump thaw. This is where a solution is frozen down in liquid nitrogen. The sample is then pumped down to pressures below 10^{-3} bar, sealed and defrosted allowing the oxygen in the gas above the solution to equilibrate with that in the solution. This process is repeated until there is no further change in the pressure when the sample is opened to the pump i.e. no further gas is left in the solution. The samples are left sealed.

3.2 Ground state spectra

3.2.1. UV/VIS spectrometry

All ground state absorption spectra of solutions in 1 cm x 1 cm quartz cells were recorded on a Hewlett Packard (8453) diode array spectrometer. The ground state diffuse reflectance spectra of solid samples were recorded using a Pye-Unicam PU8800 dual beam UV-Visible spectrophotometer with an integrating sphere (Pye Unicam). The internal surfaces of the integrating sphere were all coated with an optically thick layer of a white totally reflecting material (Kodak $BaSO_4$). The solid samples were packed into 2 cm x 1 cm cylindrical quartz cells ensuring an optically thick layer. The cell was placed in the integrating sphere and held in place with a black metal plate to reduce the specular reflection reaching the detector. The solid samples were measured against barium sulfate as standard.

3.2.2. pK_a calculations

The pK_a of the compounds were measured using the change in the ground state absorption spectra. By this method absorption spectra of the compounds are obtained in acidic and alkaline solution. The different forms of the compound in the ground state will absorb in different parts of the spectrum. If the equilibrium is taken to be

$$A\text{-}H \leftrightarrow A^- + H^+$$

The absorption spectrum seen in acidic solution will be due to the non-ionised form and the absorption spectrum seen in alkaline solution will be due to the totally ionised form. The equilibrium constant between the two forms is

$$K_a = \frac{[A^-]_{eqm}[H^+]_{eqm}}{[AH]_{eqm}} \qquad (3.4)$$

therefore,

$$[H^+]_{eqm} = \frac{K_a[AH]_{eqm}}{[A^-]_{eqm}} \qquad (3.5)$$

taking logs,

if $-\log[H^+]_{eqm} = pH$, then

$$pH = -\log K_a - \log\left(\frac{[AH]_{eqm}}{[A^-]_{eqm}}\right) \qquad (3.6)$$

The absorbance of a solution is related to the concentration by Beers law, therefore the concentrations can be calculated from the absorbance, at two different wavelengths, in the above equations. The pH of a solution depends on the ratio between acid and base so when $[AH] = [A^-]$ then $pH = -\log K_a$. Equation 3.6 is a straight line equation, therefore a plot of pH versus $\log\frac{[AH]}{[A^-]}$ should give a straight line with an intercept which is the pK_a.

3.3. Emission spectroscopy.

3.3.1 Fluorescence

The fluorescence measurements were carried out using a Spex Fluoromax spectrofluorometer. The solutions were measured at right angles to the direction of excitation as were the solid samples with the use of a front face attachment. Experiments were carried out using solutions with an absorbance less than 0.1 to reduce errors due to inner filter effects. Some of the samples being measured undergo photodegradation therefore, for the emission scan the excitation slits were reduced to half the width of the emission. All spectra were corrected for any emission from the solvent used. The fluorescence lifetimes of the compounds studied could not be measured but an order of magnitude estimate of the radiative lifetime could be found from the relationship shown in equation 3.7.

$$\tau_R = \frac{10^{-4}}{\varepsilon_{max}} \qquad (3.7)$$

Where, τ_R = Radiative fluorescence lifetime

ε_{max} = Molar absorption coefficient.

The actual fluorescence lifetime is found from the fluorescence quantum yield using equation 3.8

$$\tau = \tau_R \phi_F \qquad (3.8)$$

3.3.2 Phosphorescence

The phosphorescence measurements were carried out at 77K. This required the use of a quartz dewar to hold both the liquid nitrogen and the cell. The cells used for the phosphorescence measurements were 0.25 cm diameter quartz tubes. The solvent used was 7:3 isopentane:2-propanol[121] or 1:1 methanol:ethanol, which form good organic glasses at 77K. For these experiments the absorbances of all the solutions needed to be the same at the exciting wavelength. It was found that the absorbances varied with temperature and they therefore needed to be measured at 77 K. This was achieved by

using the diode array and putting the cell in the presence of a stream of nitrogen. The absorbances could not be exactly matched, so an adjustment was made for the small difference. The HY laser described in section 3.4.1. was used to measure the lifetime of the phosphorescence, in emission only mode exciting at 355 nm and 266 nm and fitting the traces to first order exponential kinetics.

3.3.3. Energy calculations

Using equation 1.2 from chapter 1 the fluorescence and phosphorescence can be used to find the energy of the lowest excited singlet state and the lowest excited triplet state respectively. If the fluorescence and phosphorescence are structured the 0-0 vibronic level which will have the shortest wavelength is used to calculate the energy. If however the spectra are structureless, in the case of the triplet energy the maximum of the phosphorescence is used, which will give a lower limit for the lowest excited triplet state. The singlet state energy was calculated by taking the midpoint between the maxima of the fluorescence and the maxima of the ground state absorption spectra.

3.3.4. Protein binding

These experiments were carried out only on bithionol and fentichlor on a Perkin Elmer LS50 spectrofluorimeter. The fentichlor and bithionol fluorescence could not be seen on this instrument so quenching of the fluorescence of the tryptophan molecule at 295 nm was studied. This was achieved by taking 3 ml of a fixed concentration of human serum albumin (H.S.A.), in the range $1x10^{-6}$ - $3x10^{-6}$ M in a phosphate buffer and recording the fluorescence intensity at 295 nm , then 10 μl aliquots of a fixed concentration of fentichlor were titrated into the H.S.A., and the fluorescence intensity was recorded after each addition. The solution was stirred to reduce the decomposition of the sample. This procedure was repeated until there was no change in the fluorescence intensity.

Fluorescence probe measurements were carried out on a Perkin Elmer LS50 spectrofluorimeter and the Spex Fluoromax spectrofluorimeter. The fluorescent probes were used to find at which sites the compounds were binding to the H.S.A. Dansylamide is a site 1 probe and is excited at 350 nm and emits at 485 nm.

Dansylsarcosine is a site 2 probe which is excited at 370 nm and emits at 478 nm. The experiments were carried out by taking 3 ml of $1x10^{-5}$ M of HSA with a fixed concentration of bithionol or fentichlor ($1x10^{-5}$ - $4x10^{-5}$ M), in phosphate buffer and recording the fluorescence intensity. This solution was then titrated against 10 µl aliquots of a fixed concentration of the probe and the fluorescence intensity was recorded after each addition. This procedure was repeated until there was no further change in the fluorescence intensity. This procedure was also reversed so the fluorescence probe was added to the H.S.A. at a fixed concentration and this was titrated against the fentichlor or bithionol. This showed whether the competition depended on the order in which the compounds were added. All the probe experiments were carried out using H.S.A. fraction V and using monomer H.S.A. solution to see if the presence of dimers influenced the binding parameters.

3.4. Nanosecond laser flash photolysis

3.4.1. Transmission laser flash photolysis

The nanosecond flash photolysis studies were carried out using 2 different Q switched Nd:YAG lasers, the HY200 (Lumonics) and the JK2000 (J.K.Lasers). The fundamental wavelength is 1064 nm which can be frequency mixed to produce a harmonic at 354.7 nm and frequency quadrupled to produce a harmonic at 266 nm for use as the excitation source with energies of approximately10 mJ at 354.7 nm and 18 mJ at 266 nm. The laser apparatus is set up as shown in figure 3.1.

The analysing beam used is from a 300 W xenon arc lamp (LOT Oriel Ltd) which gave a continuous beam of light through the sample at right angles to the excitation beam. Filters were placed between the lamp and the sample to cut off unwanted wavelengths of light from reaching the sample. Shutters were placed between laser and sample and analysing source and sample and these were computer controlled. The analysing shutter opened just before the laser fired and the laser shutter opened as the laser fired.

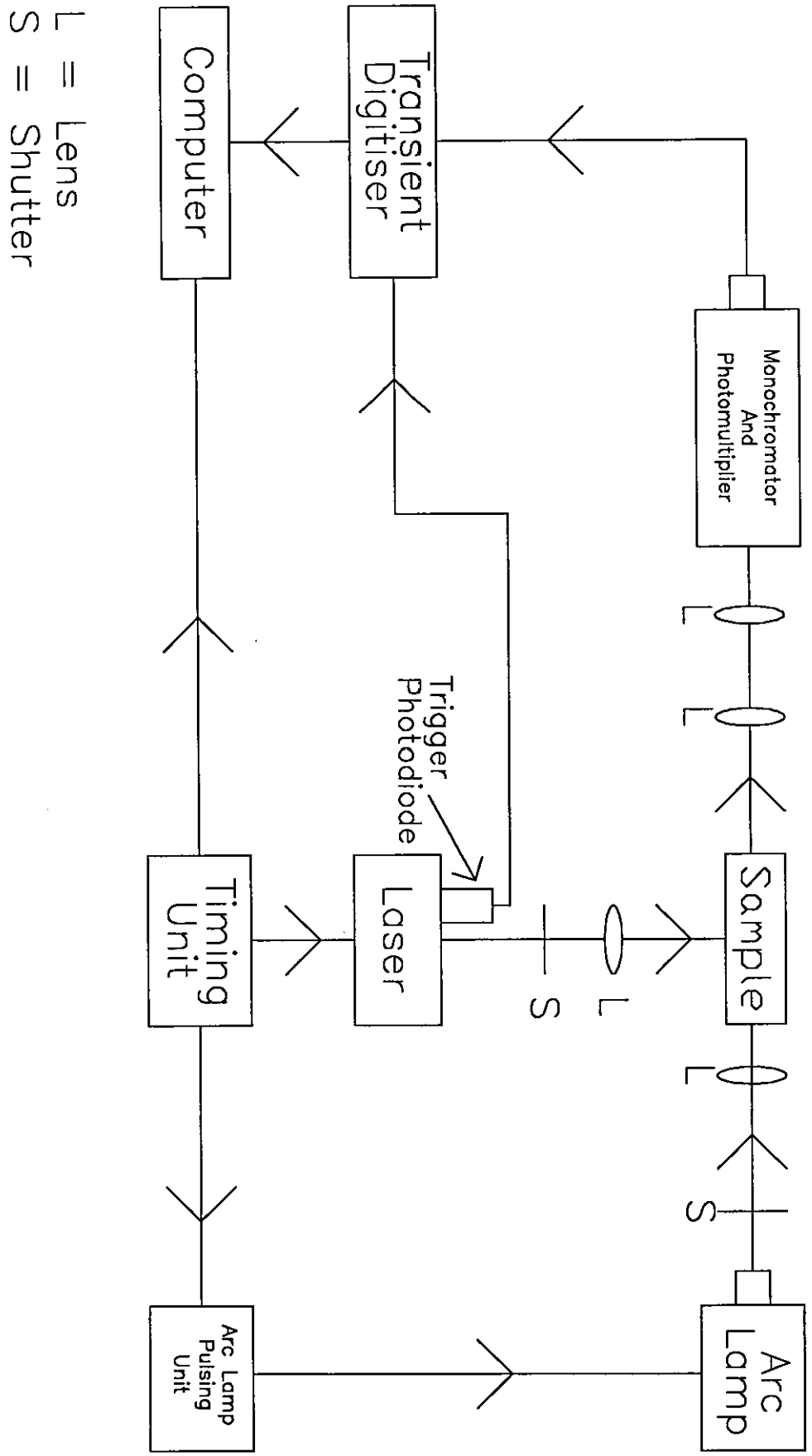


Figure 3.1 Nanosecond laser flash photolysis equipment for transmission studies

The laser flash photolysis experiments were carried out in 1 cm x1 cm quartz cells. The samples were degassed under vacuum using the freeze-pump-thaw technique. A monochromator (Applied Photophysics Ltd, F/3.4 grating) with variable slit widths collected the analysing light and the signal was detected by a R928 photomultiplier tube with a high voltage supply. The signal from the photomultiplier went into a digitiser (Tektronix TDS 420 for the JK laser and Tektronix 2432A for the HY laser) which converted the signal from an analogue to digital signal which was then sent to the computer and stored for later analysis. The digitiser was triggered by the laser either by reflecting some of the laser pulse off a glass slide (JK2000) or using a photodiode which was triggered by the 1064 nm light leaking from the end mirror of the laser cavity (HY200). The quantity measured in transmission laser flash photolysis is the change in the absorbance with time (ΔA_t) following laser excitation.

3.4.2 Diffuse reflectance laser flash photolysis

The method used to measure the excited states of solid samples is diffuse reflectance laser flash photolysis. The laser must be set up so the laser and the arc lamp irradiate the front face of the sample and only the diffusely reflected light from the sample is collected. The specular reflection must not be allowed to enter the monochromator. This is achieved by changing the geometry of the laser, arclamp and monochromator with respect to each other as shown in figure 3.2.

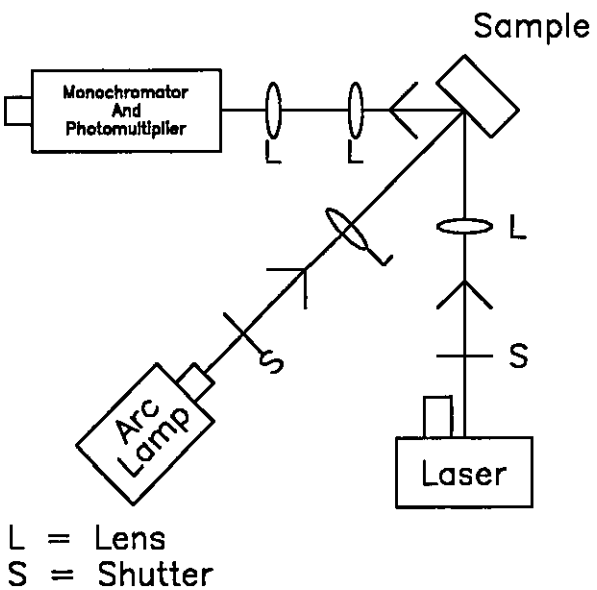


Figure 3.2: The geometry required for diffuse reflectance laser flash photolysis

The samples were measured in 2 cm x 1 cm pathlength quartz cylindrical cells (Helma Ltd) and the sample was shaken after every measurement to reduce degradation of the sample. The quantity measured in diffuse reflectance laser flash photolysis is the change in the reflectance with time (ΔR_t).

3.4.3. Collection and analysis of data from nanosecond laser flash photolysis

The traces collected from the computer are the baseline, transient absorption, emission and topline as shown in figure 3.3. These traces are all measured against time at one wavelength.

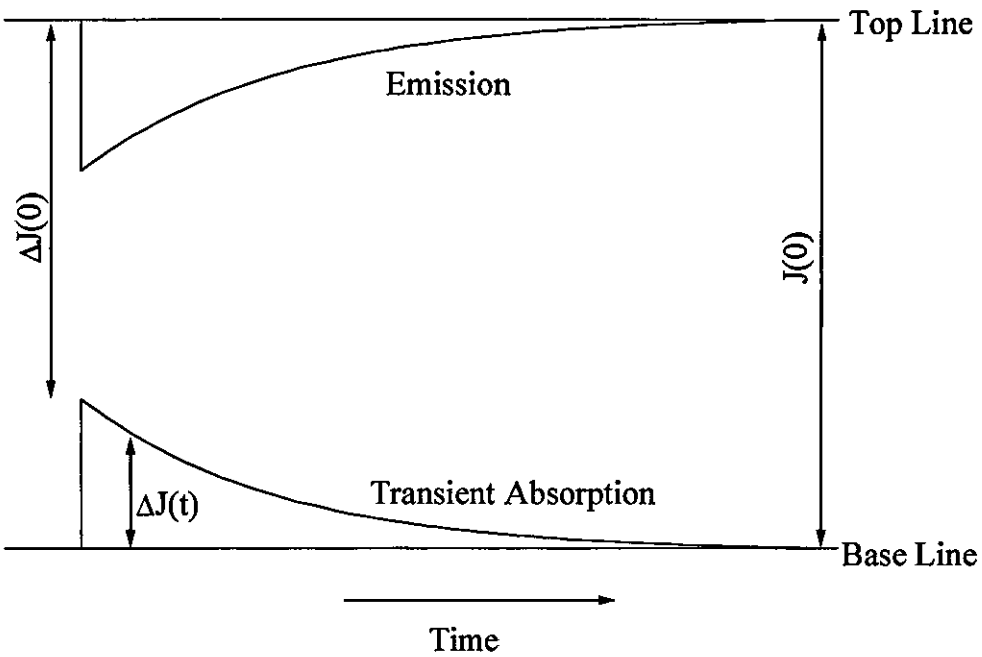


Figure 3.3 : Schematic diagram of the experimental data traces recorded for laser flash photolysis experiments.

The baseline is where only the analysing shutter is open and this represents the ground state transmittance/reflectance level. The topline is collected with both shutters closed and this represents the top level of the screen. The topline minus the baseline tell you the size of the screen. The transient absorption spectrum is measured when both the analysing and laser shutters are open and represents the change in absorption, or in the case of diffuse reflectance, the change in the reflectance due to the laser excitation of

the sample. The emission is recorded with just the laser shutter open and represents how much the sample emits. The emission trace is taken away from the transient absorption to give the final trace due to the transient absorption alone.
The value of the change in transmission is found from the equation for the size of the transmission at time t as shown in equation 3.9.

$$\Delta T(t) = 1 - \left(\frac{Transient\ absorption - Emission}{Baseline - Topline} \right) \tag{3.9}$$

From Beer's law the value for ΔA_t can be found from equation 3.10.

$$\Delta A(t) = \log_{10} \left(\frac{Baseline - Topline}{Transient\ absorption - Emission} \right) \tag{3.10}$$

The equation for diffuse reflectance measurements (3.11) is very similar to that for transmission measurements (3.9).

$$\Delta R(t) = \frac{\Delta J(t)}{\Delta J(0)} = 1 - \left(\frac{Transient\ absorption - Emission}{Baseline - Topline} \right) \tag{3.11}$$

The change in reflectance found in equation 3.11 will only show a linear relationship with the concentration if the reflectance values are kept below 0.1. The transient spectra were obtained from recordings of the decay traces at different wavelengths. The value of $\Delta A(t)$ or $\Delta R(t)$ is found for five different times in the decay traces for each wavelength. The spectrum is then plotted as change in absorption or reflectance against wavelength for each of the five different times. The transient decay traces were usually fitted to first order kinetics using equation 3.12.

$$Rate = A \exp(-kt) \tag{3.12}$$

In several cases the absorption after the end of the decay had not return to the initial absorption and a baseline needed to be added changing equation 3.12 to equation 3.13.

$$Rate = A\exp(-kt) + C \qquad (3.13)$$

3.5. Flashgun flash photolysis experiments

The equipment was the same as used for transmission laser flash photolysis, except the laser excitation source was replaced by a Mets C45-1 flashgun. The flashgun was used for bithionol and fentichlor because the amount of transient seen with the laser was very small and the lifetime of the transient was very long, so allowing a longer excitation source to be used. The difference between a laser and a flashgun is that a laser emits a pulse of high energy radiation, with a very short pulse time at one wavelength, whereas the flashgun uses a wider range of wavelengths of light, that last milliseconds, but are of a lower energy. The advantage of using the flashgun is that the samples absorb more over the range of wavelengths emitted from the flashgun than at either 355 nm or 266 nm alone. The other advantage of the flashgun is that the area of solution which can be excited is larger, therefore a 5 cm cell can be used instead of a 1 cm cell so increasing the amount of solution which can be excited.

3.6 Product analysis

3.6.1. Arc lamp degradation

The photoproducts can be analysed and the quantum yield calculated using an arc lamp with a UV interference filter to irradiate at one wavelength. The cells used, were 1 cm pathlength cylindrical cells, were put in front of the arclamp at a distance of about 30 cm. The degradation was monitored using the ground state absorption spectra. The samples used in the chromatography experiments were degraded using an ecetoc lamp with a 305 nm cut off filter.

3.6.1.1. Actinometry using potassium ferrioxalate

The quantum yield of the photoproducts was measured using potassium ferrioxalate actinometry. The $[Fe(C_2O_4)_3]^{3-}$ ions disproportionate into $[Fe(C_2O_4)]^{+}$ and $[Fe(C_2O_4)_2]^{-}$

in the acidic medium. The reaction with UV light is then as shown in equations 3.14 and 3.15.

$$[Fe(C_2O_4)]^+ \xrightarrow{h\nu} Fe^{2+} + C_2O_4^- \qquad (3.14)$$

$$\left[Fe(C_2O_4)\right]^+ + C_2O_4^- \rightarrow Fe^{2+} + C_2O_4^{2-} + 2CO_2 \qquad (3.15)$$

The quantity of ferrous ions formed during a set irradiation period is determined spectroscopically by measuring the absorbance at 510 nm of the complex formed between the ferrous ions and 1-10 phenanthroline. The absorbance should be in the region of 0.2 to 1.8. The original ferric ions do weakly complex with 1-10 phenanthroline but this complex does not absorb at 510 nm.

The actinometry was carried out by irradiating 3 ml of $K_3Fe(C_2O_4)_3$ solution for 10 minutes at 300 nm in a quartz 1cm cell. The actinometer solution (2 ml) was mixed with 1-10 phenanthroline (2 ml) and buffer (1 ml) and made up to 10 ml with water. The solution was then left for an hour before the absorbance was measured. A blank solution was prepared as above but using non-irradiated actinometer solution. The difference between the two absorbances at 510 nm was recorded and the intensity of the arclamp was calculated[122] using equation 3.16.

$$I = \frac{AV_2V_3}{\varepsilon d \phi_\lambda t V_1} \qquad (3.16)$$

Where A = absorbance at 510 nm

d = cell pathlength

ε = extinction coefficient of Fe^{2+} 1-10 phenanthroline complex - 1.11×10^4 $mol^{-1}dm^3cm^{-1}$

ϕ_λ = quantum yield of Fe^{2+} production - 1.24

V_1 = volume of irradiated actinometer solution used

V_2 = volume of actinometer solution irradiated

V_3 = volume of volumetric flask used for dilution

t = irradiation time

The quantum yield for the sample can then be calculated using equation 3.17:

$$\phi_B = \frac{\text{Number of molecules of product formed / unit time / unit volume}}{\text{Number of quanta absorbed / unit time / unit volume}} \quad (3.17)$$

This quantum yield was calculated assuming there was no product at the analysing wavelength. The quantum yields were measured after 1 minute to get the initial quantum yield where the extinction coefficient of the product would be very similar to that of the starting material. The number of molecules of product formed was calculated from the change in the absorbance using Beer's law.

3.6.2. High performance liquid chromatography (H.P.L.C)

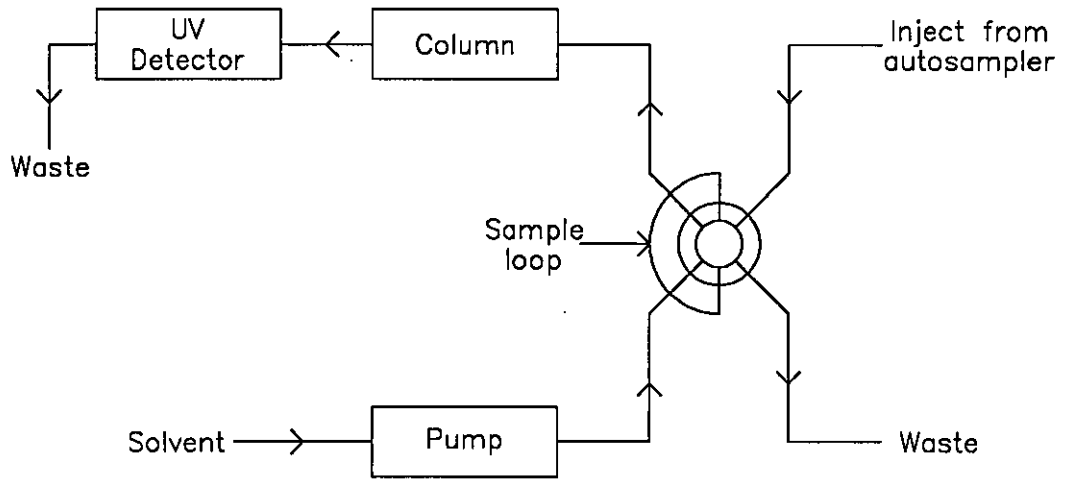


Figure 3.4: HPLC set-up

The HPLC system used consisted of a Spectraphysics P4000 pump with an AS1000 autosampler, as shown in figure 3.4. The separation was done using a C18 Whatman Partisphere reverse phase column (4.6 mm x 25 cm). An isocratic solvent system was used for the separation which was 100% water to 100% MeOH in 10 minutes, hold at 100% MeOH for 10 minutes, then 100% MeOH to 100% water in 1 minute and hold at 100% water for 9 minutes. The sample was detected using a UV1000 detector set at 290 nm. The size of the sample injected was 20 µl. The flowrate through the system was 1 ml/min. The fractions were collected manually and concentrated by evaporation.

3.6.3. Gas Chromatography-Mass Spectroscopy (GC-MS)

The samples used were 0.1% solutions bithionol and fentichlor both irradiated and non-irradiated, in methanol. The bithionol and fentichlor were injected(Hewlett Packard 7673 Autosampler 1 μl optic-2 splitless injection) into the gas chromatogram (Hewlett Packard 5890 with 15 m x 0.25 mm J + W DB-1 column). The temperature in the column was programmed to rise from 40°C to 270°C at 10°C/min before entering the mass spectrometer(VG Trio-1000 electron impact ionisation 70eV source 200 C, 150 μA) with a photomultiplier detector which scanned from m/z 50 to 500. Quantitative comparisons were made to a solution of 10 μg/ml of C_{10},C_{15}, C_{20}, C_{25} n-alkanes.

3.7. Singlet oxygen

The singlet oxygen quantum yields were found for all the compounds using the singlet oxygen detector, comprising of a Judson germanium photodiode (J16-8SP-12O5M, active diameter 0.5 cm) coupled to a Judson PA100 amplifier.[123,124] This measures the luminescence of the sample at 1270nm, as this is the wavelength at which singlet molecular oxygen emits. This is due to the emission from the lowest excited singlet state of molecular oxygen ($^1\Delta_g$)as explained in section 1.4. The samples were measured in quartz cells excited at 355 nm using a laser. Filters were placed in the laser beam to remove 1064 nm (BG38- LOT Oriel) and 532nm. For excitation at 266 nm there were no filters available to remove the 1064 nm and 532 nm light therefore, the laser beam was reflected off a 266 nm dichromic mirror at 45° to the laser. The detector was placed close to the cell face at right angles to the laser with a silicon cut off filter between the sample and the detector. The samples were recorded in acetonitrile over a timescale of 500 μs. The quantum yield of singlet oxygen produced was calculated by comparison with a standard. The intensity of singlet oxygen of the standard was measured at several different laser energies using sodium nitrite filters to lower the laser energy. A graph was then plotted of singlet oxygen intensity against laser energy to find the region of linearity. The intensity of the singlet oxygen from the sample was measured and suitable filters were used to keep the intensity within the linear region. The quantum yield could then be found from equation 3.18.

$$\phi_{sample} = \frac{S_{sample}\ \phi_{stand}}{S_{stand}} \qquad (3.18)$$

Where, S = slope of a plot of intensity against laser energy.

Chapter 4
Experiments with H.S.A.

4. Experiments with Human Serum Albumin

4.1 Dark binding of fentichlor and bithionol with H.S.A.

4.1.1. Introduction

It has been shown from previous studies that for photochemical binding to occur in T_4CS it is necessary for the compound to dark bind with the protein first. This was thought not to be true for bithionol and fentichlor, however the compounds can dark bind first and this can be used to find the position of binding on the H.S.A. Dark binding is the noncovalent binding of a compound to a protein molecule without light being used in the reaction. The dark binding of ligands has been investigated in detail using human serum albumin as the protein, as this is the most abundant and the only binding protein in blood plasma. Large numbers of low molecular weight compounds bind reversibly to HSA which is the major protein in the skin. HSA has a molecular weight of 64639. It is made up of 55 amino acid groups and it is known to have 2 major binding sites.[125,126] Most ligands are bound to a few high affinity sites as well as a number of weaker sites. The data collected from binding studies is usually analysed using the Scatchard model,[127] where it is assumed that a ligand is bound to classes of identical independent binding sites. The binding studies give the number of binding sites on the protein for the interacting species and the affinity of binding sites for the ligand concerned. The binding to albumin is affected by the concentration of ligand and albumin, pH,[128] temperature and ionic strength.

The best methods used to investigate protein binding are spectroscopic methods because the optical properties of a protein ligand complex are different to the ligand or protein alone. Fluorescence is more sensitive to the environment than absorption,[129] therefore the signal change on protein ligand binding is likely to be greater if a fluorophore is present. The advantage of fluorimetry is that results are obtained instantly and the compounds can equilibrate and be measured in one vessel, so there are no separation problems. The disadvantage of this method is the operator has no prior knowledge of how large an optical change is expected on binding, therefore it is difficult to establish a stoichiometry of protein-ligand interaction by spectroscopic methods alone. Another problem is, if multiple binding sites are present, it is assumed that the spectral change on binding the second or third ligand molecule will be

identical to that of the first.[130-137] There are several methods to investigate the binding of a compound to HSA the first is by the quenching of the tryptophan signal at 295nm.[135] For quenching experiments it has been found that it is not necessary to use very high protein concentrations[132] to determine the relationship between the concentration of bound ligand and a physical property change.

The method used to analyse the binding data, uses the titration of the compound against a fixed concentration of HSA to observe the quenching. In this method it is necessary to find the maximum fluorescence when all the ligand is bound. When the concentration of the ligand is a lot greater than the concentration of the acceptor the interception with the y axis of the graph of $1/\Delta F$ Vs 1/[ligand] gives ΔF_{max}; where, F = observed fluorescence, [138]ΔF = change in fluorescence.
The binding constants can then be calculated using the equations set out below.
The binding parameters are calculated using equation 4.1.

$$K_d = el/b \qquad (4.1)$$

where :K_d = dissociation constant of the protein- ligand complex

e = concentration of free binding sites at equilibrium

l = free concentration of added ligand at equilibrium

b = concentration of bound ligand at equilibrium

rearranged this gives equation 4.2.

$$\frac{l_0}{\alpha} = \left(\frac{K_d}{(1-\alpha)}\right) + e_0 \qquad (4.2)$$

where :n = number of binding sites per protein molecule

e_0 = total concentration of binding sites for ligand

l_0 = total concentration of added ligand at equilibrium

α = fraction of protein sites bound to ligand = $\frac{e_0 - e}{e_0}$

α is calculated using $\Delta F/\Delta F_{max}$ for each value of l_0

A plot of l_0/α Vs $1/(1-\alpha)$ will gives a slope of K_d and the intercept of e_0.[131] This should give a linear plot for 1 independent binding site. A non linear plot can occur where more than one class of binding site is present[136,139] or where the compound is affecting the fluorescent properties of binding sites to which it is not bound.[116] At high concentrations all accessible residues are quenched therefore any remaining fluorescence is independent of quencher concentration.

Enhancing the compound signal at its maximum wavelength e.g. fentichlor 341nm. is also a method which has been used to investigate the binding with protein unless as in the case of fentichlor and bithionol the fluorescence is too weak to be measured accurately.[134,140]

Another method which has been used to investigate the binding of drugs to H.S.A., was by using a fluorescent probe. A fluorescent probe is defined as a compound which undergoes a change in one or more of its fluorescent properties when bound to certain proteins.[141,142] The change in fluorescence characteristics associated with these probes binding to a protein, are similar to the change seen when they are transferred from a polar to a non polar environment. The probes bind to the hydrophobic binding sites on proteins causing the fluorescence to shift to the blue, increasing the quantum yield.[143] The compounds used to displace them are assumed to have a hydrophobic area at the binding site. The relative strength of the binding of fentichlor and bithionol to a protein has been studied by the ability of these compounds to decrease the fluorescence of the probe bound to the protein. Probes with a single binding site or two sites with widely separated dissociation constants could be used as specific probes of H.S.A. binding sites.

There are said to be two sites for binding on H.S.A. which can be probed called site 1 and site 2. These binding sites lie in the subdomains IIA and IIIA which are hydrophobic pockets accessed through 10 Å openings. Subdomain IIA (site 1) spans from Lys 199 to Glu 292 and subdomain IIIA (site 2) is from Pro 384 to Phe 488. The position and character of binding depends on the structure of the compound. The class of probes generally used are the dansylamino acids. Two probes which are commonly

used are dansylamide which is a site 1 probe and dansylsarcosine which is a site 2 probe.

In this method the compound of interest was bound to the H.S.A. initially then the H.S.A. and compound were titrated against the probe. This was then compared to values obtained when the compound wasn't present. The study relies on competition of the binding between the compound and the fluorescent probe. Competition is only observed if the compound binds at the same site as the probe.[144] The model used to interpret the data is the Scatchard model[127] expressed in equation 4.3.

$$\frac{r_d}{[A]} = K_d n - K_d r_d \qquad (4.3)$$

Where: r_d = no of probe molecules per mole of HSA

K_d = binding constant of probe molecule

[A] = concentration of unbound probe molecules

n = number of binding sites

Using a plot of r/[A] against r you obtain a slope of $-K_d$ and an intercept of nK_d
Inverting the above equation gives equation 4.4.

$$\frac{1}{r_d} = \frac{1}{K_d[A]n} + \frac{1}{n} \qquad (4.4)$$

using a plot of 1/r against 1/[A] should give a straight line plot with a slope of $1/K_d n$ and an intercept of 1/n. The parameters for equations 4.3 and 4.4 were calculated from the fluorescence intensity using the equations below

$$c_b = \frac{F}{F^{lim}} * [probe_{lim}] \qquad \text{therefore,} \quad r_d = \frac{c_b}{[HSA]}$$

Therefore the unbound ligand concentration can be calculated[145,146] using

$$[A] = [c_t - c_b]$$

In the presence of these compounds equation 4.3 could be expanded as shown in equation 4.5. to find binding constants for the compounds rather than the probes.

$$K_d = \frac{r_d}{[A](n - r_d - r_f)} \qquad (4.5)$$

where, r_f = no of fentichlor molecules per mole of HSA

The inverse of equation 4.5 gives equation 4.6 which should again give a straight line plot.

$$r_d = \frac{1}{K_d[A](n - r_f)} + \frac{1}{(n - r_f)} \qquad (4.6)$$

In equations 4.5 and 4.6 there is the term r_f which cannot be measured and one must therefore be able to calculate it from the parameters already known. One method to find the value of r_f is to solve the quadratic equation 4.7.

$$K_d = \frac{r_f}{([f^0] - r_f[HSA])(n - r_d - r_f)} \qquad (4.7)$$

rearrange to get

$$r_f^2[HSA]K_d + K_d[f^0](n - r_d) = r_f(1 + K_d[HSA]_n - r_d[HSA]K_d + K_d[f^0])$$

where f^0 = concentration of fentichlor added

solving using $-b \pm \frac{\sqrt{b^2 - 4ac}}{2a}$

which gives the equation 4.8 for r_f

$$-(1+K_d[HSA](n-r_d)+K_d[f^0])\pm\frac{\sqrt{(1+K_d[HSA](n-r_d)+K_d[f^0])^2-4K_d^2[HSA][f^0](n-r_d)}}{2[HSA]K_d} \quad (4.8)$$

The quenching of the probe-HSA fluorescence observed could be less than expected if the quencher did not penetrate into the interior of the protein. This could be due to a surface only effect which is characterised by a downward curve of the graph of 1/r against 1/[A].

Where the probe has bound, an indication of the strength of the binding can be calculated from

$$\% \text{ probe bound} = F_0/F_b \times 100$$

where F_b = bound fluorescence

F_0 = observed fluorescence

$F_2/F_1 \times 100$ = fluorescence as % initial fluorescence.[147,148]

All the above methods of analysing dark binding data were used to find the binding parameters of both bithionol and fentichlor binding to H.S.A.

4.1.2. Dark binding results

4.1.2.1. Fluorescence quenching of tryptophan fluorescence by bithionol and fentichlor.

Binding studies were initially carried out by the quenching the fluorescence of tryptophan residue in the H.S.A. The quenching data obtained was analysed using the Scatchard equation 4.2. From this equation a plot of I_0/α against $1/(1-\alpha)$ should give a straight line for a compound which binds to 1 independent binding site on H.S.A. Bithionol and fentichlor however both gave a curved plot of I_0/α v's $1/(1-\alpha)$,

suggesting the presence of more than 1 class of binding site,[136,139] or the presence of many weaker binding sites as seen by other groups with photochemical binding, or that bithionol or fentichlor affects the fluorescent properties of binding sites to which they are not bound possibly by disrupting the conformation of the H.S.A. molecule.[149] This suggests that the sites are not identical or independent which is an assumption made with the Scatchard equation.

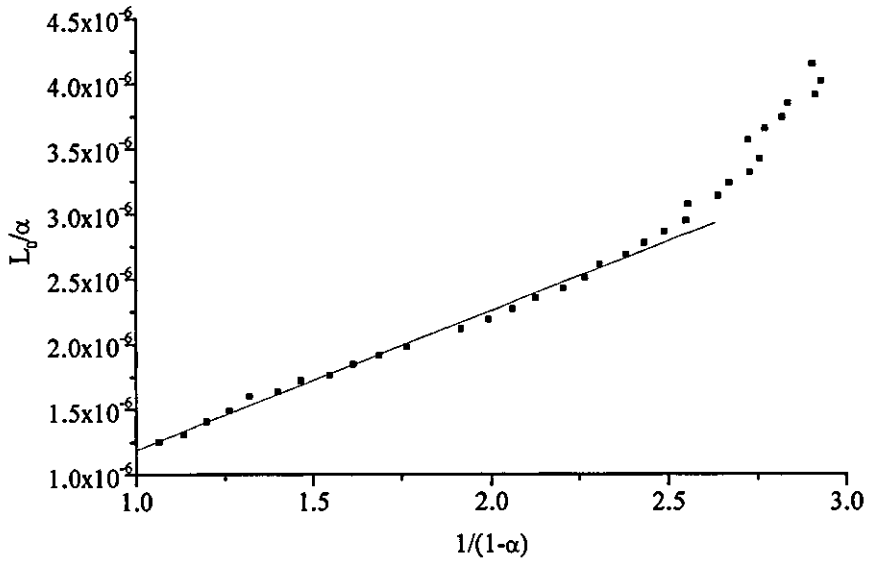


Figure 4.1: The binding of increasing concentrations of bithionol to $1x10^{-6}$ M H.S.A.

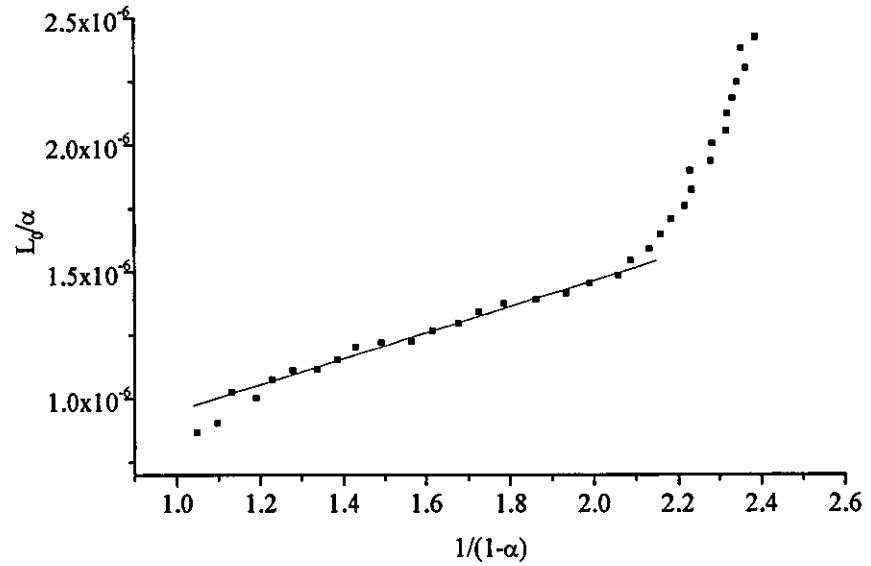


Figure 4.2: The binding of increasing concentrations of fentichlor to $1x10^{-6}$ M H.S.A.

The plot of fluorescence quenching is curved therefore it is difficult to derive binding parameters from the plots as binding at different sites may cause varying degrees of quenching.[145] By fitting the initial part of the curve a value can be obtained for the binding parameters of the strong sites when multiple sites are present. The initial part of these curves were fitted to a straight line and were found to give the binding parameters listed in table 1.

Compound	n	$K_d/mol^{-1}l$
Fentichlor	0.16	$1.1x10^{-6}$
Bithionol	0.44	$5.1x10^{-6}$

Table 1: Binding parameters obtained by the quenching of the tryptophan signal.

The value for n, the number of binding sites, should be an integer because it is not possible to have a fraction of a binding site. Therefore there are obviously problems with the values obtained. There is no explanation with this theory for the number of binding sites being lower than 1, however previous studies have approximated all values above 0.5 to be 1.[150] It could however be that the compounds are binding to several sites at one time or affecting the fluorescence of several sites to which the photoallergens are not bound. The problem with using equation 4.2 is that the double reciprocal plot of $1/\Delta F$ against 1/[ligand] to find ΔF_{max} is not always perfectly linear and therefore the answer for ΔF_{max} is not very accurate. A small change in the gradient causes a large change in ΔF_{max}, which will cause a large change in the values of n and k. One explanation for the lowering of the binding constant was due to the use of commercial H.S.A. which is shown later in this thesis to be partly dimerised and it is only the monomer H.S.A. which is bound. The use of monomer H.S.A. should more than double the binding constants and therefore the values could be approximated to 1 as seen previously.[150]

The second part of the trace may be due to several weaker binding sites. Other methods of analysing the data,[151] including fitting the raw data directly to the equation 4.2, were carried out but were unsuccessful. These experiments have proved that binding possibly occurs between both fentichlor and bithionol and H.S.A. and that the

binding occurs initially strongly to 1 class of sites before binding to many weaker binding sites. It has also shown that the Scatchard model is not a good model for these compounds as the binding sites are probably not independent or identical.

4.1.2. DNSA and dansylsarcosine as fluorescent probes

DNSA and dansylsarcosine are used as fluorescent probes for drugs binding to H.S.A.. DNSA binds to site 1 and Dansylsarcosine binds to site 2 on H.S.A. The experiments were carried out on both commercial H.S.A. and monomer H.S.A..[130] Monomer H.S.A. gives a different result for the binding parameters to the results obtained with commercial H.S.A. possibly due to dimerization occurring in commercial H.S.A.. Since binding only takes place with the monomer H.S.A.. it is better to remove the dimer from any H.S.A. used.

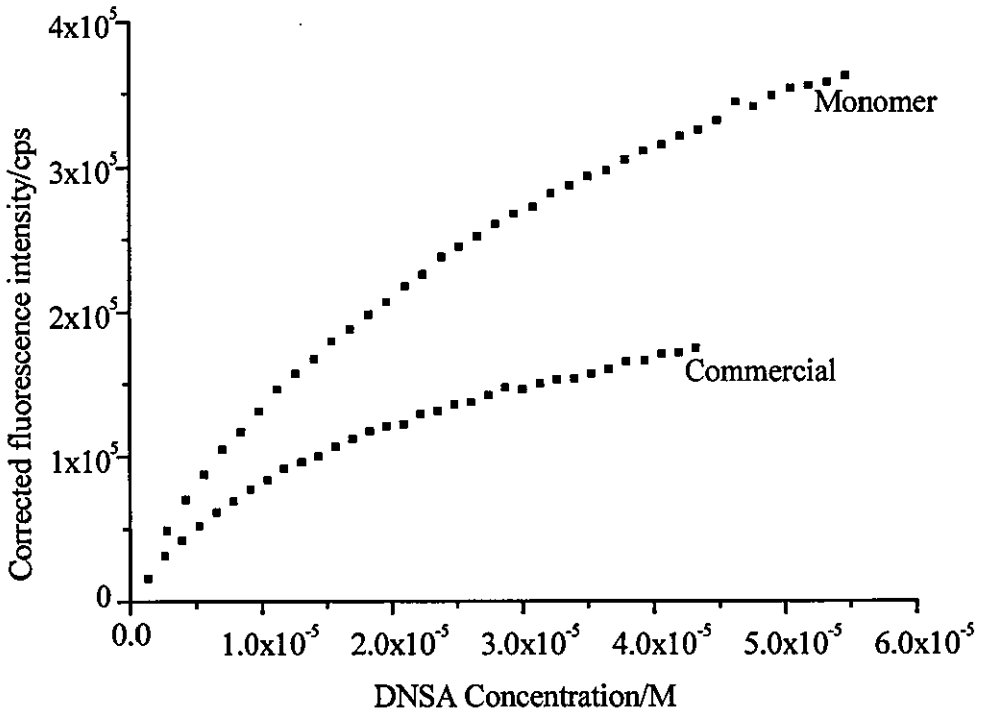


Figure 4.3: The increase in the fluorescence with increasing DNSA concentration for commercial and monomer H.S.A($1x10^{-6}$ M).

Figure 4.3 shows the difference in the binding between monomer and commercial H.S.A. The initial experiments were carried out with just the probe and H.S.A. present. Equation 4.3 was used to analyse the data and was found to give a straight line plot for

r/A against r as found by Sudlow et al.[146] The double reciprocal plot of 1/r against 1/[A] from equation 4.4 also gave a straight line as found in previous work with various drugs.[146] The fentichlor was added to the H.S.A., to competitively bind with the probes, and this mixture was titrated against the probe. Several different concentrations of fentichlor were used but the concentration of H.S.A. used was always the same ($1x10^{-5}$ M). The graphs of r/A against r in the presence of fentichlor were no longer linear but the double reciprocal plot of 1/r against 1/A still gave a straight line as shown in figures 4.4 and 4.5. and seen by Sudlow et al,[146] allowing binding parameters to be calculated.

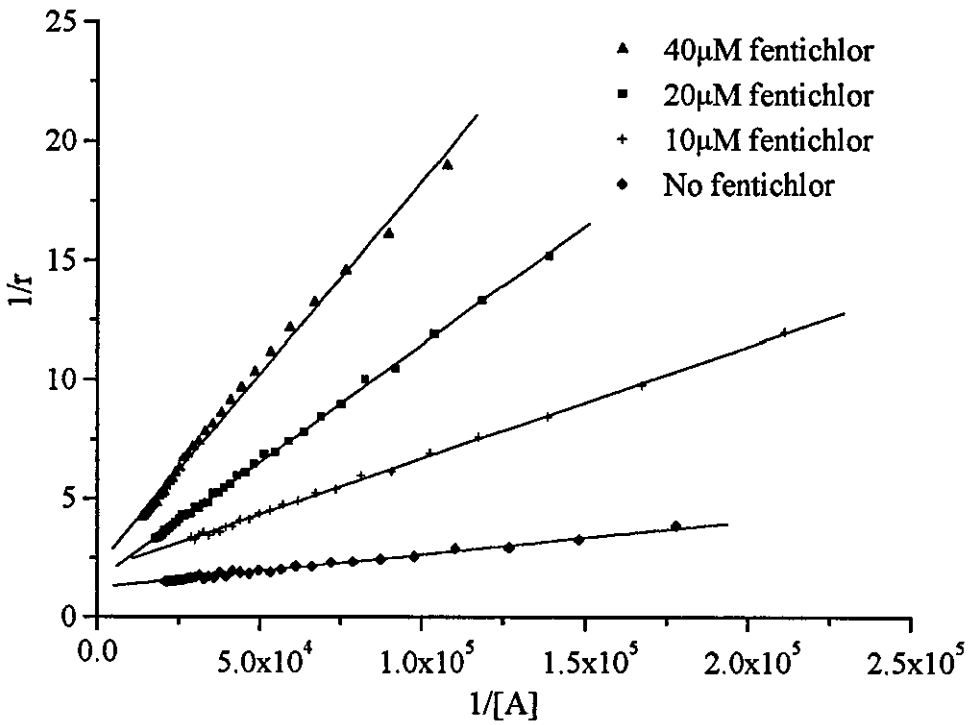


Figure 4.4: The competitive binding between fentichlor and DNSA to $1x10^{-6}$ M H.S.A.

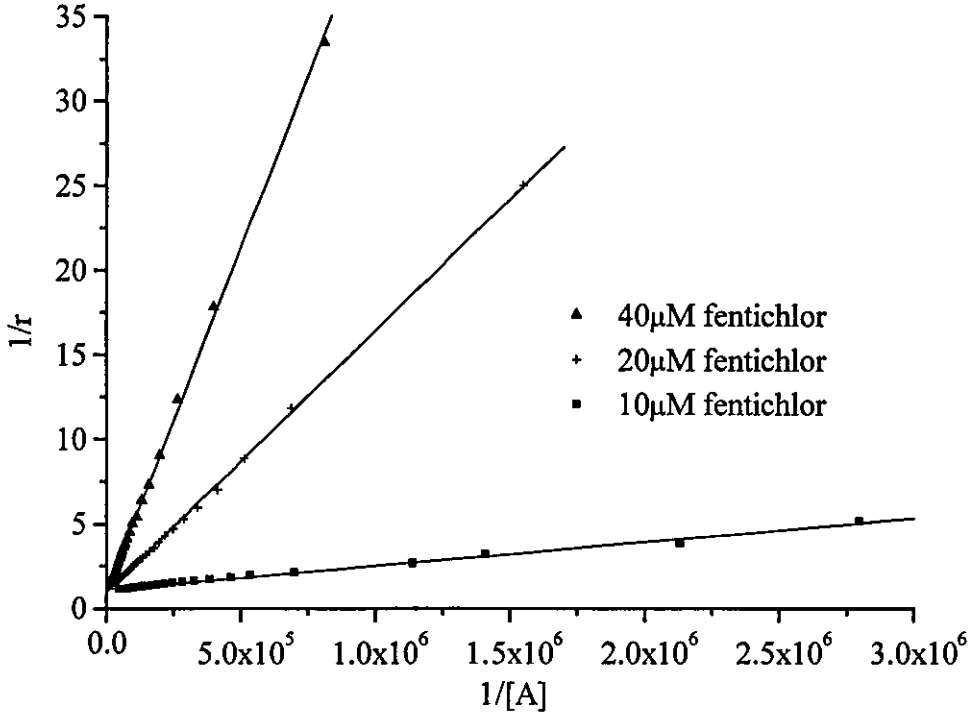


Figure 4.5: The competitive binding between fentichlor and dansylsarcosine to $1x10^{-6}$ M H.S.A.

From these plots of 1/r against 1/[A], from equation 4.4, values of n and K could be found, where the slope = $1/nK_{dd}$ and the intercept = 1/n which are shown in tables 2,3 and 4. The 40 μM titration of H.S.A. with fentichlor and H.S.A. shows a move away from linearity perhaps due to the H.S.A. binding sites being saturated. In these tables the K_{dd} represents the binding constant for the probe and K_{df} is the binding constant for the fentichlor.

Fentichlor concentration/μM	n	$K_{dd}/mol^{-1}l$
0	0.37 ± 0.02	$8.71x10^4 \pm 200$
0	0.37 ± 0.01	$7.84x10^4 \pm 200$
10	0.23 ± 0.005	$5.43x10^4 \pm 100$
10	0.26 ± 0.04	$6.17x10^4 \pm 100$

Table 2: Binding parameters obtained from titrating DNSA with commercial H.S.A.

Compound	Compound concentration /μM	n	K_{dd}/Mol^{-1}l	K_{df}/Mol^{-1}l
None	0	0.662 ± 0.007 0.763 ± 0.008	1.79x10^5 ± 1000 1.04x10^5 ± 1000	0
Fentichlor	10	0.51 ± 0.01 0.524 ± 0.008	4.66x10^4 ± 350 5.17x10^4 ± 400	8.4x10^5 ± 4000 6.1x10^5 ± 3500
Fentichlor	20	0.64 ± 0.01	1.59x10^4 ± 100	6.3x10^5 ± 3000
Bithionol	5	0.56 ± 0.01	3.32x10^4 ± 300	1.761x10^6 ± 5000

Table 3: Binding parameters obtained from titrating DNSA with monomer H.S.A..

Compound	Compound concentration /μM	n	K_{dd}/Mol^{-1}l	K_{df}/Mol^{-1}l
None	0	0.9 ± 0.1 0.84 ± 0.08	6.10x10^5 ± 1200 1.171x10^6 ± 1300	0 0
Fentichlor	10	0.75 ± 0.05 0.81 ± 0.06	5.34x10^5 ± 1000 3.213x10^5 ± 800	1.13x10^5 ± 1500
Fentichlor	20	1.03 ± 0.02	6.31x10^4 ± 200	8.90x10^5 ± 2000
Fentichlor	40	1.09 ± 0.03	2.24x10^4 ± 100	1.112x10^6 ± 5000
Bithionol	5	0.86 ± 0.06	2.482x10^5 ± 900	1.914x10^6 ± 5000

Table 4: Binding parameters obtained from titrating Dansylsarcosine with monomer H.S.A.

There is a difference in the results obtained from commercial H.S.A. and monomer H.S.A. as seen in tables 2,3 and 4. This shows that only the monomer H.S.A. is binding and that the commercial H.S.A. has a large amount of dimer present. Therefore the values of n and K_{dd} are lower in commercial H.S.A. than in monomer H.S.A.. Fentichlor and bithionol compete with both dansylsarcosine and DNSA to bind to H.S.A. which shows they bind to H.S.A. in both the site 1 and the site 2 positions. The

number of binding sites in each case is ≈1.[145] The K_{dd} decreases with increasing concentration of fentichlor, however the value of n is always similar as seen with drugs.[147,148] Figure 4.6 shows the change in the binding constant with change in the mole ratio of fentichlor and H.S.A..

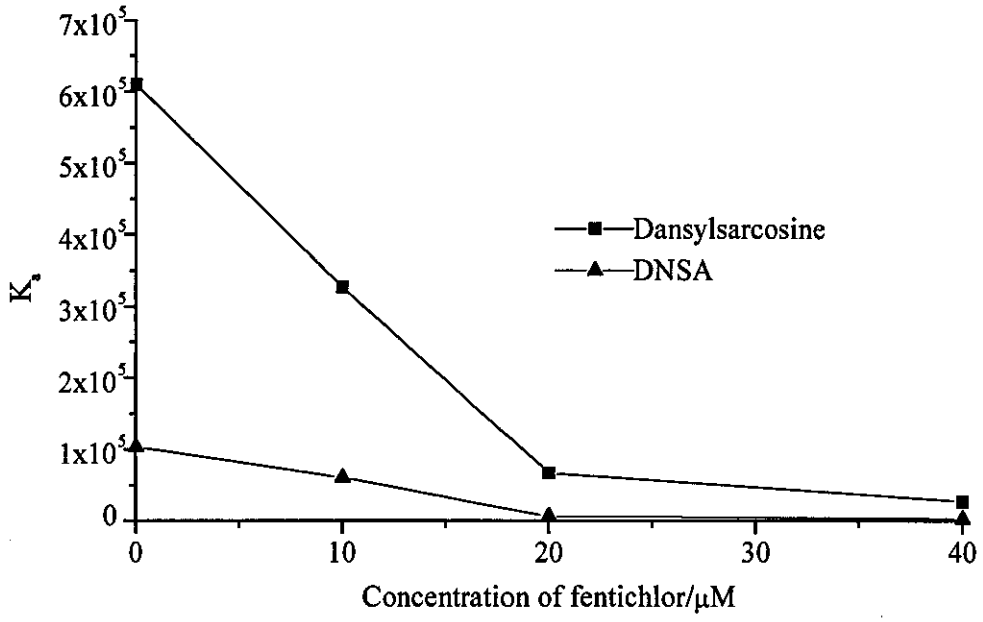


Figure 4.6: The effect of fentichlor concentration on the binding constant.

The increase in the fentichlor concentration causes a decrease in binding constant probably with more sites being filled by the fentichlor. This shows there is more competition for the binding sites at higher fentichlor concentrations. This would suggest that the concentration of fentichlor added was not enough to saturate all the DNSA binding sites possibly due to the fentichlor binding to another site.

When the titration is performed the other way around n is still equal to 1 but K_{dd} is almost double the value. It has been assumed that this reaction is in equilibrium between the photoallergen and the fluorescent probes for the binding sites on the H.S.A.. This may not be true as the protein binding may actually not be totally due to competitive displacement but may also result from conformationally mediated interactions between distinct binding sites. Therefore a high degree of conformational mobility means that binding sites for exogenous components are unlikely to be

independent [145,148] Also the fentichlor and bithionol may bind to other sites on the H.S.A. in addition to or instead of the sites being probed. Other groups have noted that when a compound binds to both site 1 and site 2 it could also bind to a third site. It has also been speculated that the compounds may in fact not be bound to sites 1 and 2 but be bound to another site close by which is affecting the fluorescence.[146] Also drugs were shown to bind to H.S.A. but not to either site 1 or site 2 showing the presence of 1 other major site or several other minor binding sites.[146] The binding of some drugs to site 2 caused changes in the structure of site 1.[148] This is further evidence of the possible presence of multiple binding sites seen in the tryptophan quenching experiments with fentichlor and bithionol.

The use of fluorescent probes has found the binding parameters for the probe DNSA and dansylsarcosine but still gives no indication of the binding parameters for the fentichlor. A method to find the binding parameters was found using the equation 4.8 and the results for K_{df}, the binding constant of fentichlor and bithionol, are shown in tables 2,3 and 4. The value obtained for K_{df} for fentichlor were in the region 6 - $8x10^5$ for site 1 and $1x10^5$ - $1x10^6$ for site 2 and for bithionol K_{df} were $1.78x10^6$ for site 1 and $1.91x10^6$ for site 2.

The strength of binding to a site on H.S.A. can be estimated from a plot of % fluorescence as a function of initial fluorescence against mole ratio as explained earlier. As can be seen from figure 4.7 the % fluorescence as a function of initial fluorescence dropped with an increase in the fentichlor concentration which is probably due to there being less freely available sites where DNSA could bind. This suggests that less DNSA or dansylsarcosine is bound due to there being more competition by the fentichlor. The % displacement of the DNSA probe with 10 μM fentichlor is 20% and for dansylsarcosine is 15% which shows that the binding to these sites by the compounds is not very strong and therefore these may be minor sites or that bithionol and fentichlor could be binding to other sites. Photochemical binding suggests binding to many weaker sites rather than 1 major site[40] and the same may be true for dark binding.

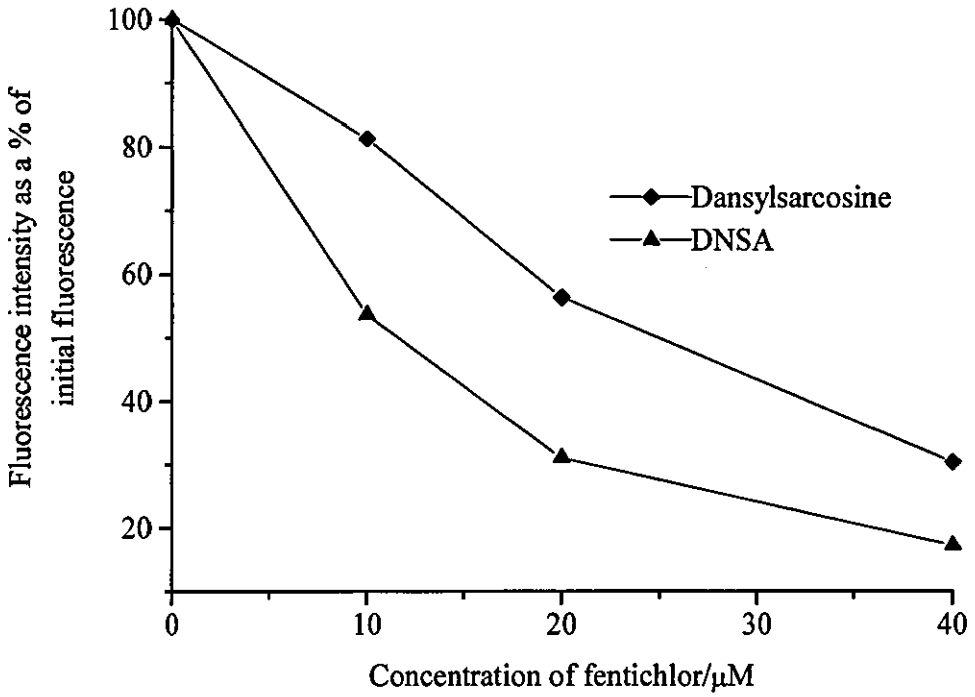


Figure 4.7: The effect of fentichlor concentration and 10 μM H.S.A. on the % fluorescence intensity.

Summary

Quenching of the tryptophan signal on H.S.A. by fentichlor and bithionol showed that dark binding occurs between fentichlor or bithionol and H.S.A.. These showed the possible presence of 1 class of binding sites to which fentichlor and bithionol bind initially and the presence of many weaker sites to which they bind randomly. These sites are probably not independent or identical and therefore the Scatchard equation would no longer be valid for the analysis of the binding data. The use of fluorescent probes to investigate the dark binding showed that both fentichlor and bithionol bind to site 1 (Lys199-Glu292) and site 2 (Pro384-Phe488) on H.S.A.[146] Binding constants for bithionol was found to be 1-10x10^5 and for fentichlor to be 6-8x10^5. The competitive reaction between the fentichlor and the probes was found to be not in equilibrium, which suggests that the fentichlor binds to other sites or affects the fluorescence of other sites on the H.S.A. Fentichlor and bithionol only bound weakly to site 1 and site 2 which suggests they are minor rather than major sites. This may suggest the presence

of another major site or the presence of several minor sites as seen with photobinding.[40] There is a need for a different method of analysing the binding data, which does not make the assumption that the binding sites are independent and identical.

4.2. Photochemistry of H.S.A.

The photochemistry of the H.S.A. was measured in pH 7.4 buffer. The ground state absorption spectrum of H.S.A. shows 1 peak with a λ_{max} at 280 nm. On the addition of H.S.A. to the photoallergens the resulting absorption spectrum was due to the sum of the two compounds.

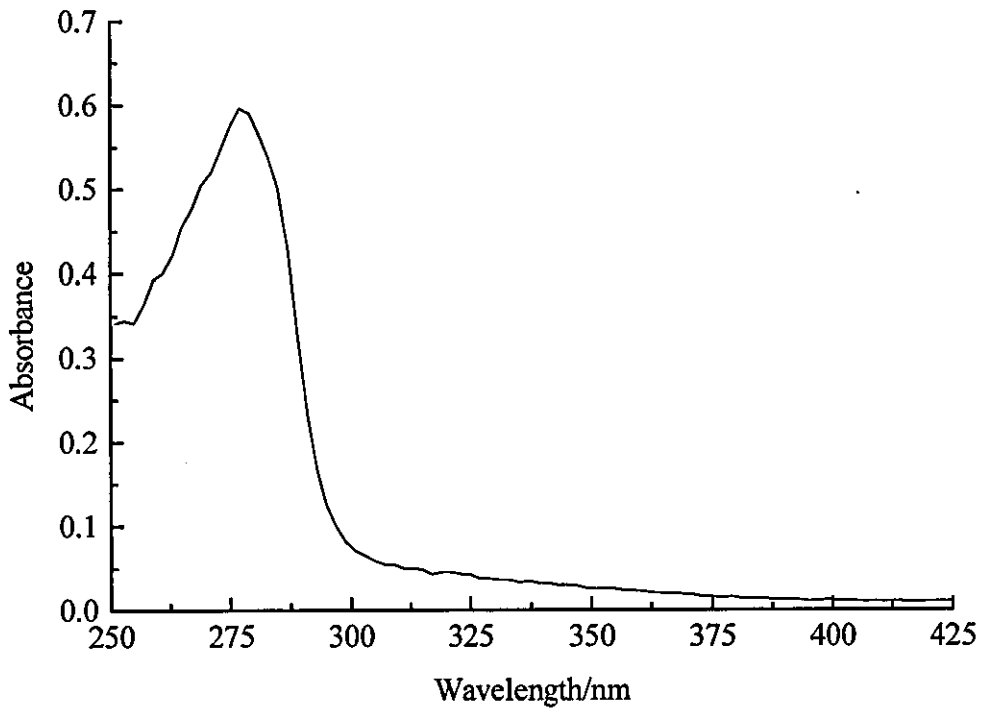


Figure 4.8: Ground state absorption spectrum of H.S.A. in pH 7.4 buffer.

The fluorescence spectrum of H.S.A. showed 1 peak with a λ_{max} at 345 nm when exciting the tryptophan residue at 295 nm.

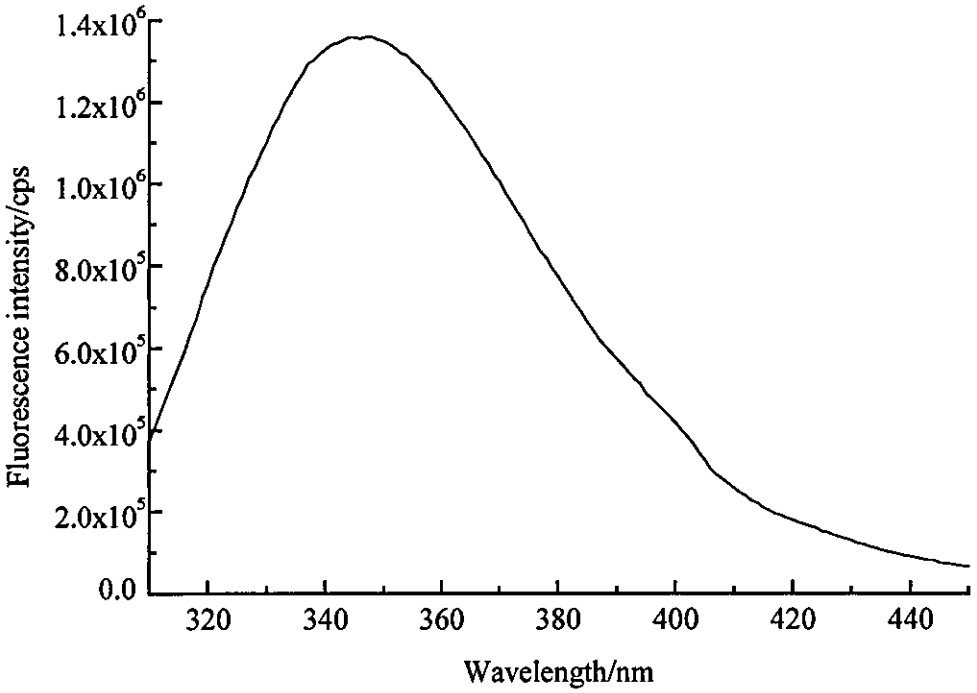


Figure 4.9: Emission spectrum of H.S.A. at room temperature exciting at 295 nm with excitation slits = 1 mm, and emission slits = 2 mm.

There was no transient absorption detectable after laser excitation at 355 nm or 266 nm, at any of the experimental parameters used in this thesis.

Chapter 5
Results and Discussion

5. Results and discussion

The purpose of this thesis was to find the definition of a photoallergen. In the previous studies of photoallergens a single compound has been studied by a variety of methods including E.S.R., laser flash photolysis and by in vivo methods. In each case the photochemistry observed has been assumed to be the cause of the photoallergy as with T_4CS[81-87], or not , as with chlorpromazine and promazine.[104-110] These reports have not attempted to find what actually constitutes a photoallergen. This thesis sets out to investigate the photochemistry of 8 separate photoallergens to try and find a connection between all the photoallergens and therefore find what constitutes a photoallergen. This should allow photoallergens to be identified without the need for animal tests.

This chapter is concerned with the determination of the photochemistry of these compounds and the discussion of their photochemistry as individual compounds. The comparison of the photochemistry and any connections made with photoallergy are made in the last chapter. The photochemistry of the photoallergens were measured in three different situations. The basic photochemistry was carried out in solution. Various different solvents were used in order to understand the full photochemistry of the compounds, as the photochemical properties of the compounds sometimes varied with solvent. The main solvent used was either water or $\leq$ 10% (v/v) ethanol in water as it was assumed that the photoallergy reaction would be occurring in aqueous solutions in the body.

Once the basic photochemistry of these compounds had been established, these results were compared with the photochemistry of the compounds adsorbed onto cellulose. These experiments were carried out to investigate any differences in the photochemistry seen due to adsorption onto surfaces. The cellulose ground state experiments were recorded against barium sulfate. However it was found that barium sulfate showed some deviation from 100% reflectance below 300 nm. There were also problems with emission from both the photoallergens and the cellulose below 300 nm causing the intensity of the ground state spectra to be lower than expected at wavelengths below 300 nm.

The final experiments carried out were in aqueous solutions with the addition of H.S.A. to investigate the effect the presence of a protein had on the photochemistry of the photoallergens. This was considered necessary as it has been shown from in vivo studies that the photoallergens bind, either covalently or non covalently, to the proteins in the skin before causing a photoallergic reaction.[46-55] The measurement of the phosphorescence at 77 K in aqueous solutions meant the glass formed was very cracked and therefore the results were not as accurate as would be liked. The quantum yields measured in the presence of H.S.A. showed the maximum change in the quantum yields obtainable with H.S.A..

5.1. Bithionol and Fentichlor

Fentichlor and bithionol are structurally related compounds as shown in section 2.2.1 and would therefore be expected to have a similar photochemistry. Fentichlor and bithionol are only soluble in aqueous solution above pH 8 but they are soluble in most organic solvents. The solvent chosen for these studies therefore, was an ethanol water mixture usually consisting of 10% ethanol to 90% water.

5.1.1. Ground state absorption spectra

Ground state absorption spectra for fentichlor and bithionol in ethanol are shown in figures 5.1.

The λ_{max} which is due to a $\pi \rightarrow \pi^*$ transition was located at 305 nm in both bithionol and fentichlor. The ground state absorption spectra were taken for these compounds in toluene, propanol and acetonitrile and little difference was found in the position of the λ_{max} between the different solvents. The molar absorption coefficient at the peak was calculated and found to be 6300 $mol^{-1}cm^{-1}dm^3 \pm 200$ $mol^{-1}cm^{-1}dm^3$ for bithionol in 10% ethanol and 90% water at pH 7.4 and 6000 $mol^{-1}cm^{-1}dm^3 \pm 250$ $mol^{-1}cm^{-1}dm^3$ for fentichlor in ethanol and water at pH 7.4. As explained later the spectra of bithionol and fentichlor are very sensitive to pH changes and therefore these values for the extinction coefficient will vary with pH.

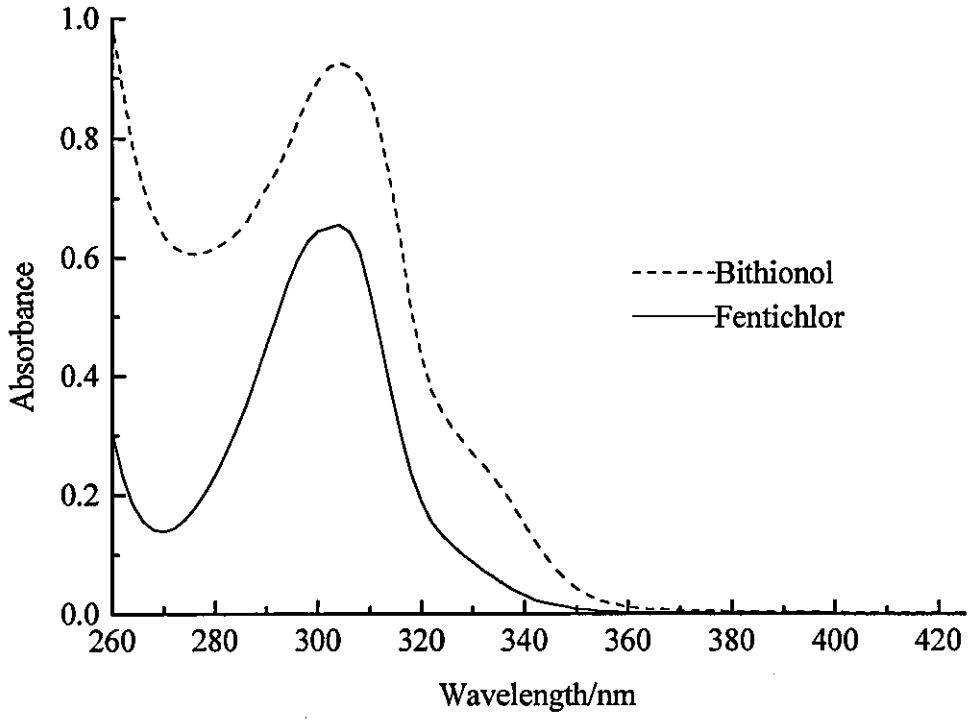
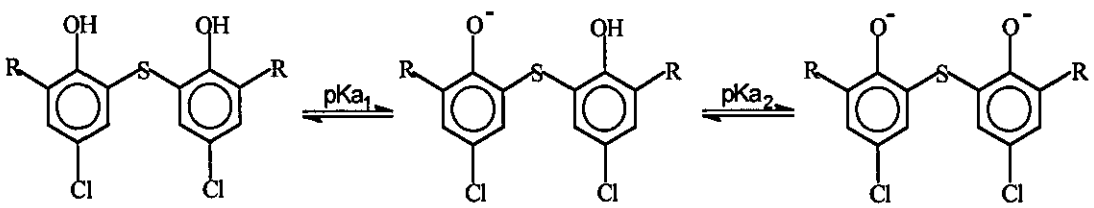


Figure 5.1 : Ground state absorption spectra of 8.2×10^{-5} M fentichlor in ethanol and 1.0×10^{-4} M bithionol in ethanol.

The ground state absorption spectra of bithionol and fentichlor are known to change with changing pH[59-61] in 95 % ethanol solutions this effect is shown in figures 5.2 and 5.3. The increase in the pH leads to a red shift in the position of the λ_{max}. This is due to the deprotonation of the hydroxyl groups.



Where R = Cl or H.

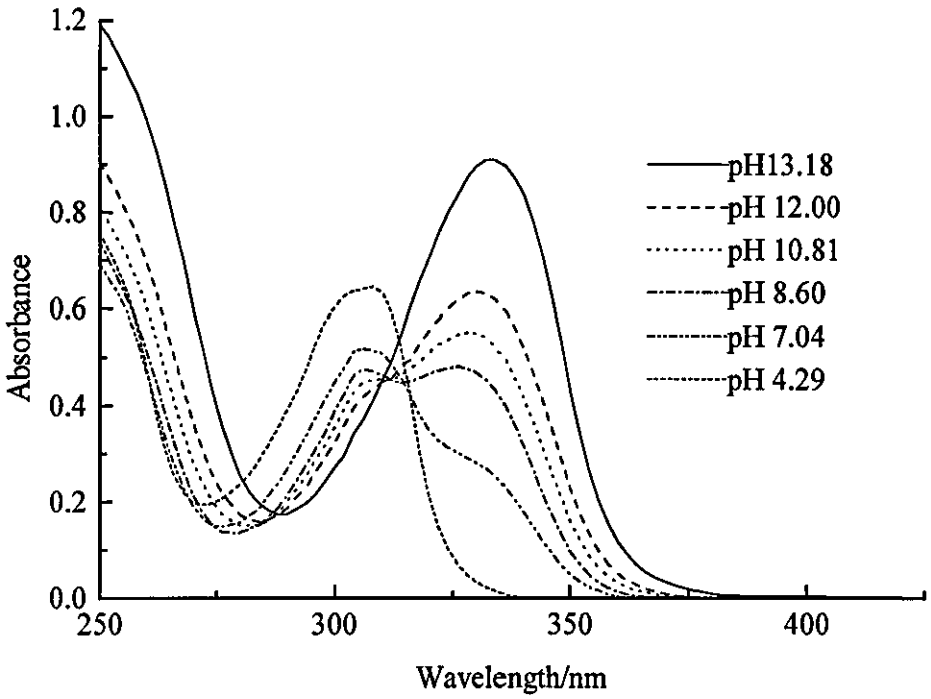


Figure 5.2: Ground state absorption spectra of bithionol, $1.0x10^{-4}$ M, at various pH's in 10% ethanol 90 % water solution

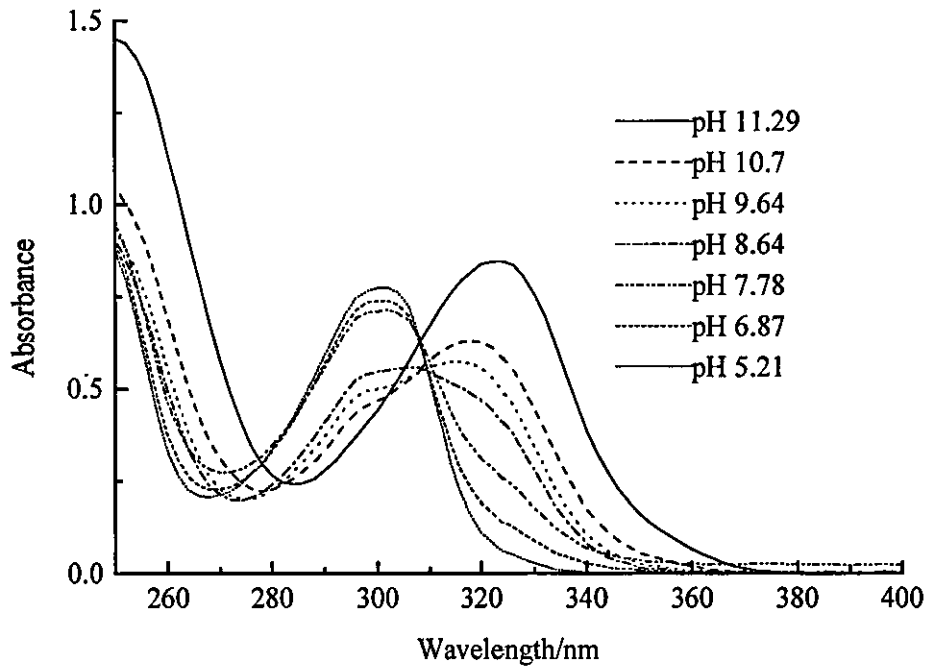


Figure 5.3: Ground state absorption spectra of fentichlor $9.9x10^{-5}$ M at various pH's in 10% ethanol 90 % water solution.

The peak at 330 nm for bithionol and at 324 nm for fentichlor are due to the non protonated form of the compounds and have molar absorption coefficients of 9300 $mol^{-1}cm^{-1}dm^3 \pm 525$ $mol^{-1}cm^{-1}dm^3$ for fentichlor and 10000 $mol^{-1}cm^{-1}dm^3 \pm 650$ $mol^{-1}cm^{-1}dm^3$ for bithionol at pH 11.4. These values are very similar to the values of 8000 and 13000 for fentichlor and bithionol respectively at pH 9 found by Chignell et al.[60] The peaks at 305 nm for fentichlor and bithionol are for the protonated forms of the compound.

There are two sets of isosbestic points in the spectra which show that there are three protonation states involved in this reaction and therefore there are two pKa's. There were no more changes seen even though the ground state absorption spectra were measured down to pH 1. This result does not agree with Chignell et al[60] who claim that there is only one isosbestic point present at these pH's and therefore there is only one protonation reaction occurring. They believe the two species involved are the partially protonated form and the deprotonated form. However it does not seem likely that this compound would be in the partially protonated form at pH's as low as pH 1 rather than the fully protonated form. Inspection of the spectra presented in this paper[60] also show imperfect isosbestic points which may indicate the presence of two isosbestics very close together which would agree with this work where two of the isosbestic points were very close together at 314 nm and 315 nm for fentichlor and 312 nm and 313 nm for bithionol. The loss of one hydrogen occurs over a very small range of pH's and the second hydrogen is lost very quickly after the first.

An accurate value for the pKa cannot be found due to the isosbestic points on the absorption spectra being very close together and also due to the presence of ethanol in the solvent causing slight inaccuracies in the measurement of the pH values. An estimate of the pKa's was made for both compounds and it was found that pKa_1 was 8 and 7 and pKa_2 was 8.5 and 8 for fentichlor and bithionol respectively. These values are close to the pKa value of 9.4 found for the same protonation reaction with 4-chlorophenol by Brown et al.[152]

The ground state diffuse reflectance (Kubelka Munk) spectra were measured for bithionol and fentichlor adsorbed onto cellulose. The spectra were found to be very similar to the ground state absorption spectra of these compounds in polar solvents shown in figure 5.4, which are due to the protonated form of the sample.

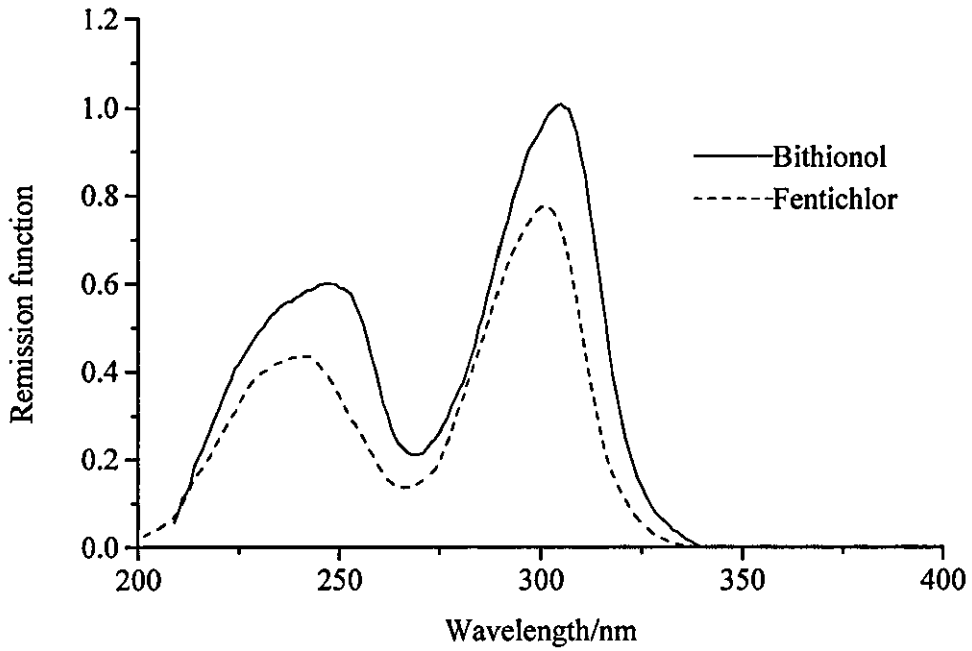


Figure 5.4: Ground state diffuse reflectance (Kubelka Munk) spectrum of 5 mg/g fentichlor and 12 mg/g bithionol

The λ_{max} occurs at 305 nm for bithionol and 300 nm for fentichlor compared to 305 nm for both compounds in ethanol. The peaks are broader when adsorbed onto cellulose than in solution due to the inhomogeneity of the cellulose. There is a change in the ratio of the peaks with adsorption of the compounds onto cellulose. This effect causes the adsorption at higher wavelengths to be greater than the adsorption at lower wavelengths probably due to the emission from bithionol and fentichlor as explained earlier.

5.1.2. Emission spectroscopy

5.1.2.1. Fluorescence

These compounds were found to be only weakly fluorescent when excited at 314 nm. They show a λ_{max} at 400 nm for bithionol and 430 nm for fentichlor at pH 7.4 in ethanol and water as shown in figure 5.5.

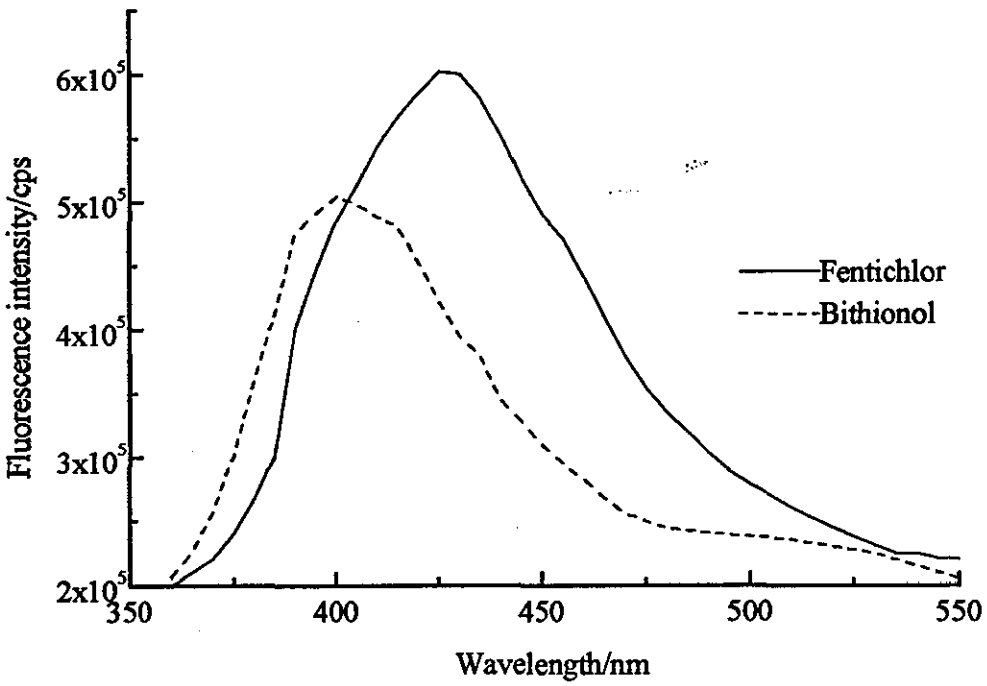


Figure 5.5: Emission spectra of 4.1×10^{-5} M bithionol and 3.02×10^{-5} M fentichlor in 10% ethanol solution exciting at 314 nm with excitation slits = 1mm and emission slits = 2 mm..

The position of the emission was found to be independent of the exciting wavelength. The energy of the lowest excited singlet state could be calculated from the midway position between the λ_{max} of the fluorescence and ground state absorption as described in chapter 3 and was found to be 326 $kJmol^{-1} \pm 4$ $kJmol^{-1}$ for fentichlor and 339 $kJmol^{-1} \pm 5$ $kJmol^{-1}$ for bithionol. The fluorescence spectra were recorded with and without the presence of oxygen and were found not to be affected by the presence of oxygen and therefore the fluorescence measurements could be carried out in aerated solutions. This suggests the lifetime of the fluorescence was too short to be quenched by molecular oxygen. The lifetime however, can not be found experimentally in our laboratory due to the low quantum yield of the fluorescence, but an estimate of the

radiative lifetime, using the method described in chapter 3, is ~$1.6x10^{-8}$ s. The quantum yield could not be measured accurately but was taken to be approximately $1x10^{-4}$ and from this the real fluorescence lifetime was calculated to be ~$1.6x10^{-12}$s. The excitation spectrum was found to be the same as the ground state for both compounds showing there were no other impurities present.

The fluorescent emission spectra for both compounds were measured at different pH's exciting at 314 nm, which was the isosbestic point of the ground state absorption spectra with changing pH. This exciting wavelength was chosen so the absorbances of all the compounds would be the same value for the same concentration of the compounds. The fluorescence spectra show a red shift with increasing pH which is the same effect as seen with the ground state absorption spectra which is the same as the red shift of the fluorescence with increasing pH seen with 4-chlorophenol.[152] However the fluorescence spectra of bithionol and fentichlor are very weak and noisy so no further information about the protonation reactions of the excited states was gained from these spectra. This would suggest the excited singlet state exists in all the same protonated forms seen in the ground state. This agrees with the results found for 4-chlorophenol where the excited state pKa was found from the fluorescence spectra to be 9.4 which is identical with the ground state pKa.[152]

The emission spectra of fentichlor and bithionol adsorbed onto cellulose was very weak but showed 1 peak with a λ_{max} at 360 nm for both compounds. This emission is at a lower wavelength than in solution at pH 7.4 as shown in figure 5.6 but is the same as seen in alcoholic solutions which is probably due to the compounds being initially dissolved in alcohol.

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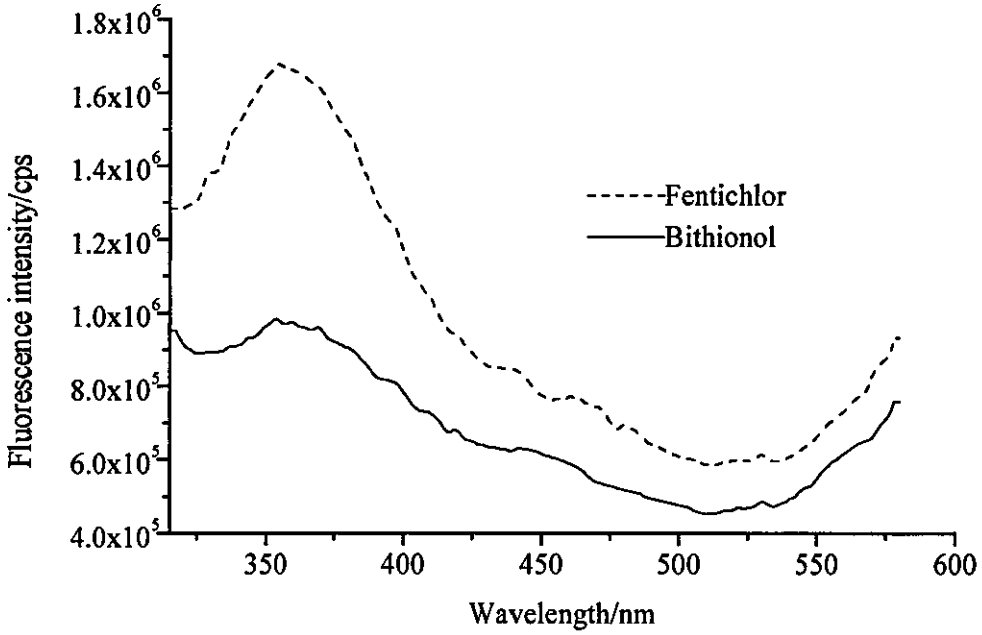


Figure 5.6: Emission spectra of 12 mg/g bithionol and 5 mg/g fentichlor absorbed onto cellulose, Slit widths Ex = 1 mm, Em = 2 mm.

The addition of H.S.A. to solutions of bithionol and fentichlor greatly enhanced the fluorescence and caused a red shift in the fluorescence as shown in figures 5.7 for bithionol with and without H.S.A. This enhancement of the fluorescence is quite often used to study the dark binding of a compound to H.S.A., as seen in chapter 4 as the H.S.A.-photoallergen complex is found to be far more fluorescent than the photoallergen alone. There are several possible explanations for the increase observed in the fluorescence.

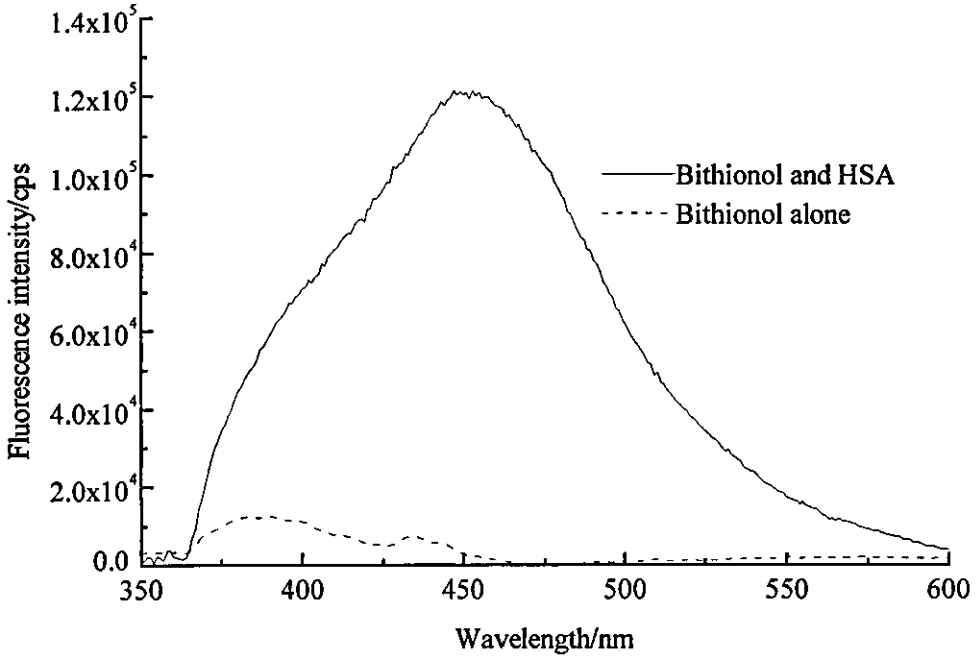


Figure 5.7: Emission spectra of bithionol with and without $5.0x10^{-6}$ M H.S.A. exciting at 314 nm with excitation slits = 0.75, emission slits = 1.5 mm in pH 7.4 buffer.

Other research has suggested that the quenching seen in the tryptophan residue fluorescence on addition of compounds to be bound is due to radiative energy transfer.[150] However energy transfer cannot be used to explain the reverse reaction as the ground state absorption spectra of bithionol and fentichlor do not overlap the fluorescence emission spectrum of H.S.A. and the enhancement of bithionol and fentichlor is seen even when the excitation wavelength is in a position where the H.S.A. does not absorb. Another explanation for the increase in the fluorescence could be that the binding of the bithionol and fentichlor to the H.S.A. forms a complex which decreases the competing nonradiative transitions from the excited singlet state or the dissociative decay of the excited singlet state.

5.1.2.2. Phosphorescence

Bithionol and fentichlor showed no phosphorescence at room temperature which was probably due to the phosphorescence having a long radiative lifetime and therefore being quenched in solution as explained in chapter 1. The samples were therefore cooled to 77 K to form an organic glass of 3:7 2-propanol:isopentane.[153] The samples were excited at 310 nm and showed a very broad and structureless emission spreading form 350 nm to 550 nm.

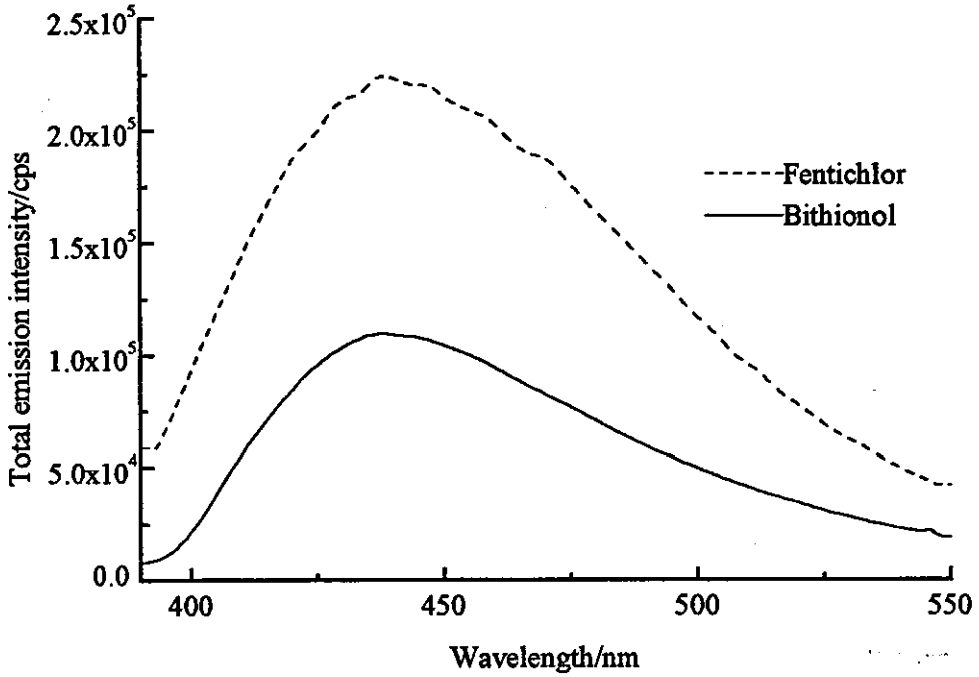


Figure 5.8:Emission spectra of bithionol and fentichlor in 7:3 isopentane:2-propanol at 77K exciting at 310 nm with emission slits = 0.3 mm, excitation slits = 0.15 mm.

The λ_{max} for the phosphorescent emission was found to be 445 nm for fentichlor and 450 nm for bithionol as shown in figure 5.8. The difference in the wavelength being attributed to the red shift due to the extra chlorines on the bithionol. The lower limit of the lowest excited triplet state energies could be calculated from the position of the λ_{max} of phosphorescence as described in chapter 3 and was found to be ≥ 268 kJmol^{-1} $\pm$ 3 kJmol^{-1} and for bithionol to be ≥ 265 kJmol^{-1} $\pm$ 3 kJmol^{-1}. The separation between the lowest excited triplet state and the lowest excited singlet state was found to be $\leq$ 57 kJmol^{-1} $\pm$ 7 kJmol^{-1} for fentichlor and ≤ 73 kJmol^{-1} $\pm$ 8 kJmol^{-1} for bithionol. The

quantum yields were measured against benzophenone whose quantum yield is known to be 0.84 in non polar solvents,[154] and they were found to be 0.56 for fentichlor and 0.34 for bithionol. The phosphorescence quantum yield of fentichlor is a lot higher than that of bithionol which is unexpected as the bithionol has more chlorine atoms than the fentichlor. It would therefore be expected that the bithionol would have a greater rate of intersystem crossing than the fentichlor due to the heavy atom effect, thereby increasing the phosphorescence. However chlorine is not a very heavy atom and the rate of intersystem crossing is already large, which is seen by the large phosphorescent quantum yields, and therefore the presence of 2 extra chlorines may have very little effect on the rate of intersystem crossing.

The phosphorescent lifetimes were measured using the HY laser in emission only mode exciting at 266 nm and were found to be 6.3 ms ± 0.25 ms for bithionol and 8.8 ms ± 0.08 ms for fentichlor.

The energy gap may suggest that the transition from the singlet to the triplet state is a $\pi \rightarrow \pi^*$ transition, however the low ϕ_F and high ϕ_P together with the millisecond lifetime of the phosphorescence is more typical of a $^3(n\pi^*)$. However with no obvious chromophores to support this transition. The triplet state is more likely to be a $^3(l,\pi^*)$ state probably due to the lone pairs on the sulfur.

There was no change in the intensity or lifetime of the phosphorescence of either compound on the addition of H.S.A. showing that binding to H.S.A. does not affect the triplet state of the molecule. This result may be considered surprising as an increase was observed in the fluorescent quantum yield which was assumed to be due to a decrease in the nonradiative transitions including the intersystem crossing. This can be explained where the quantum yield of fluorescence is defined in terms of the rate of fluorescence, rate of intersystem crossing and rate of internal conversion as shown below.

$$\phi_F = \frac{k_F}{k_F + k_{IC} + k_{ISC}}$$

Where, ϕ_F = Quantum yield of fluorescence of bithionol or fentichlor alone.

k_F = Rate of fluorescence of fentichlor or bithionol alone.

k_{IC} = Rate of internal conversion of fentichlor or bithionol alone.

k_{ISC} = Rate of intersystem crossing of fentichlor or bithionol alone.

If it is assumed that in solution $k_F << k_{IC} + k_{ISC}$

Then, $\phi_{ISC} = \frac{k_{ISC}}{k_{IC} + k_{ISC}}$

Where, ϕ_{ISC} = Quantum yield of intersystem crossing.

When the fentichlor and bithionol are complexed to H.S.A. the fluorescence increases therefore the non radiative transitions decrease i.e.

$$k'_{IC} + k'_{ISC} < k_{IC} + k_{ISC}$$

where, k'_{IC} = Rate of internal conversion in the presence of H.S.A.

k'_{ISC} = Rate of intersystem crossing in the presence of H.S.A.

If ϕ_{ISC} does not vary much with complexation to H.S.A. it follows that $k_F < k'_{IC} + k'_{ISC}$. Therefore a large change in the rate of fluorescence will only result in a small change in the intersystem crossing. Also the fluorescence measurements were carried out at room temperature whereas the phosphorescence measurements were carried out at 77 K and therefore these results could only be compared if the rate of internal conversion and the rate of intersystem crossing were independent of temperature.

5.1.3. Nanosecond laser flash photolysis

The samples were excited with the laser at 266 nm and 355 nm in an attempt to observe the transient absorption spectra for these compounds. However only a weak transient absorption was recorded at all wavelengths above 370 nm, following 355 nm

laser excitation, in both degassed and aerated solutions, which was too noisy to be analysed. The weak transient absorption could be due to a long lived transient (>10 ms) or the formation of a product. The lack of transient absorption assignable to the triplet state could be due to the triplet having a very low quantum yield or the lifetime of the triplet being too short to be measured using our apparatus. The former could be overcome by sensitisation of the triplet, using a suitable triplet sensitiser. The sensitiser chosen was benzophenone which has a triplet energy[154] of 287 kJmol $^{-1}$ which is higher than the triplet energies of bithionol or fentichlor which were calculated from the phosphorescence data. These experiments were carried out in acetonitrile and the samples were degassed using the freeze pump thaw technique described in chapter 3 to ensure that the triplet state of the sensitiser or the compound would not be quenched by oxygen. The samples were found to show a large amount of degradation during the laser experiments, therefore it was necessary to shake and replace the solutions frequently. Following laser excitation at 355 nm of the compounds in the presence of benzophenone a weak transient absorption was produced with a λ_{max} at 375 nm for bithionol and 360 nm for fentichlor as shown in figures 5.9 and 5.10.

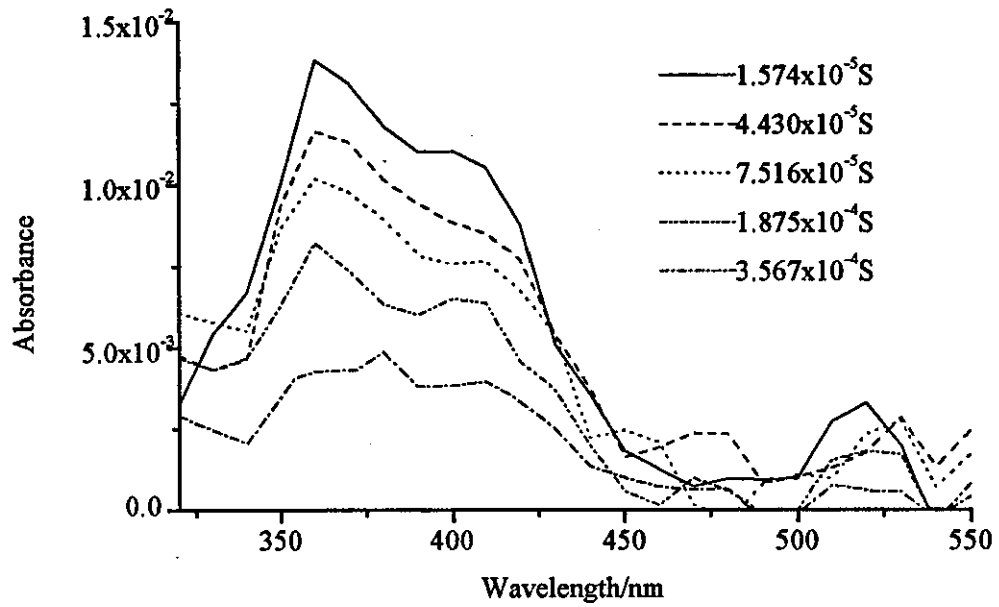


Figure 5.9:Transient absorption spectrum of 2.54x10^{-3}M bithionol sensitised by 2.0x10^{-3} M benzophenone in acetonitrile exciting at 355 nm

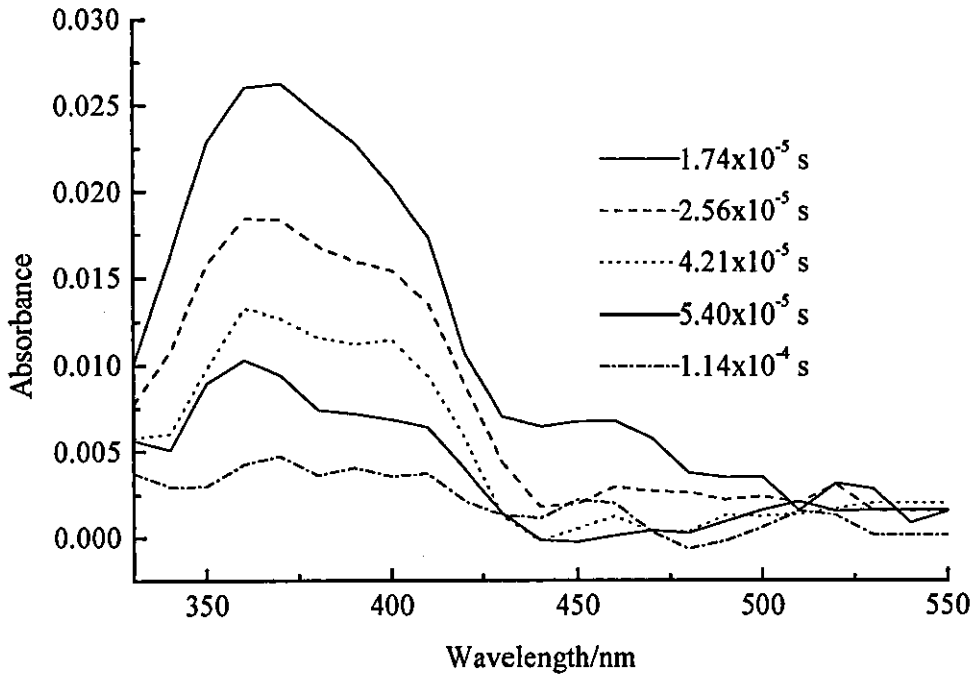


Figure 5.10: Transient absorption spectra of 1.83×10^{-3}M fentichlor sensitised by 2.0×10^{-3} M benzophenone in acetonitrile exciting at 355 nm.

In addition to the bithionol and fentichlor transient being observed at 370 nm the benzophenone triplet at 530 nm was reduced in the presence of fentichlor and bithionol. This confirms that the transient species observed is either a triplet or is formed directly from the triplet. The transient absorption spectrum does not fully return to the original absorption suggesting the presence of a longer lived species or the formation of a product. The kinetic decay traces recorded at 370 nm were fitted to first order kinetics and a baseline to give lifetimes of 7.0×10^{-5} s $\pm$ 3×10^{-6}s and 6.5×10^{-5} s $\pm$ 4×10^{-6}s for fentichlor and bithionol respectively. These lifetimes confirm that the reason the triplet species or species formed from the triplet can not be seen, is not that the lifetimes are too short for the instruments but that the quantum yield of bithionol and fentichlor triplets are very low. The phosphorescence data however showed a high triplet quantum yield, at 77 K in an organic glass, which suggests that there is a singlet state reaction occurring in the fluid solution which reduces the triplet quantum yield.

5.1.4. Flash Photolysis.

Previous work on these compounds using methods such as E.S.R. have shown the formation of radicals upon irradiation with light.[59,60,65-71] It is these radical species which have been said to cause photoallergy. Excitation at the two wavelengths of the laser showed the presence of a weak long lived transient which could not be measured accurately using the laser as the excitation source. The sensitisation experiments showed the presence of triplet species or a species formed from the triplet but it also showed the presence of longer lived species. These can be investigated over longer timescales and using a different excitation source. The laser was replaced with a flash lamp which gave a wider range of exciting wavelengths. The 1 cm cell used for laser flash photolysis was replaced with a 5 cm pathlength cylindrical cell which increased the area being excited. This showed the presence of a transient which was initially assigned as a radical species, as reported in E.S.R. studies,[59,60,67-71] due to the loss of one chlorine which is a usual reaction to form aryl radicals.[155] These results can be compared with those found for 4-chlorophenol by E.S.R.[71,75] and by laser flash photolysis.[72,156] There has been much controversy about the mechanism by which 4-chlorophenol reacts with light. Older reports show the presence of an aryl radical species due to dechlorination, which then reacts with the solvent to form a product which was identified by H.P.L.C..[73,76,77,152,157] More recent reports seem to favour the formation of a carbene by loss of HCl which in aerated aqueous solutions reacts with molecular oxygen to form a peroxy radical which can then go onto form products.[72,156] This would suggest that the species found from bithionol could also either be a radical or a carbene species.

The transient absorption spectra were recorded in both degassed and aerated solutions. The intensity of the absorption change seen in degassed solution was slightly less than that seen in aerated solutions which suggests that oxygen must play some part in the formation of the product. The lifetime however showed no change with a change in oxygen concentration. There has been some controversy in the literature over whether the products formed after irradiation, detecting with E.S.R., depend on oxygen. Some groups have reported different products in aerated solutions to those found in degassed solutions[59] whereas others say the difference seen in the products was due to the differences in the solvent used.[60,69,70] This can be compared with 4-chlorophenol

where its reaction with oxygen depends on whether the transient is assigned as a radical or a carbene.[79,152] If the transient is assigned as a radical its lifetime and abundance is not affected by oxygen. However with the carbene model molecular oxygen affects the final products seen and reduces the lifetime of the carbene.[72,156] The reduction in the amount of product seen with the carbene model is due to the molecular oxygen in the air reacting with the carbene and therefore this model could be used to explain the differences seen with bithionol and fentichlor. The effect of oxygen on the product formation of bithionol and fentichlor will be investigated further later. The transient spectra of the compounds were taken at two different pH's, pH 7.4, the physiological pH, and pH 11.4 , where the fully ionised form of the compound is being excited. The results at pH 11.4 are shown in figures 5.11 and 5.12.

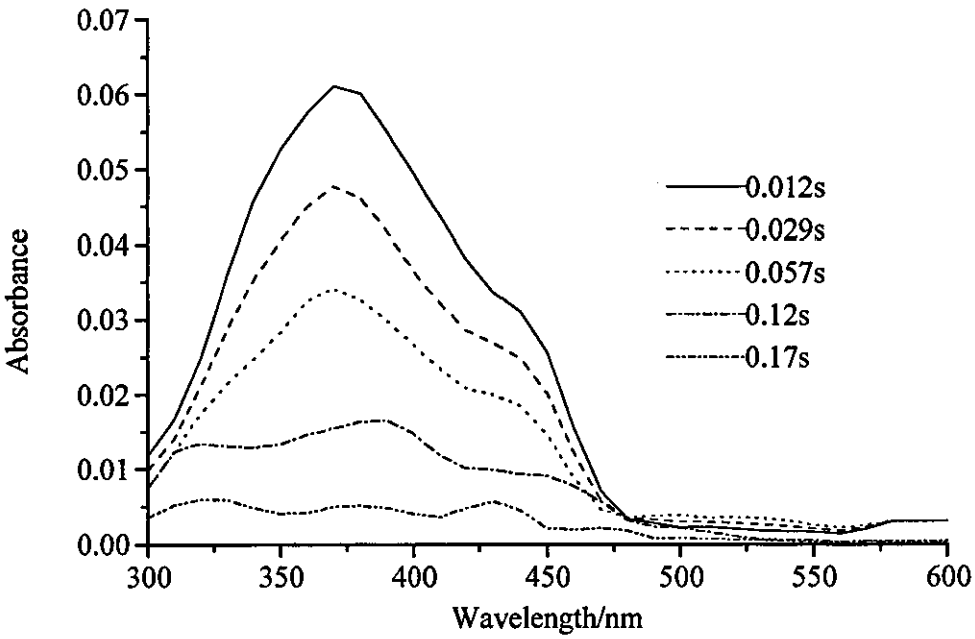


Figure 5.11: Transient absorption spectra of $7x10^{-5}$M fentichlor in 10% ethanol 90% water solution at pH 11.4 exciting with a flashlamp.

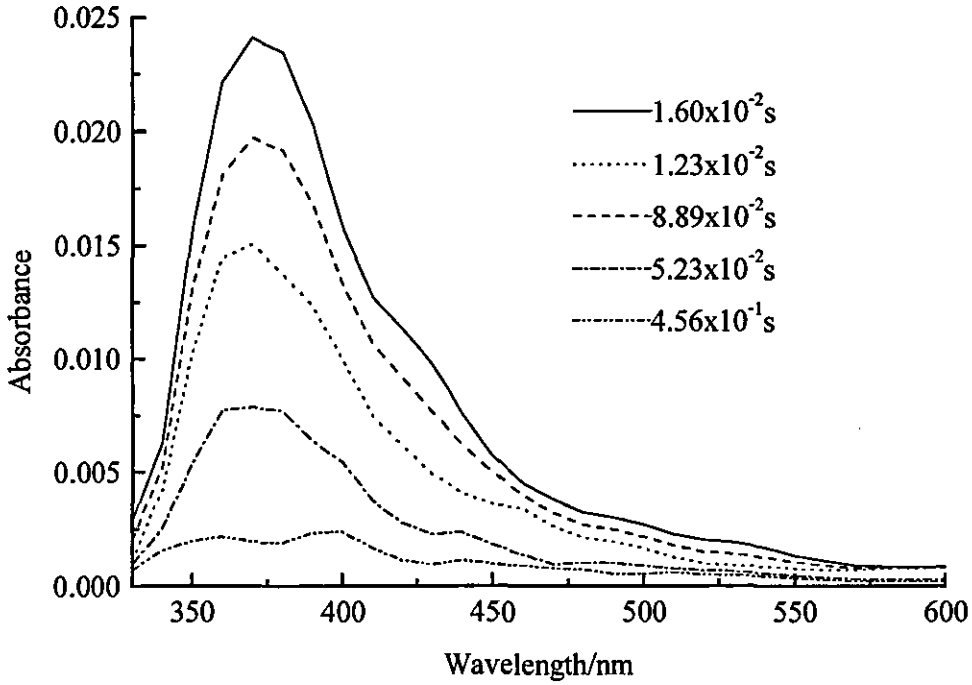


Figure 5.12: Transient absorption spectra of $8x10^{-5}$ M bithionol in 10% ethanol 90 % water solution at pH 11.4 exciting with a flashlamp.

The transient absorption spectra for both compounds showed only 1 peak whose λ_{max} are listed in table 5

Compound	pH	λ_{max}/nm	Lifetime/s
Fentichlor	11.4	370	0.10 ± 0.02
Fentichlor	7.4	370	0.050 ± 0.007
Bithionol	11.4	370	0.12 ± 0.04
Bithionol	7.4	350	0.062 ± 0.008

Table 5 : Transient absorption data from flash photolysis experiments on bithionol and fentichlor at two different pH's.

The end of trace transient decay trace at 370 nm for both compounds shows the presence of a permanent absorption change after the decay of the transient at each pH. This absorption is almost certainly due to the formation of a product. The lifetime of the initial transient varied between the two pH's and was found by fitting the transient decay traces to first order kinetics with a baseline which gave the values listed in

table 5. The lifetime of the transient is affected by the pH and this may be due to the transient being in different protonation states. It was seen earlier in the fluorescence studies that the excited singlet state showed two distinct protonated forms of the compound. If the radical is formed from the singlet state it is highly likely that the radical will also be in two different protonated forms. The intensity of the absorption at the end of the trace is also be reduced by a drop in the pH. This effect has also been reported by Chignell et al working with E.S.R.[60] who suggest that the product distribution varies with pH.

The transient was suspected to be a radical species due to the previous work on the subject using E.S.R. and the fact that the transient was not quenched in oxygen. From an inspection of the structures of fentichlor and bithionol, the radical species most likely to be formed would be due to loss of the chlorine to form substituted phenyl radicals. Phenyl radical scavangers carbon tetrabromide[158] and THF[153] were mixed with the compound to see whether they quenched the transient absorption signal. They were found to quench the transient signal by reducing the lifetime of the transient linearly with increasing quencher concentration giving the k_q values listed in table 6. This would suggest that the transient seen is a radical species and not a carbene as seen with 4-chlorophenol,[72,156] this may be due to extra stability from the sulfur and second phenol group. However it is not known if carbontetrabromide quenches carbenes, and further work would have to be done to find this out. The quenchers also reduced the formation of the product in the same proportion as they quenched the radical.

Compound	pH	k_q from $CBr_4/M^{-1}s^{-1}$	k_q from $THF/M^{-1}s^{-1}$
Fentichlor	11.4	$1.5x10^5 \pm 5x10^4$	$2.76x10^3 \pm 300$
Bithionol	11.4	$5.4x10^4 \pm 9x10^3$	$1.75x10^3 \pm 250$
Fentichlor	7.4	$2.6x10^5 \pm 3x10^4$	$6.47x10^3 \pm 400$
Bithionol	7.4	$3.3x10^5 \pm 3x10^4$	$1.34x10^4 \pm 550$

Table 6: Radical quenching constants for bithionol and fentichlor.

This suggests that the product is formed from the radical rather than directly from the triplet which corroborates the fact that the product could be seen in both aerated and degassed solutions.

The transient difference spectrum of bithionol and fentichlor adsorbed onto cellulose was measured and gave 1 peak with a λ_{max} at 390 nm for fentichlor and at 370 nm for bithionol as shown in figure 5.13 and 5.14. The peaks are in similar positions to the transient species seen in solution for the radical species of fentichlor and bithionol respectively at pH 7.4 and the triplet species seen at 375 nm and 360 nm for bithionol and fentichlor respectively.

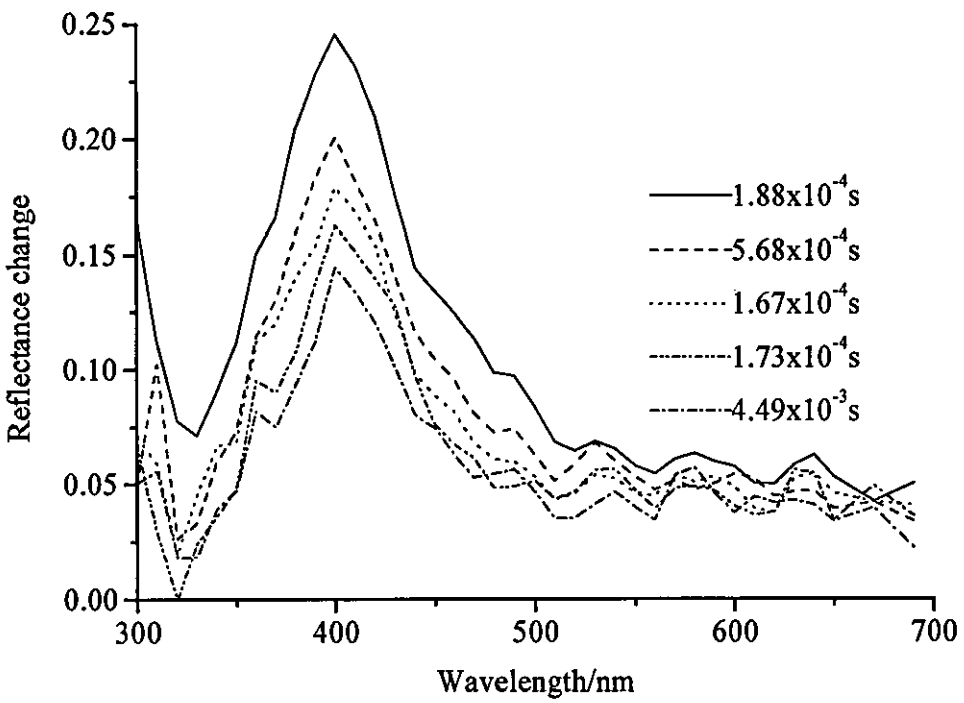


Figure 5.13: Transient difference spectrum of fentichlor 5 mg/g adsorbed onto cellulose, exciting at 266 nm.

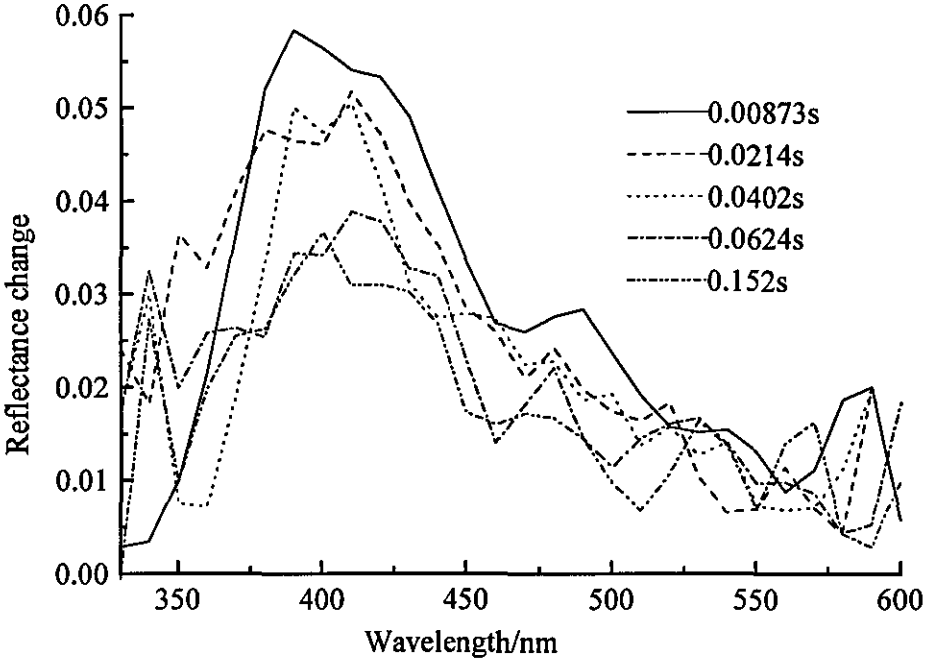


Figure 5.14: Transient difference spectrum of bithionol 12 mg/g adsorbed onto cellulose, exciting at 266 nm

The transients seen when bithionol and fentichlor are adsorbed onto cellulose are probably due to the radical species, even though the triplet absorbs in the same region, as the lifetime of the transient is very long. However in the case of bithionol there are signs of absorption at 350 nm which could be due to the triplet species. The lifetimes were found by fitting the transient decay traces to first order kinetics and were found to be 0.11s for fentichlor and 0.09 s for bithionol. These lifetimes were found to be higher than for fentichlor and bithionol in solution due to the greater rigidity and high hydrogen bonding capacity of cellulose which can protect the excited states from quenchers as explained in chapter 2.

The λ_{max} for the transient absorption was in the same place with H.S.A. present, for experiments using the flash lamp as the excitation source. The H.S.A. alone was not affected by the flash lamp therefore any changes seen in the transient absorption were due to the compounds or their complexes with H.S.A.. The similarity in the shape of the spectra shows that these transient species are the same as the transients seen in solution. The intensity of the end of trace absorption increased with the addition of

H.S.A. for fentichlor, which suggests the formation of more product. This is in contrast to bithionol where the intensity of the laser induced time dependent difference spectrum decreased on addition of H.S.A., showing the formation of less product. Both compounds showed an increased transient lifetime from 0.05 s to 0.082 s for bithionol and from 0.06 s to 0.085 s for fentichlor. The increased lifetime may be expected if the product is formed from the singlet state as there has already seen to be a decrease in the non radiative processes from the singlet state, shown by an increase in the fluorescence. The difference in the behaviour of the end of trace absorption cannot be so easily explained and experiments to study the products with and without H.S.A. need to be carried out.

5.1.5. Singlet Oxygen

Singlet oxygen was measured using an I.R. detector described in chapter 3 exciting solutions of fentichlor and bithionol at 355 nm with the JK2000 laser. Singlet oxygen yields were measured in acetonitrile with the absorbances of the solutions all being about 0.5. The quantum yield of singlet oxygen production was measured relative to the yield for 2-acetonaphthone, whose quantum yield is known to be 0.79, by the method described in section 3.7. Fentichlor produced virtually no singlet oxygen. Bithionol however produced enough singlet oxygen to be measured and the singlet oxygen quantum yield was found to be 0.03. This singlet oxygen is formed from the quenching of the triplet state as the singlet state i.e. fluorescence was not quenched by oxygen. This value is very low suggesting that this probably plays little or no part in the photoallergy process as there are several stronger photochemical processes occurring.

5.1.6. Product analysis

The compounds were seen to form a product when excited with the laser, these products needed to be identified to help define the mechanism. The compounds were initially degraded in front of an arclamp with a 300 nm interference filter. The absorption spectra were recorded at regular time intervals until the spectra no longer changed. The ground state absorption spectra showed a blue shift of the λ_{max} as the irradiation time increased at both pH 11.4 and pH 7.4 as shown in figures 5.15 and 5.17 and 5.16 and 5.18 for fentichlor and bithionol respectively.

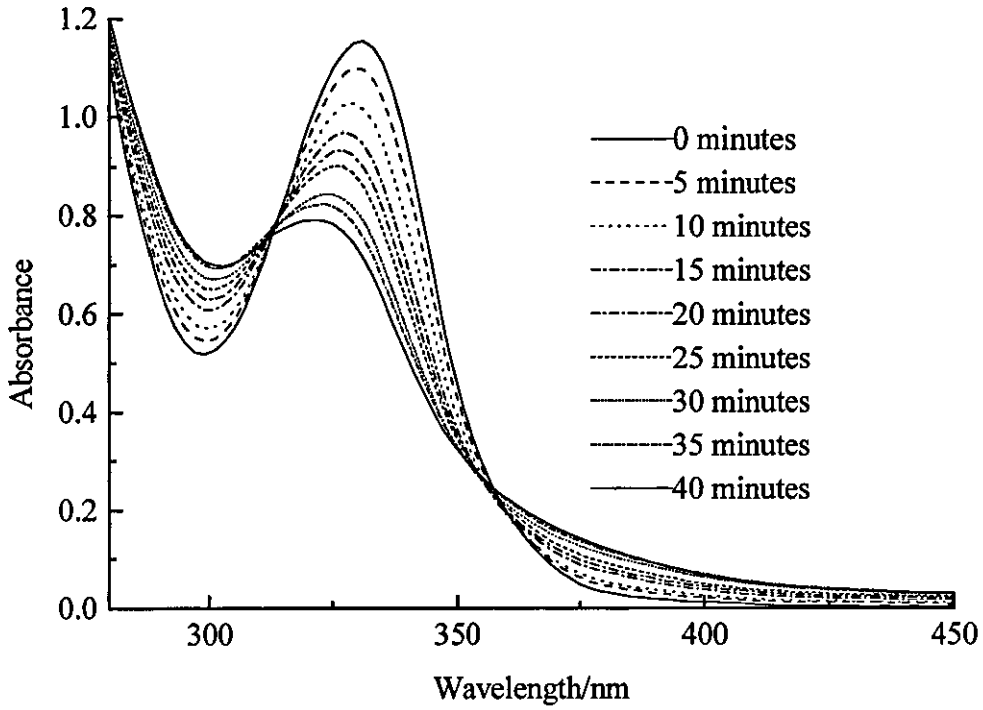


Figure 5.15: 1.2×10^{-4} M Bithionol at pH 11.4 irradiated over time at 300 nm

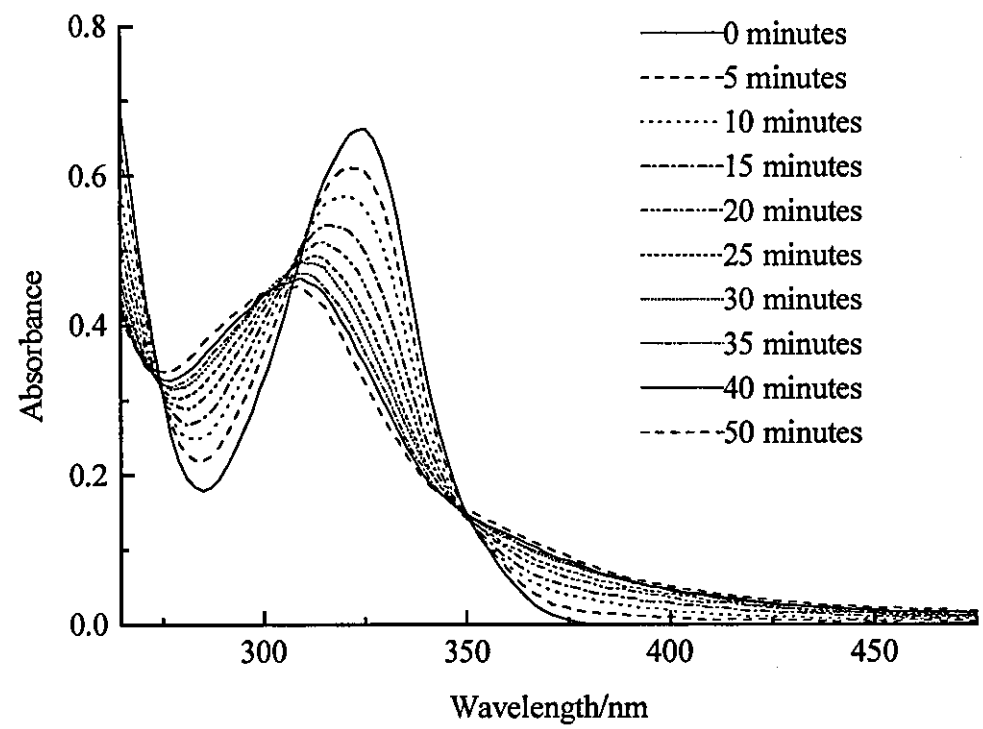


Figure 5.16: 9.9×10^{-5} M Fentichlor at pH 11.4 irradiated over time at 300 nm

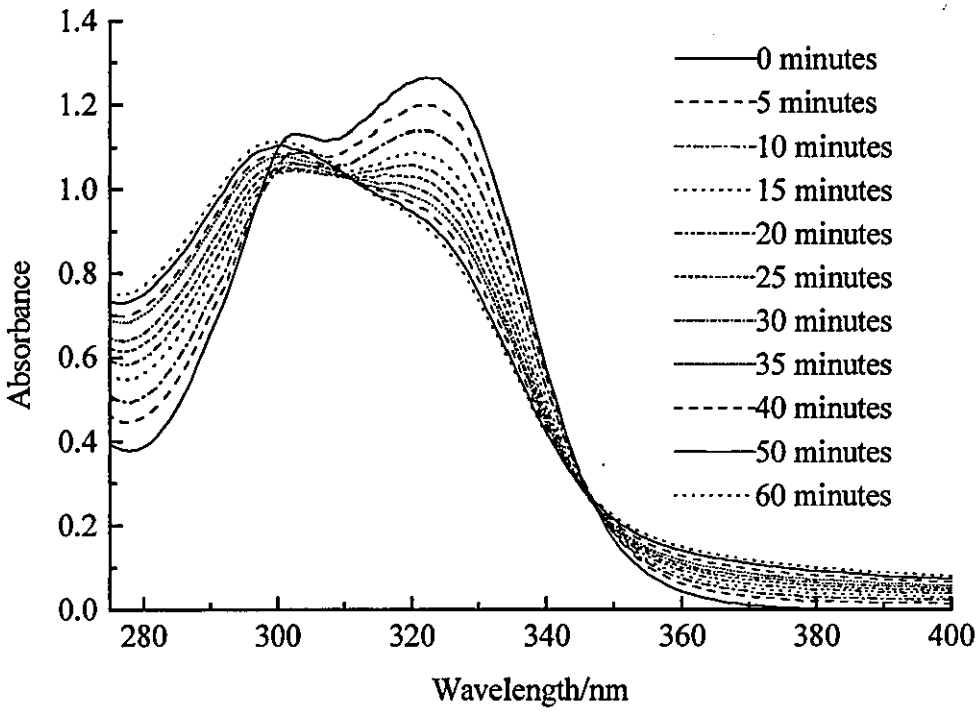


Figure 5.17: 2.1×10^{-4} M Bithionol at pH 7.4 irradiated over time at 300 nm.

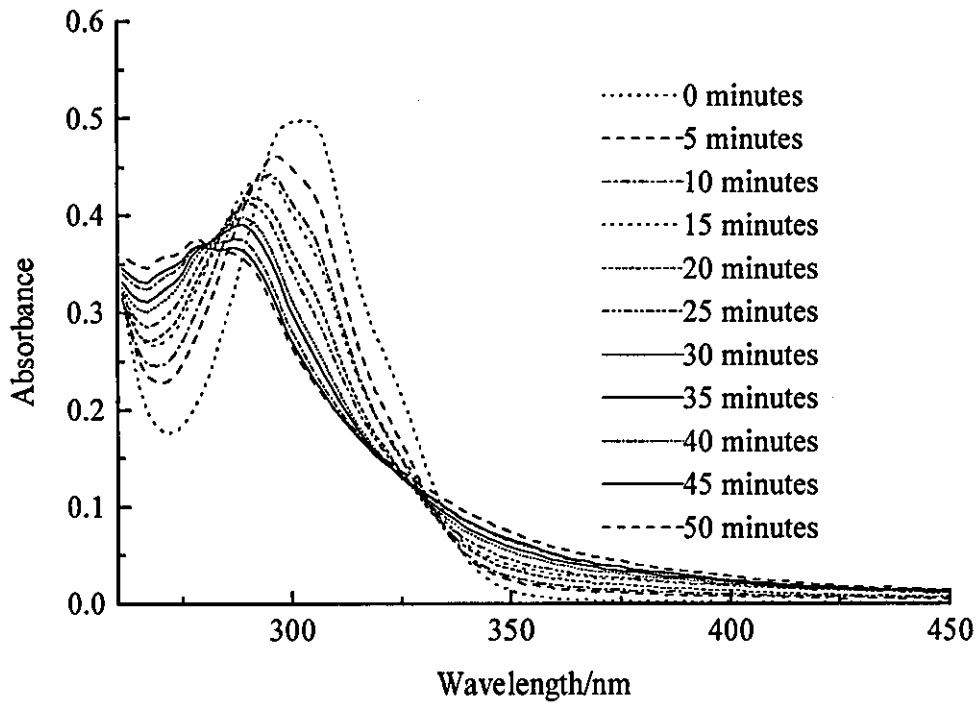


Figure 5.18: 1.4×10^{-4} M Fentichlor at pH 7.4 with irradiation with time at 300 nm.

The spectra of the products show that λ_{max} moves to lower wavelengths which are given in table 7. The quantum yield of initial degradation was calculated using ferrioxalate actinometry as described in chapter 3 and the quantum yields were found to vary with pH as shown in table 7. The variation in the quantum yields with pH may be due to the difference in the ground state properties due to protonation of the compounds or to the difference in the substitution reactions of the solvent. In pH 11.4 solutions even with ethanol present there is an excess of OH^- ions which may cause hydroxyl ions from the water to add to the radical, whereas at pH 7.4 the radical may abstract a hydrogen atom from the ethanol in the solvent.

Compound	pH	Product λ_{max}	ϕ
Fentichlor	11.4	310	0.458
Fentichlor	7.4	290	0.253
Bithionol	11.4	320	0.239
Bithionol	7.4	295	0.253

Table 7: Quantum yields of degradation for bithionol and fentichlor at different pH's and the λ_{max} of the product.

It has been suggested by previous workers, using TLC, that both compounds lose chlorines upon irradiation and that bithionol forms fentichlor[59,62-64] upon irradiation by loss of the ortho chlorines. This could not be proven from the irradiation studies alone but it can be seen that there is the removal of an auxochromic group which could be the chlorine ion. The compounds were found to degrade less with degassed solutions again showing the dependence of the product formation on the presence of oxygen.

The degradation of the fentichlor is different with the presence of H.S.A. On irradiation at 300 nm the ground state absorption spectra showed a blue shift in the λ_{max} , however there was little change in the amount of degradation seen. Bithionol showed a substantial degradation alone but with the addition of H.S.A. the amount of degradation was reduced and with an excess of H.S.A., degradation was stopped, i.e. reducing the formation of the product. These results agree with the laser flash photolysis results for the end of trace absorption but are surprising as it would be expected that the degradation would be affected in the same way for both compounds. However the difference could be due to different products being formed with the

presence of H.S.A. to those formed without H.S.A. which have different molar absorption coefficients. This seems likely in the case of fentichlor where a shift in the spectra could also be seen. However it could also be due to the position of the binding preventing the loss of the ortho chlorines in bithionol but freely allowing the loss of the para chlorines in fentichlor.

5.1.6.1. High Performance Liquid Chromatography

The system was set up as described in section 3.6.2. The concentrations of all the irradiated samples were 0.1% before irradiation. The fentichlor and bithionol non-irradiated samples were 0.025% each and were run together to show that a separation could be achieved between the two compounds. The peaks were seen at 14'35 mins for bithionol and 13'36 mins for fentichlor which is as expected since bithionol is less polar than fentichlor. Samples of each compound were irradiated at 305 nm for 1.5 hours to form the products. Although the product distribution changed with time, this irradiation time was chosen to show a large yield of initial photoproducts as well as some secondary photoproducts. These samples were then run through the column separately to obtain the chromatograms for the products of fentichlor and bithionol. These chromatograms show the presence of extra peaks compared to the non-irradiated compound.

The irradiated bithionol shows peaks at 14'08, 13'35 and 12'39 as well as the original bithionol peak. Comparing these retention times with those for the standards of fentichlor and bithionol it is found that the peak at 13'35 is probably due to fentichlor. This suggests that the peak at 14'08 would be due to loss of 1 chlorine as it is at a longer retention time therefore it must be less polar than fentichlor. This confirms the literature[67,68] data which dictates that bithionol goes to fentichlor by systematic loss of the ortho chlorines upon irradiation. Also it is well documented that the ortho chlorines to sulfur, oxygen or phenyl groups cleave first and faster than the meta or para chlorines.[159] The peak at 12.39 is the result of further degradation this could be better identified by looking at the irradiated fentichlor chromatogram which shows peaks at 12'53, 12'19, 11'37, 2'44 as well as the non irradiated fentichlor peak. The peaks at 12'53 and 12'19 are probably due to the systematic loss of the para chlorines as they

are at shorter retention times therefore they are more polar compounds but this can not be confirmed by this method.

5.1.6.2. Gas Chromatography- Mass Spectrometry

The results obtained using HPLC are a good indication of the mechanism for bithionol photodegrading to fentichlor in an alcohol based solvent but they cannot identify the mechanism by which fentichlor photodegrades. Therefore it was decided to use GC-MS where the products could be identified from their mass spectra. All the compounds concentrations were 0.1% so a comparison of amounts can be made from the peak areas. The bithionol was run through the GC-MS and gave a chromatogram on which a few minor impurities could be identified but the main peak was due to bithionol which was confirmed by its mass spectrum. The irradiated bithionol was run through the GC-MS and it gave a chromatogram which showed the presence of additional peaks and a reduction in the bithionol peak. The results are listed in table 8 and the major products were identified as due to the loss of 1 ortho chlorine, to fentichlor, and to chlorophenol.

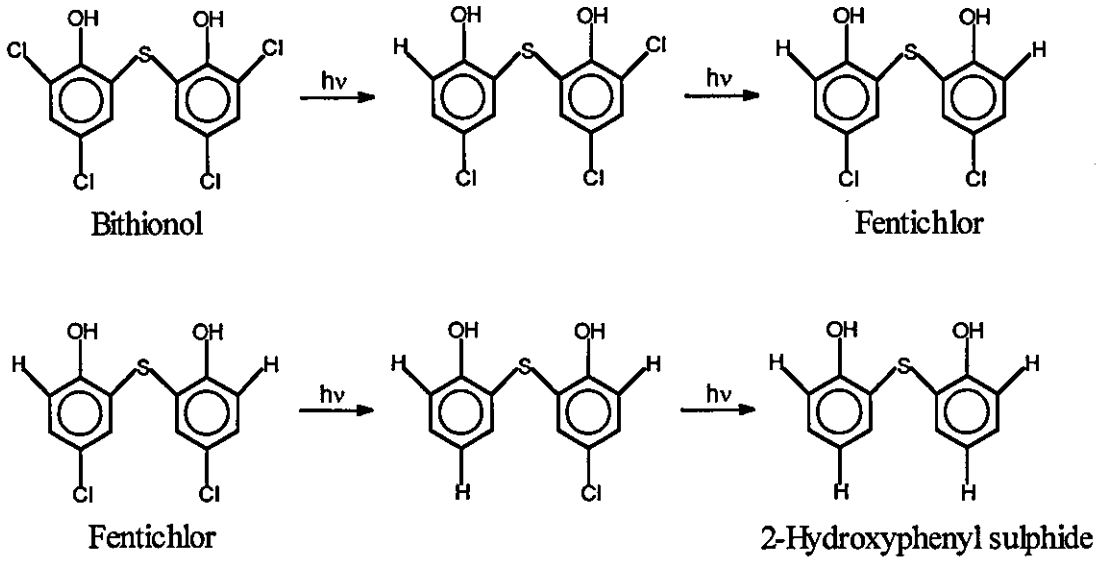
Starting material	R_t of peaks/mins	Molecular Weight	Product
Non-irradiated Bithionol	20.78	354	Bithionol
Irradiated Bithionol	20.78	354	Bithionol
	19.38	320	Bithionol - Cl
	18.41	286	Fentichlor
	6.31	128	p-Chlorophenol
Non-irradiated Fentichlor	18.53	286	Fentichlor
Irradiated Fentichlor	18.53	286	Fentichlor
	16.66	252	Fentichlor - Cl
	14.63	218	Fentichlor - 2Cl
	5.89	128	p-Chlorophenol

Table 8: GC-MS results from irradiated bithionol and fentichlor.

A sample of non irradiated fentichlor was run through the GC-MS and was found to give both fentichlor and the o,p chlorine derivative which suggests the sample is not isomerically pure and therefore there may be products present due to both isomers.

The distribution between the p-p fentichlor and o-p fentichlor was 1o-p :20 p-p. The irradiated sample of fentichlor gave a chromatogram which showed peaks due to loss of 1 and 2 para-chlorines as well as para-chlorophenol.

These results show the stepwise mechanism for formation of the products is probably as in scheme 3.



Scheme 3 : Stepwise mechanism for the reaction of bithionol with light.

The GC-MS results also show that the sulfur bridge can be broken during irradiation to give para-chlorophenol but it is unknown at which stage of the mechanism this occurs. The flash photolysis work and the degradation studies both showed the formation of more product in aerated solutions than degassed solutions. Some previous studies of these compounds using E.S.R. have stated that the products vary with the presence of oxygen and in aerated solutions it would therefore be expected to see the substitution of an hydroxyl group rather than a hydrogen atom to form the semiquinone type radicals. However this GC-MS work which was carried out in aerated solutions of methanol which shows the presence of only hydrogen substitution causing me to agree with those[60,70] who have stated that the type of products seen depends on the solvent used and not whether oxygen is present. The substitution of hydrogen in the above reactions is from the methanol which is used as the solvent, in an aqueous solvent there

would be the substitution of hydroxyl radicals to get different products. This is the same effect as seen with 4-chlorophenol as both the radical and the carbene have been shown to be proton sensitive, when the proton is present in high concentrations.[68,72] The basic mechanism for the formation of products from bithionol and fentichlor in aqueous solution however, will probably be the same as seen in methanol.

Summary of the photochemical properties of bithionol and fentichlor

The ground state absorption spectra of these compounds show λ_{max} at 305 nm in ethanol which has been assigned as a $\pi \rightarrow \pi^*$ transition. The ground state absorption of these compounds varied with pH showing a shift to the red with increasing pH. This effect has been seen before but no attempts were made to calculate the pKa of the reaction or to assign the peaks to the different protonated forms of the compounds. Previous work has suggested that only 1 protonation reaction is occurring.[60] This work however, has shown that the peak seen at higher wavelengths are due to the non protonated form of the compounds and at the lower wavelengths are due to the fully protonated form of the compounds. There is no obvious spectra for the partially protonated form showing the loss of one hydrogen ion is followed very quickly by the loss of the second hydrogen ion. The adsorption of bithionol and fentichlor onto cellulose did not alter the position of the ground state absorption peaks, but it did alter the ratio of the molar absorption coefficients of the peaks. When adsorbed onto cellulose these compounds showed greater absorption at longer wavelengths.

These compounds were found to weakly fluorescence with a λ_{max} around 400 nm with quantum yields $\leq 10^{-4}$. The addition of H.S.A. to bithionol and fentichlor in aqueous solutions was found to have a large effect on the singlet state seen by an increase in the fluorescence quantum yield. This is possibly due to a reduction in the nonradiative transitions from the singlet state.

The phosphorescence emission from these compounds at 77 K was strong with quantum yields of 0.56 and 0.34 for fentichlor and bithionol respectively and lifetimes of 6.3 ms and 8.8 ms for bithionol and fentichlor respectively. The triplet state was assigned as $^3(l,\pi)^*$ from the lifetime and quantum yield of the phosphorescence, due to the lone pairs on the sulfur.

Transient absorption spectra showed the presence of a triplet species, or a species formed from the triplet, in the presence of a triplet sensitiser with lifetimes of $7.0x10^{-5}$ s and $6.5x10^{-5}$ s for fentichlor and bithionol respectively. With a millisecond excitation source the transient absorption showed the presence of another species at the same wavelength with lifetimes in the 50 - 100 ms region depending on the pH. This was identified as a phenyl radical, via radical quenching experiments, due to dechlorination of the fentichlor and bithionol. When bithionol and fentichlor were adsorbed onto cellulose the position of the peaks remained the same suggesting the species was the same as seen in solution. The lifetimes increased due to the cellulose protecting the excited state from quenchers as explained in chapter 2.

Chromatographic analysis was carried out on the products of these compounds to help identify the mechanism of degradation. These found that the initial products were due to systematic loss of a chlorine, in the case of bithionol the ortho chlorines to form fentichlor. Then the fentichlor lost the para chlorines to form 2-hydroxyphenylsulfide. The products were then formed by abstraction of a hydrogen atom from alcoholic solutions or a hydroxyl group in aqueous or alkaline solutions. The products were found to be both solvent dependent and oxygen dependent, although the mechanism by which oxygen affects the product is still unknown. This mechanism confirms those postulated by previous workers from experiments carried out using E.S.R. to identify the radicals involved.[60,70]

5.2. Tribromosalicylanilide (TBS)

The structure of TBS is very similar to T_4CS therefore it is expected that the photochemistry will also be very similar. T_4CS photochemistry has been studied in detail before[56] by laser flash photolysis, gas chromatography[81] and E.S.R.[160] and will be used as a model to compare these results with. This compound would not dissolve in water therefore most experiments are done with a small amount of ethanol added.

5.2.1. Ground state absorption spectra

The ground state absorption spectrum of TBS shows two peaks with λ_{max} at 360 nm and 275 nm as shown in figure 5.19 in 10% ethanol: 90% water at pH 7.4. This was assigned as a $\pi \rightarrow \pi^*$ transition. The molar absorption coefficient of TBS at 360 nm was found to be 8700 $mol^{-1}dm^3cm^{-1} \pm 320$ $mol^{-1}dm^3cm^{-1}$.

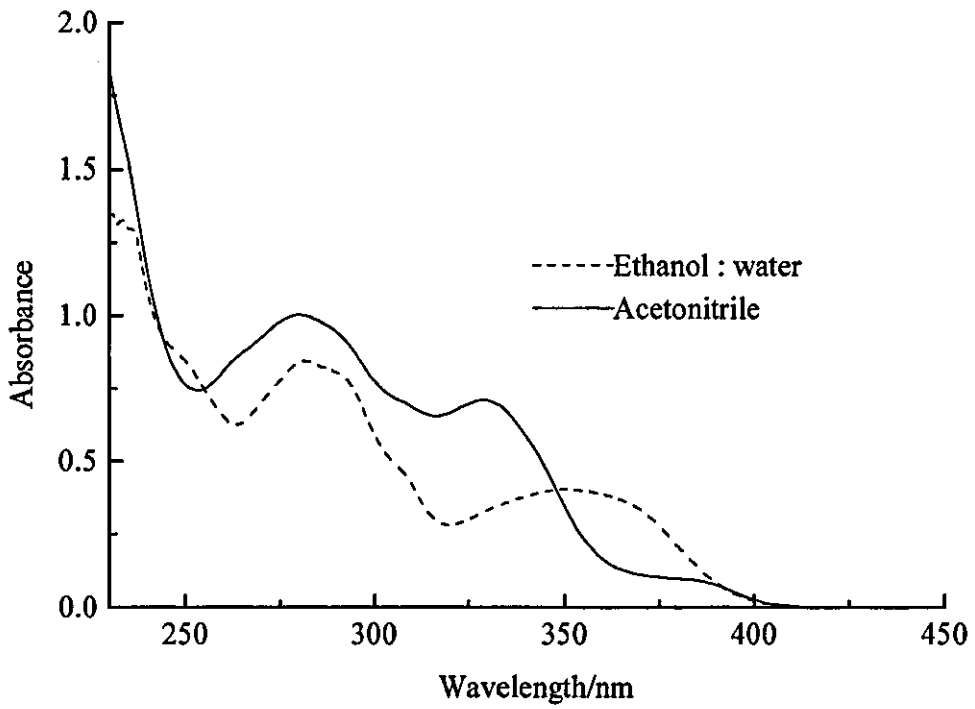


Figure 5.19: Ground state absorption spectra of $4x10^{-5}$M TBS in acetonitrile and ethanol:water at pH 7.4.

The ground state absorption spectrum shows some change in the position of the λ_{max} with different solvents. In protic solvents there is an absorption band at 360 nm where as in aprotic solvents, such as acetonitrile the absorption band is at 330 nm. This is the

same effect as seen with T_4CS where there is a peak at 360 nm in alcohols and a peak at 330 nm in solvents such as acetonitrile. For this transition to be a $\pi \rightarrow \pi^*$ transition the absorption spectra of TBS in an aprotic solvent should be red shifted in relation to TBS in a protic solvent. There is however another peak present when TBS is in acetonitrile at 375 nm which is due to the $n \rightarrow \pi^*$ nature of this transition. The absorption spectra show a difference in the position of the λ_{max} with a change in pH as shown in figure 5.20.

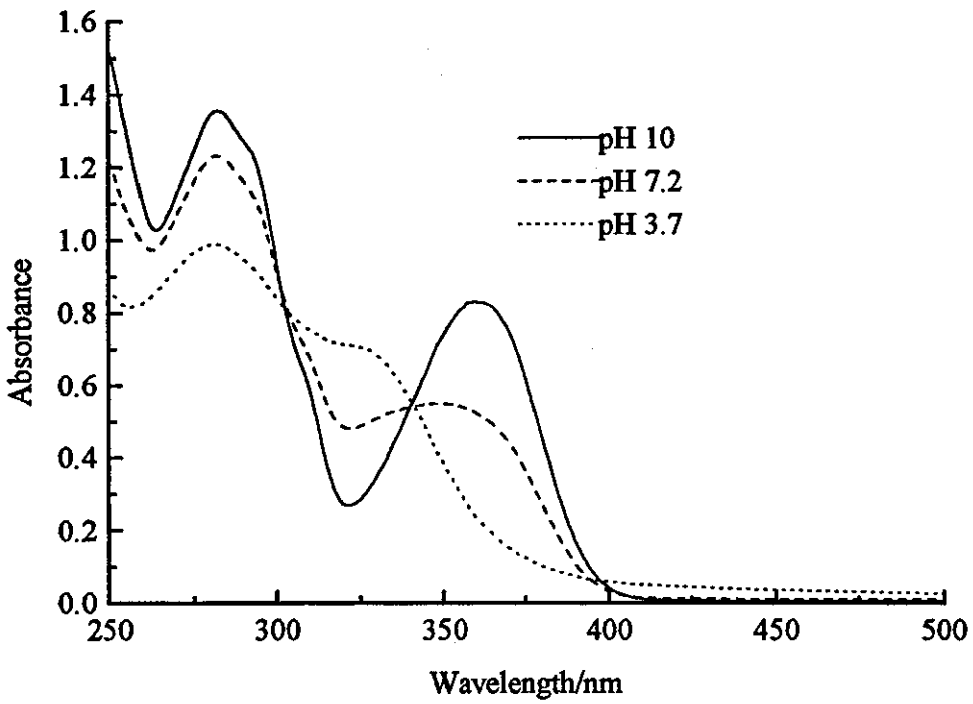
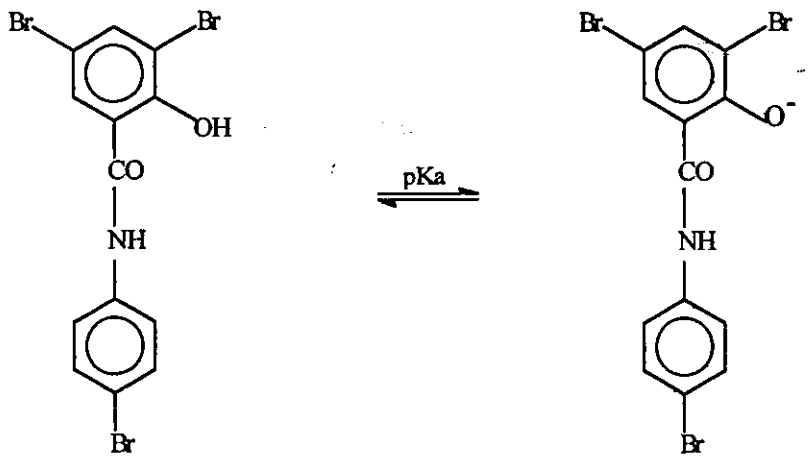


Figure 5.20:Ground state absorption spectra of 4.4×10^{-5} M TBS at different pH's

The increase in the pH leads to a red shift in the position of λ_{max} from 320 nm to 360 nm. The molar absorption coefficient at 320 nm was found to be 6500 $mol^{-1}dm^3cm^{-1} \pm$ 200 $mol^{-1}dm^3cm^{-1}$. The pKa was calculated according to the method explained in chapter 3 and was found to be 6.9. This is due to the deprotonation reaction below.



Non-ionised Ionised

It follows from the value of the pKa that in experiments performed at pH 7.4, the physiological pH, the TBS will be in the non protonated form in the ground state. The pKa is higher than that of T_4CS of 5.5.[56]

The ground state diffuse reflectance (Kubelka Munk) spectrum of TBS adsorbed on cellulose appears a different shape compared to the ground state absorption spectrum in solution as seen in figure 5.21.

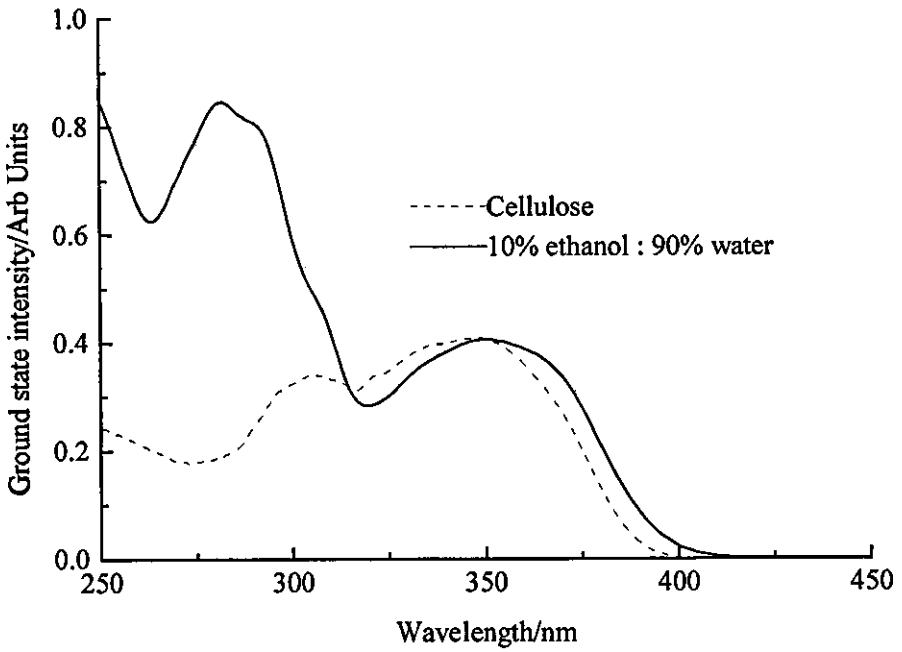


Figure 5.21: Ground state spectra of TBS adsorbed onto cellulose and in 10% ethanol 90 % water solution.

The peak at 275 nm has been reduced in intensity relative to the peak at 360 nm, this may show that the compounds have an increased absorption of longer wavelengths of light. It may also be an indication of some absorption of the light by the barium sulfate below 300 nm as explained earlier. The peak at 275 nm in solution has been red shifted to 310 nm when adsorbed onto cellulose.

5.2.2. Emission spectroscopy

5.2.2.1. Fluorescence

The solution fluorescence measurements were carried out in a 10% ethanol and water mixture. The λ_{max} for fluorescence emission was seen at 440 nm when exciting at 355 nm as shown in figure 5.22 which is similar to the emission maximum of T_4CS at 438 nm. The excitation spectrum was found to be the same as the ground state absorption spectra showing the emission to be due to the TBS alone.

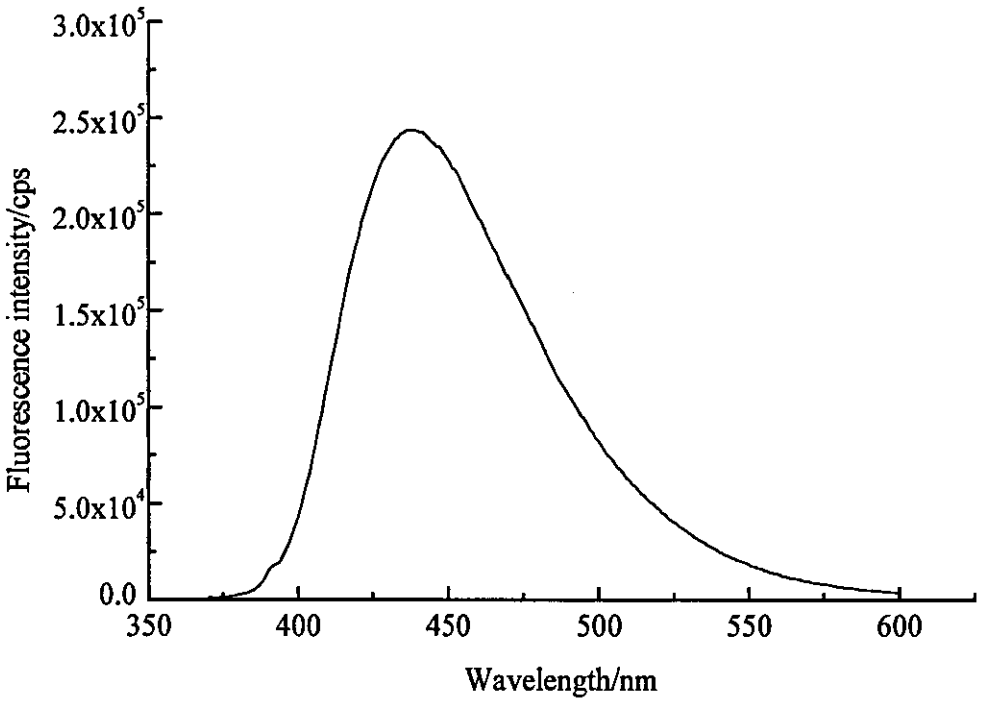


Figure 5.22: Emission spectrum of TBS in 10% ethanol 90 % water solution with excitation slits = 0.2 mm and emission slits = 0.4 mm exciting at 330 nm.

The energy of the lowest excited singlet state was calculated from the mid position between the λ_{max} of fluorescence and the λ_{max} of the ground state absorption as

explained in chapter 3 and it was found to be 299 $kJmol^{-1} \pm 5\ kJmol^{-1}$. The quantum yield of fluorescence was calculated in ethanol using 9,10 diphenylanthracene as a fluorescent standard whose quantum yield is known to be 0.9 in polar solvents.[161] This gave a value for the fluorescence quantum yield of 0.065. The fluorescence quantum yield of T_4CS is 0.34[56] and the difference is probably due to the internal heavy atom effect. The bromines of TBS are heavier atoms than the chlorines of T_4CS therefore increasing the intersystem crossing to the triplet state and quenching the fluorescence. The fluorescence was not affected by the presence of oxygen this suggests the lifetime is very short. The lifetime could not be measured but the radiative lifetime was calculated using the method described in chapter 3 to be $1.2x10^{-8}$s. The estimated lifetime found using the quantum yield of fluorescence was $7.5x10^{-10}$ s. The fluorescence of TBS showed a red shift with decreasing pH, as shown in figure 5.23.

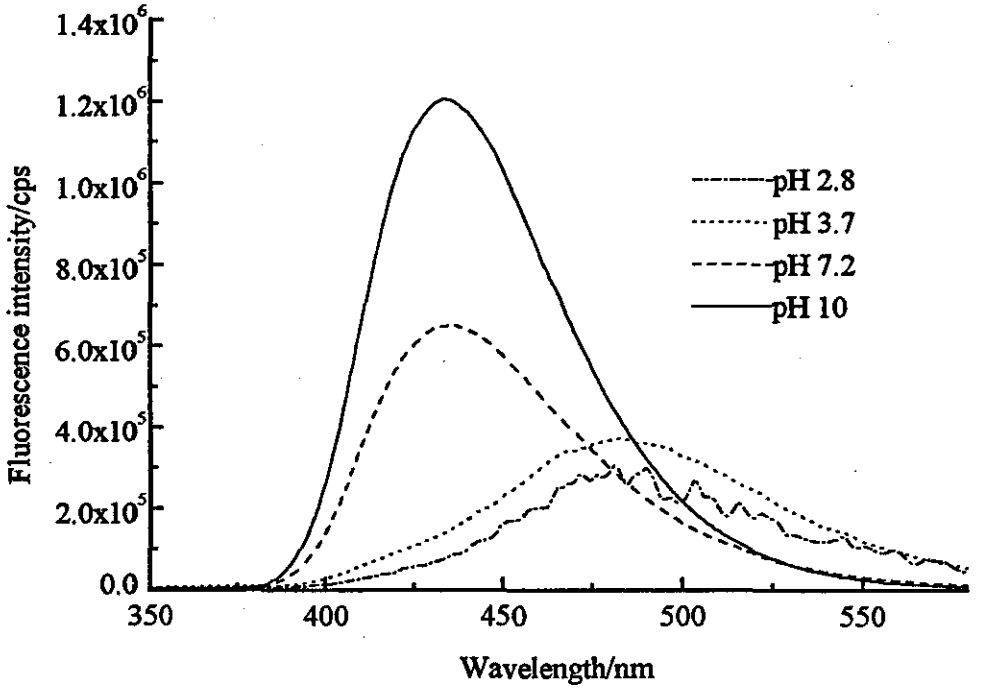


Figure 5.23: Emission spectra of TBS with variation in the pH with excitation slits = 0.3 mm and emission slits = 0.6 mm when exciting at 330 nm.

This is as a result of the same protonation reaction which caused the change in the ground state absorption spectra. There however was no clear isosbestic point of these spectra which may be due to problems with solubility at low pH's.

It can then be seen that the λ_{max} for TBS adsorbed onto cellulose is at 480 nm which is the same as seen in polar solvents. There is a slight broadening of the fluorescence peak when the TBS is adsorbed onto cellulose compared to the fluorescence observed in solution which is expected for compounds absorbed onto cellulose due to the inhomogeneity of the surface as explained in chapter 2.

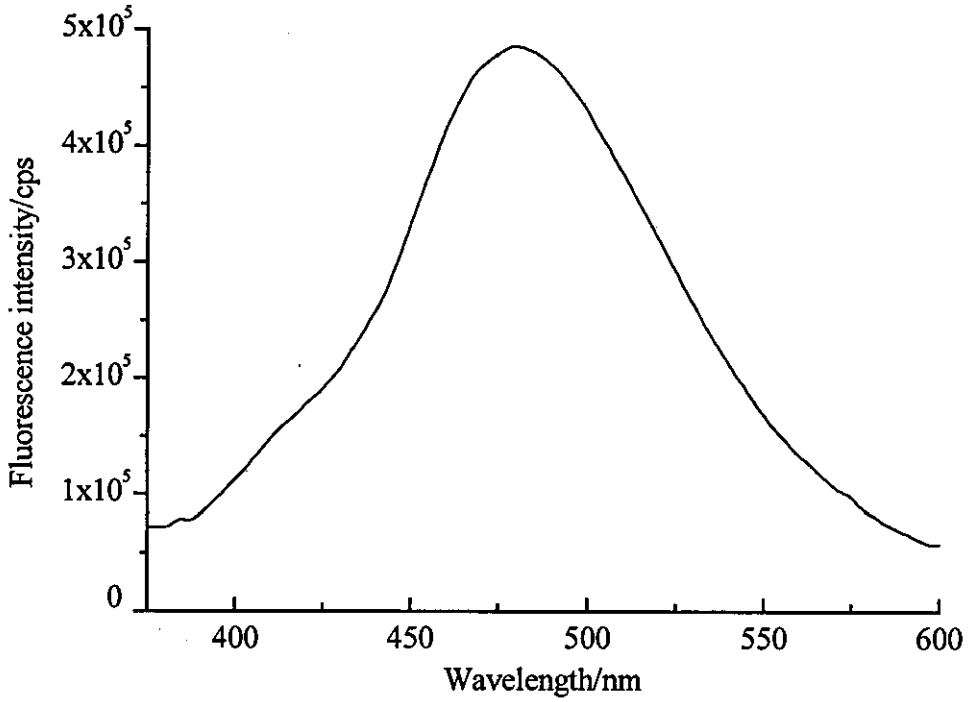


Figure 5.24: Emission spectrum of 1 mg/g TBS adsorbed onto cellulose with excitation slits = 0.3 mm, emission slits = 0.6 mm, exciting at 360 nm.

On the addition of H.S.A. the quantum yield decreased and the position of the peaks showed a slight blue shift as shown in figure 5.25.

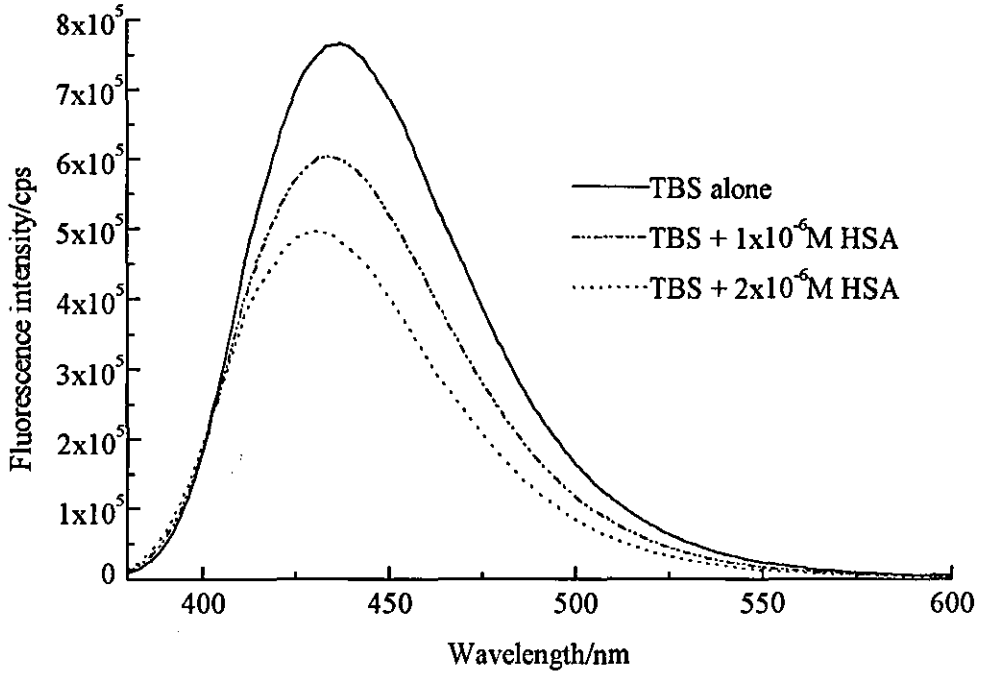


Figure 5.25: Emission spectra of TBS without and with H.S.A. exciting at 360 nm with excitation slits = 0.4 mm, and emission slits = 0.8 mm in 10% ethanol 90% pH 7.4 buffer.

The fluorescence quantum yields measured in pH 7.4 buffer were lower than measured in ethanol and the fluorescence quantum yields in the presence of H.S.A. were measured at the maximum change in fluorescence. The decrease in the fluorescence quantum yield was small, changing from 0.019 without H.S.A. present to 0.014 with H.S.A. present. However in repeated experiments the decrease was found to always be present, showing a distinct difference on the addition of H.S.A. This effect has been reported for many compounds including T_4CS[56] and is often used for measuring the dark binding of compounds to H.S.A.[138] The decrease in fluorescence quantum yield varied with concentration of H.S.A. down to a minimum value for the fluorescence, where the fluorescence no longer decreased with H.S.A. concentration. This was the value used to measure the quantum yield in the presence of H.S.A. Changes in the quantum yield in the past have been suggested to be due to radiative energy transfer,[150] however that can not be the case here as the fluorescence spectrum of TBS does not overlap the ground state absorption spectrum of H.S.A. The most likely explanation for a decrease in the fluorescence intensity is due to an increase in the rates of non

radiative transitions from the singlet state probably by the formation of a protein-TBS complex.

5.2.2.2. Phosphorescence

The TBS showed a phosphorescence emission with a λ_{max} at 460 nm in an organic glass of ethanol at 77 K when excited at 360 nm as shown in figure 5.26.

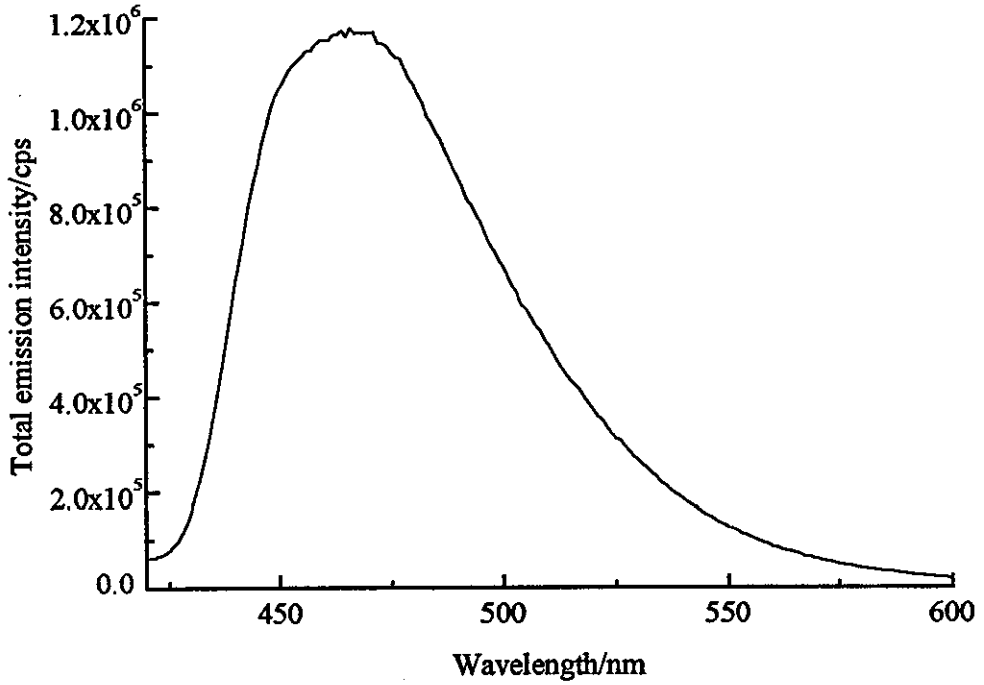


Figure 5.26: Emission spectrum of TBS in ethanol at 77K exciting at 360 nm with excitation slits = 0.2 mm and emission slits = 0.4 mm.

The phosphorescent emission was broad and structureless. The energy of the lower limit of the lowest excited triplet state was calculated to be 260 $kJmol^{-1} \pm 4$ $kJmol^{-1}$ from the λ_{max} of the phosphorescence. This results in the energy gap between the singlet and triplet excited states being 39 $kJmol^{-1} \pm 9$ $kJmol^{-1}$. The quantum yield of phosphorescence was calculated using benzophenone as a phosphorescent standard, whose quantum yield is known to be 0.84 in polar solvents[154] and found to be 0.59 in polar solvents. The lifetime of the phosphorescent decay was found to be 8.8ms ± 0.1 ms using the transient emission trace observed following excitation at 355 nm. The low energy gap between the singlet and triplet state together with the millisecond

phosphorescent lifetime and the large phosphorescence quantum yield suggest that the transition from the ground state to the triplet excited state is a $n \rightarrow \pi^*$ transition. However the presence of bromine atoms in this compound could contribute to a heavy atom effect and therefore the lowest triplet state could actually be $^3(\pi\pi^*)$.

The phosphorescence intensity of TBS was increased with the presence of H.S.A. This suggests that the presence of H.S.A promotes intersystem crossing to the triplet state.

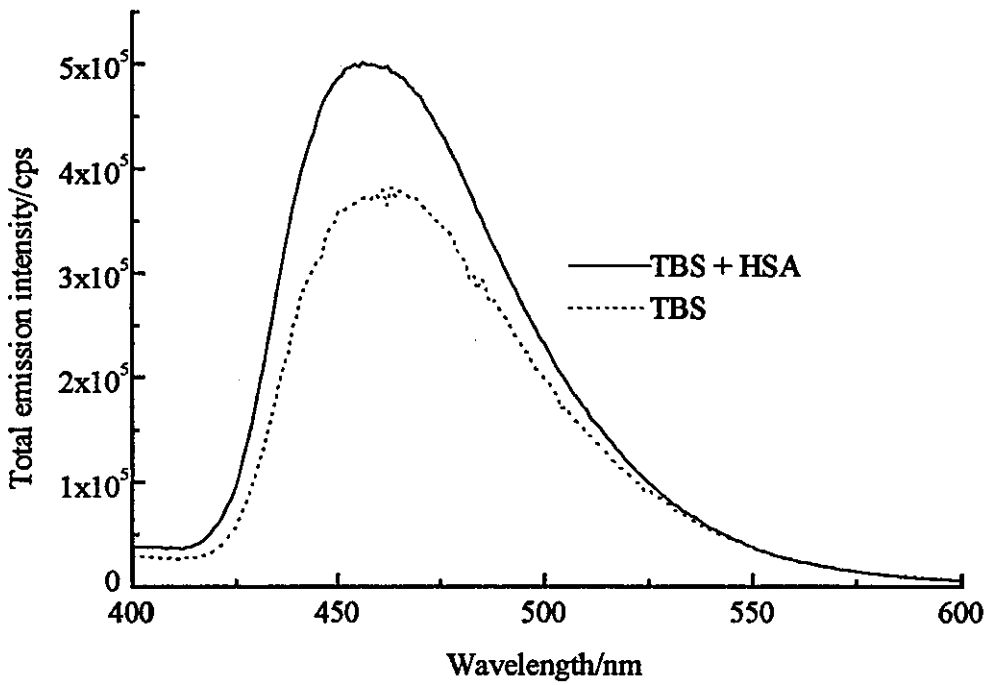


Figure 5.27: Emission spectra of TBS with and without the presence of $2x10^{-5}$ M H.S.A at 77K exciting at 350 nm with excitation slits = 0.15 mm and emission slits = 0.3 mm.

This agrees with the decrease seen in the fluorescence and corroborates the theory that the change in the quantum yields is due to increased nonradiative transitions including intersystem crossing. However, the phosphorescence and fluorescence data may not be compared directly as the fluorescence experiments were carried out at room temperature whereas the phosphorescence experiments were carried out at 77 K and the rates of intersystem crossing and internal conversion may not be the same at both temperatures. The peak also showed a slight blue shift as shown in figure 5.27 this is the opposite of the red shift seen in many compounds when their fluorescence is

enhanced in the presence of H.S.A. The lifetime of the phosphorescence also showed a slight increase with H.S.A. present.

5.2.3. Laser flash photolysis

The TBS was excited at 355 nm and the transient difference spectrum was recorded. TBS in a degassed solution of acetonitrile showed two broad overlapping peaks as shown in figure 5.28

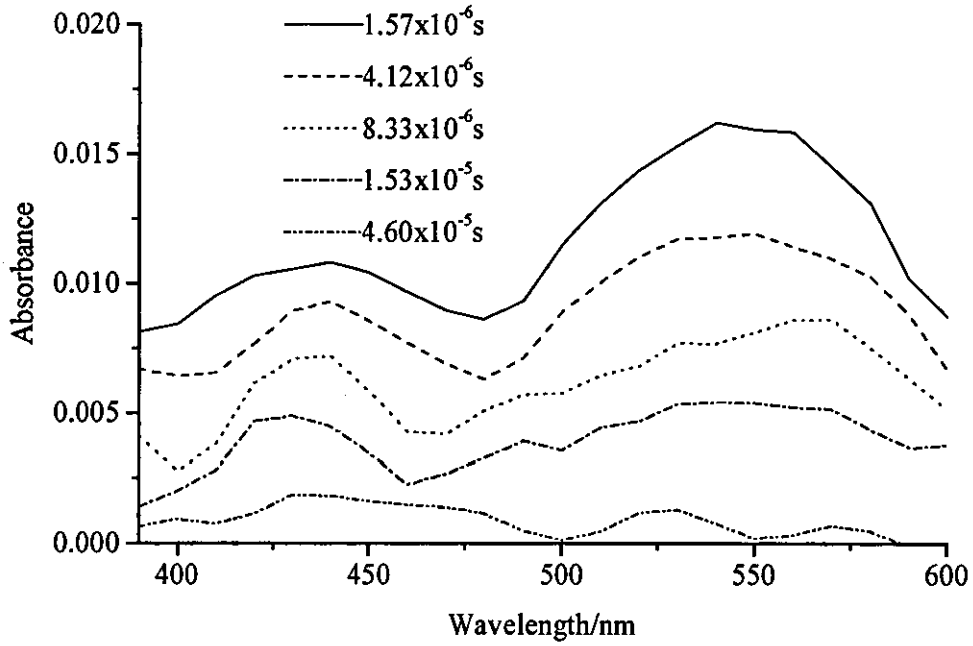


Figure 5.28: Transient absorption spectra of $8x10^{-5}$ M TBS in acetonitrile exciting at 355 nm.

The λ_{max} for these peaks was found to be 440 nm and 550 nm. The lifetime of the peak at 550 nm was found to be $8.5x10^{-6}$ s $\pm$ $5x10^{-7}$ s and for the peak at 440 nm to be 9.6 $x10^{-6} \pm 6x10^{-7}$ s. The peak at 550 nm may be due to a triplet species as it is quenched in the presence of oxygen. The peak at 550 nm was confirmed as a triplet by using a triplet quencher. A suitable quencher was found to be naphthalene which has a triplet energy[162] of 253 kJ/mol in nonpolar solvents which is lower than the triplet energy of TBS calculated from the phosphorescence data. The decay rate constant of the triplet species was found to increase linearly with increasing concentration of quencher as shown in figure 5.29 to give a k_q of $2.1x10^9$ $M^{-1}s^{-1} \pm 2x10^8$ $M^{-1}s^{-1}$.

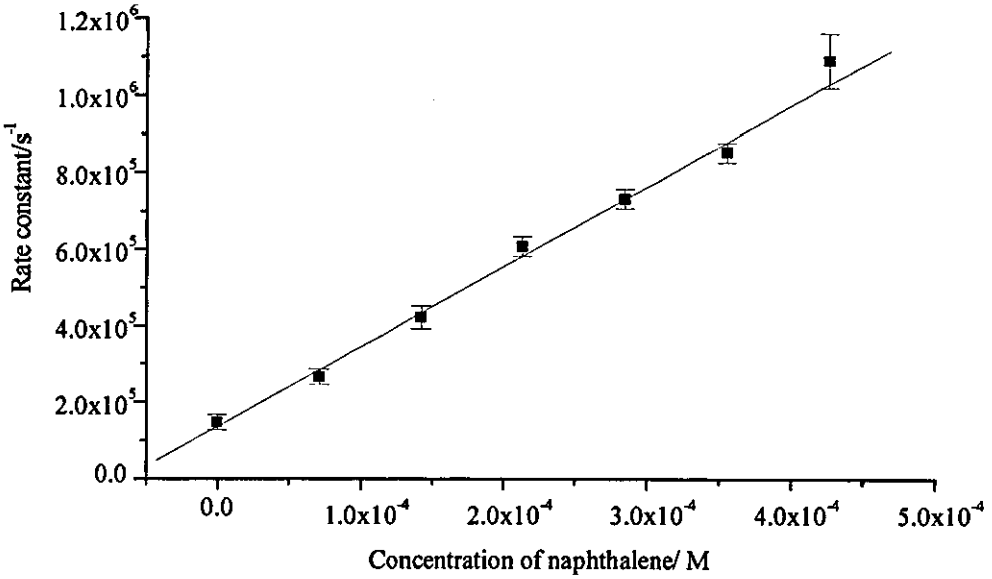


Figure 5.29: A graph showing the quenching by naphthalene of the transient absorption at 550 nm observed following laser excitation of 9.2×10^{-5} M TBS in acetonitrile

In addition to the decrease seen in the TBS transient there was also an increase in the naphthalene transient seen at 415 nm. This confirms that the species seen at 550 nm is due to the triplet or a species formed directly from the triplet. The peak at 440 nm was probably also due to the triplet species due to the similarity in the lifetime with the species at 550 nm. However in aerated solutions there was still a very weak absorption at 440 nm suggesting the presence of another species. The presence of 2 species was difficult to confirm in degassed solution due to the large absorption by the triplet species. The species present at 440 nm in aerated solutions maybe a radical species as it is not quenched by oxygen. With T_4CS a peak was found at 460 nm in 10% ethanol 90 % pH 7.4 buffer in both degassed and aerated and was identified as due to the radical and the triplet species therefore it is likely the peak at 440 nm on TBS is also partly due to a radical species. The TBS peak at 440 nm was investigated in more detail by changing the solvent to a 10 % ethanol 90 % water solution, where it was found that the triplet species could no longer be observed, possibly caused by a reaction with the solvent. In this solvent there is only one peak present with a λ_{max} at 440 nm as shown in figure 5.30 which is seen in both degassed and aerated solutions in the same amounts. The lifetime of the radical in this solvent was found to be 3.2×10^{-6} s.

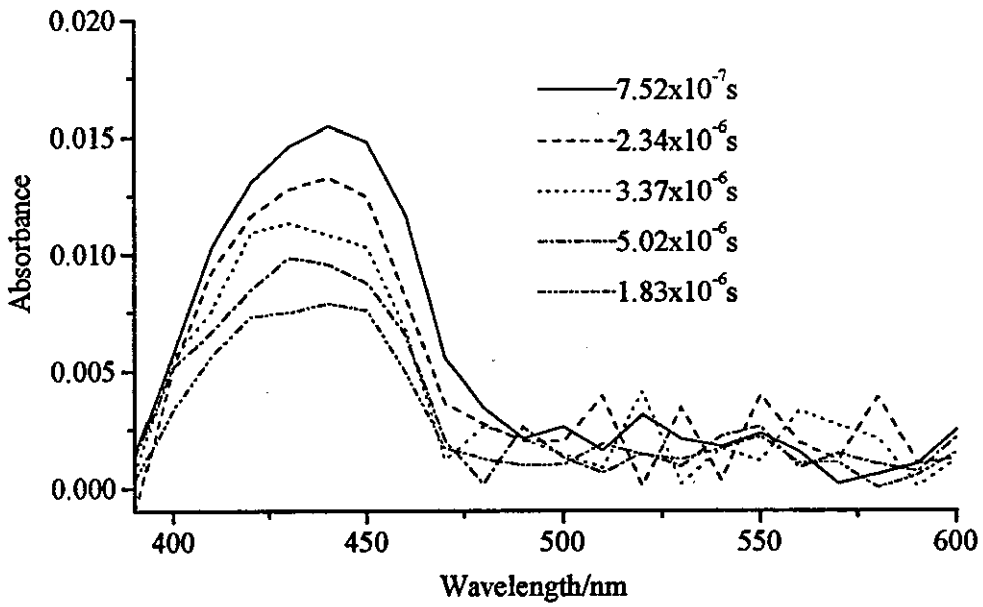


Figure 4.30: Transient absorption spectrum of 9.4×10^{-5}M TBS in 10% ethanol solution exciting at 355 nm.

The radical is suspected to be due to the loss of the bromine atom so experiments were carried out using the phenyl radical quencher carbontetrabromide.[158] This showed total quenching at high concentrations of the quencher confirming the species as being a phenyl radical. These laser flash photolysis results for TBS agree with the results found with T_4CS where the peak at 660 nm was assigned as a triplet and the peak at 460 nm was assigned as due to the radical and triplet species.[56] The difference between the transients seen in the two different solvents is probably due to the difference in the protic/aprotic nature of the solvent. There was a residual absorption present at the end of the decay trace in both aerated and degassed solutions. This shows the formation of a product which is probably formed from the radical species as it is seen in the same quantities in both aerated and degassed solutions, whereas the triplet species is only seen in degassed solutions. This product is also seen in the same region of the spectrum as the radical species. The radical and the product are probably formed from the singlet state which is not quenched by oxygen, whereas the triplet state has been shown in the above experiments to be quenched by oxygen.

The transient difference spectrum of TBS adsorbed on cellulose was obtained using a laser excitation wavelength of 355 nm. This spectrum again shows the presence of the two peaks seen in the acetonitrile solution spectrum.

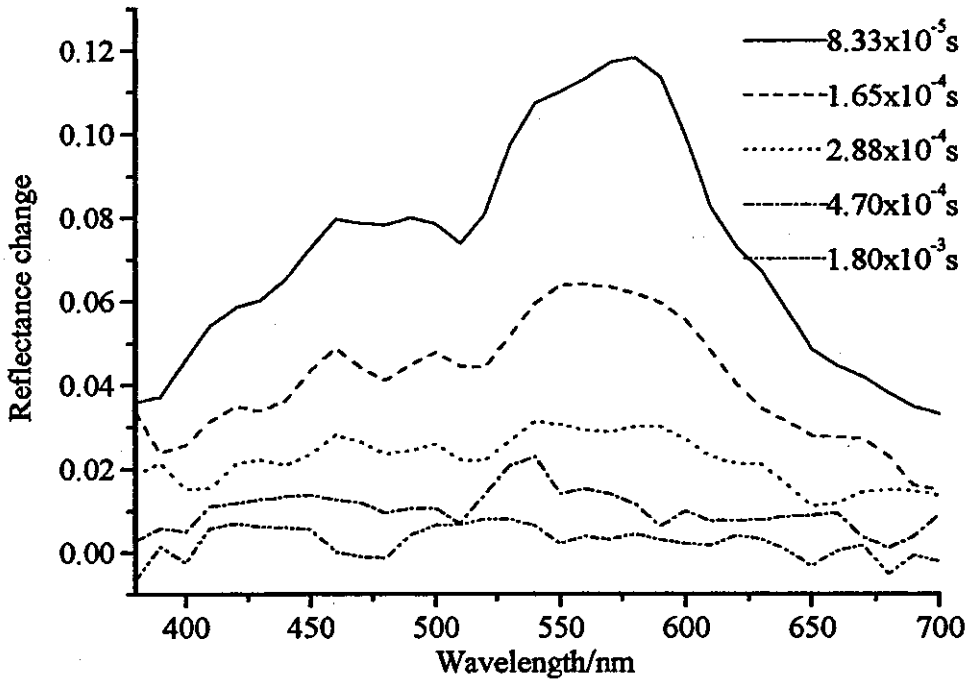


Figure 5.31: Transient difference spectrum of 1 mg/g TBS adsorbed onto cellulose, exciting at 355 nm.

The two peaks have λ_{max} at 570 nm and 470 nm, which is the same as seen for TBS in acetonitrile. The spectrum of TBS on cellulose should be the same as seen in a polar solvents[113] as shown by other work on cellulose and therefore the presence of the radical species should be clearly seen. The peak at 550 nm was assigned to a triplet species in solution and was therefore assumed to be the same when TBS was adsorbed onto cellulose. This transient was fitted to first order kinetics to give a lifetime of 1.62×10^{-4} s which is longer than the value of 8.85×10^{-6} s seen in solution. The second peak at 470 nm has been shown in solution to be a phenyl radical species in polar solvents. The lifetime of the species at 470 nm was found to be 1.82×10^{-4} s which is the same lifetime as seen at 570 nm which would suggest that the species at 470 nm on cellulose is due to the triplet species agreeing with the results obtained in acetonitrile.

However some radical species may still be present absorbing at this wavelength as it was seen in solution when the triplet species was not there to mask it.

In an aqueous solution at pH 7.4 the only transient seen is the radical at 460 nm which has been assigned as a substituted phenyl radical. On the addition of H.S.A. to TBS the intensity of this radical species decreases. These results provide more evidence for increased intersystem crossing to the triplet state on the addition of H.S.A.. This agrees with the fluorescence and phosphorescence data, assuming the radical species is formed from the singlet state of the compound. There is a slight decrease in the lifetime from $1.3x10^{-5}$ s $\pm$ $6x10^{-7}$ s without H.S.A present to $8.0x10^{-6}$s $\pm$ $3x10^{-7}$ s with H.S.A. present. There is still no sign of the triplet species in this solvent even with increased nonradiative transitions from the singlet state including intersystem crossing to the triplet state.

5.2.4. Singlet oxygen

Singlet oxygen was detected from a solution of TBS in acetonitrile after excitation at 355 nm. The quantum yield was found to be 0.04 when measured compared to 2-acetonaphthone whose quantum yield is known to be 0.79 in acetonitrile by the method described in chapter 3. This value shows that the triplet quantum yield must have a value of at least 0.04. This is because the singlet oxygen must be formed from the triplet state alone as the fluorescence was shown earlier not to be quenched by oxygen. This is a low value therefore it is likely that the production of singlet oxygen does not play a major role in the photoallergy process.

The quantum yield of singlet oxygen production was reduced in D_2O in the presence of H.S.A.. This cannot be explained by the increased intersystem crossing as an increased triplet quantum yield should increase the singlet oxygen quantum yield. Therefore this effect is probably due to the H.S.A. protecting the triplet species from molecular oxygen, which is similar to the effect seen by adsorbing these compounds onto cellulose, therefore preventing the formation of singlet oxygen. The singlet oxygen was measured in D_2O as the signal from water would be too quick to measure. The lifetime is markedly shorter and the quantum yield smaller in water than in acetonitrile which is

expected as the oxygen concentration in water is a factor of ten less than in acetonitrile and the triplet decay was not detected in water.

5.2.5. Photodegradation

The laser transient decay traces showed the formation of long lived species. A comparison made of the ground state absorption spectrum before and after laser flashing shows a permanent difference in the spectrum. This was investigated further by placing the TBS in front of an arc lamp using a 350 nm interference filter. The quantum yield of initial degradation, assuming there was no absorption of the product at the analysing wavelength of 380 nm, was found using the method described in chapter 3 to be 0.12 which shows there is a large amount of degradation of TBS. The ground state absorption spectra show a decrease in the absorption at 360 nm and 275 nm as shown in figure 5.32. There is however an increase in the absorption at 325 nm. There was no difference seen in the degradation between aerated and degassed solutions.

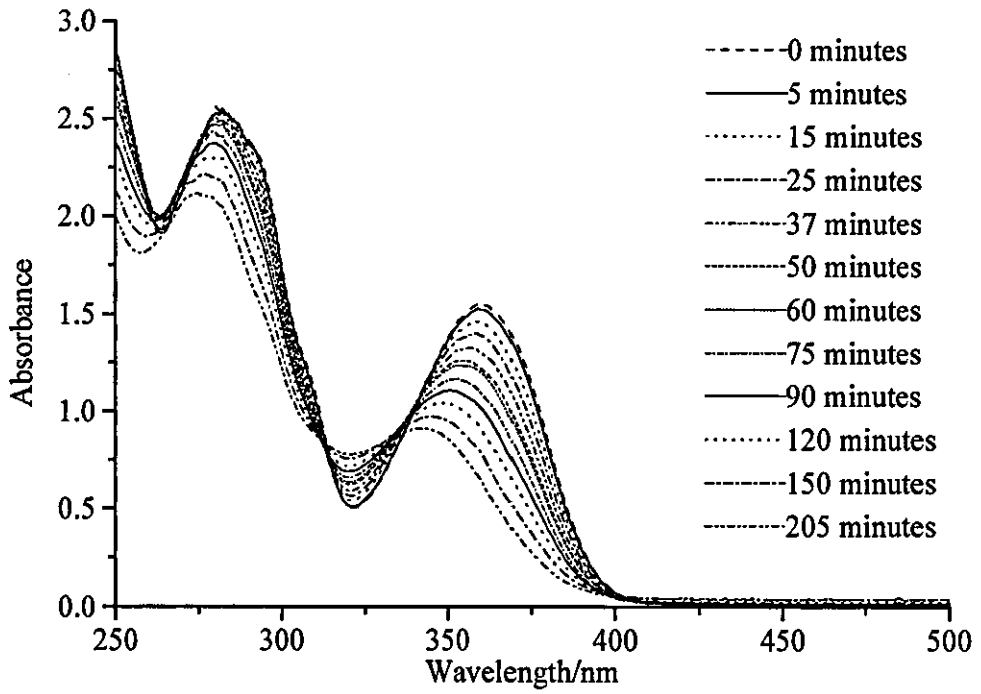


Figure 5.32: 1.5×10^{-4}M TBS at pH 7.4 with irradiation over time at 350 nm.

Irradiation moves the position of the peak at 360 nm to 340 nm. This blue shift is probably due to the stepwise loss of the bromine ions which would agree with the E.S.R work carried out by others[81,87] showing the systematic loss of 3, 5,4' bromines from TBS and their being substituted with a hydrogen from the solvent as seen with T_4CS when studied by gas chromatography.[81] This substitution has not been shown to be dependent on solvent from this or previous work on either TBS or T_4CS by E.S.R.,[81-86] however the work carried out on bithionol and fentichlor showed some solvent dependence and it is therefore possible that the TBS radical may abstract different groups from different solvents in the same manner. The effect of solvents on TBS would need further investigation using methods such as HPLC and GC-MS.

Irradiation of TBS in the presence of H.S.A. was found to give considerably less degradation than without H.S.A. present and with an excess of H.S.A. present it was found to be almost non existent. This may suggest that the product is from the singlet state as this was found to decrease as shown by the fluorescence data.

Summary of the photochemical properties of TBS

TBS has been shown here to have a very similar photochemistry to T_4CS which has been studied previously.[56] The ground state absorption spectra showed a peak at 360 nm in water which was assigned as a $\pi \rightarrow \pi^*$ transition. In acetonitrile a peak was also seen at 380 nm which was assigned as an $n \rightarrow \pi^*$ transition. The ground state absorption of TBS varied with pH showing a pKa of 6.9 which is higher than the value obtained for T_4CS of 5.5[56]. The ground state spectra of TBS adsorbed onto cellulose was the same as that seen in polar solutions except for a greater relative absorption at longer wavelengths.

TBS has shown a fluorescence emission with a quantum yield of 0.065 which was lower than the 0.34 found with T_4CS[56] due to the bromine atoms on the TBS increasing the intersystem crossing to the triplet state. The phosphorescence emission at 77 K gave a quantum yield of 0.59 and a phosphorescent lifetime of 8.8 ms. The triplet state was assigned as a $^3(n\pi^*)$ state due to the small energy gap between the excited singlet and triplet state and the millisecond lifetime of the phosphorescence.

However the bromine atoms could contribute to a heavy atom effect and this transition could therefore be a $\pi \rightarrow \pi^*$ transition. The addition of H.S.A. to the aqueous solution of TBS showed an increase in the intensity of the triplet state and a decrease in the fluorescence intensity from the singlet state.

Laser flash photolysis studies have shown that in acetonitrile there are two peaks present at 550 nm and 440 nm which were both assigned as the triplet species by triplet quenching experiments with naphthalene. However in aerated solutions it was found that a fraction of the peak at 440 nm was still present. This showed the possible presence of a radical species at 440 nm underlying the triplet species. This radical was investigated further by changing the solvent to 10 % ethanol: 90 % water where the triplet was no longer observed and it was found that in this environment the radical was the only species present and had a lifetime of 3.2×10^{-6} s. The radical was identified as a phenyl radical due to the loss of a bromine, using carbontetrabromide as a phenyl radical quencher. This agrees with the photochemistry found for T_4CS[56] where a triplet species was found to give an absorption change at 660 nm and 460 nm and the radical was found to absorb at 460 nm which was due to the loss of the 3 chlorine from the phenyl ring.[81-85]

On cellulose only the triplet species of TBS was observed in the same place as in solution but it was found to have a longer lifetime than seen in solution. The addition of H.S.A. in aqueous solutions was found to decrease the lifetime of the radical and the amount of radical seen. This would agree with the data found for the changes seen in the fluorescence and phosphorescence on adding H.S.A. if the radical were formed from the singlet state. It seems likely that the radical is formed from the singlet state as it is not quenched by oxygen whereas the triplet state has been shown that it is quenched by oxygen.

Singlet oxygen production showed a low quantum yield of 0.04 in D_2O which would suggest that it is not the main pathway for photoallergy to occur in this compound. However this does tell us that the quantum yield of the triplet species is at least 0.04,

as the singlet oxygen must come from the triplet species as the fluorescence is not quenched by oxygen.

The degradation studies carried out on TBS showed the formation of a product or several products. Work carried out by others using E.S.R.[81,87] have shown that bromines are lost stepwise from the 3,5,4' positions with increasing amounts of radiation. The products are then formed from the radicals by hydrogen abstraction from the solvent.

5.3. 6-Methylcoumarin

Coumarins have been studied in detail before[91] but very little work has been carried out on 6-methylcoumarin. 6-methylcoumarin has been shown to be a photoallergic compound whereas coumarin shows no photoallergic capability. Photobiological studies have shown[89] that a substitution of an alkoxy or methyl group on the 6 or 7 position on the benzopyran ring will confer photoallergic capability.[88,89]

5.3.1. Ground state absorption spectra

The ground state absorption spectra of 6-methylcoumarin was recorded in ethanol as shown in figure 5.33.

The position of the absorption maximum, which is due to a $\pi \rightarrow \pi^*$ transition, is at 320 nm.[62] Although it has been predicted by Becker et al[91] that a $n \rightarrow \pi^*$ transition should occur due to the lone pair on the oxygen of the carbonyl it could not be found at any concentration in the present work or by Becker et al.[91]

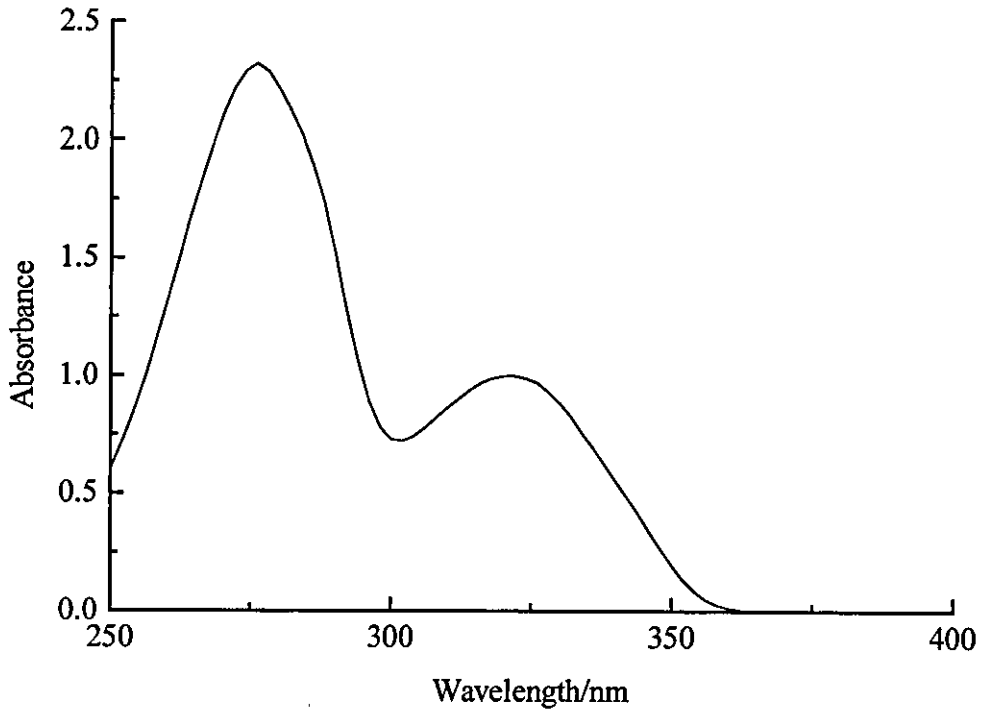


Figure 5.33: Ground state absorption spectra of 2.1×10^{-4} M 6-methylcoumarin in ethanol.

The molar absorption coefficient measured at 320 nm in ethanol was found to be 4700 $dm^3mol^{-1}cm^{-1} \pm 250$ $dm^3mol^{-1}cm^{-1}$. The molar absorption coefficient was measured in different solvents and was found to increase with increasing polarity as shown in table 9.

Solvent	Molar absorption coefficient/$dm^3mol^{-1}cm^{-1}$
Acetonitrile	4300 ± 200
Propanol	4700 ± 200
water	5000 ± 200

Table 9 : Molar absorption coefficients of 6-methylcoumarin at 320 nm in different solvents

The ground state diffuse reflectance (Kubelka Munk) spectrum of 6-methylcoumarin adsorbed onto cellulose showed peaks at 325 nm and 280 nm showing a slight red shift from those observed in ethanol. The size of the peaks relative to each other has changed, as shown in figure 5.34.

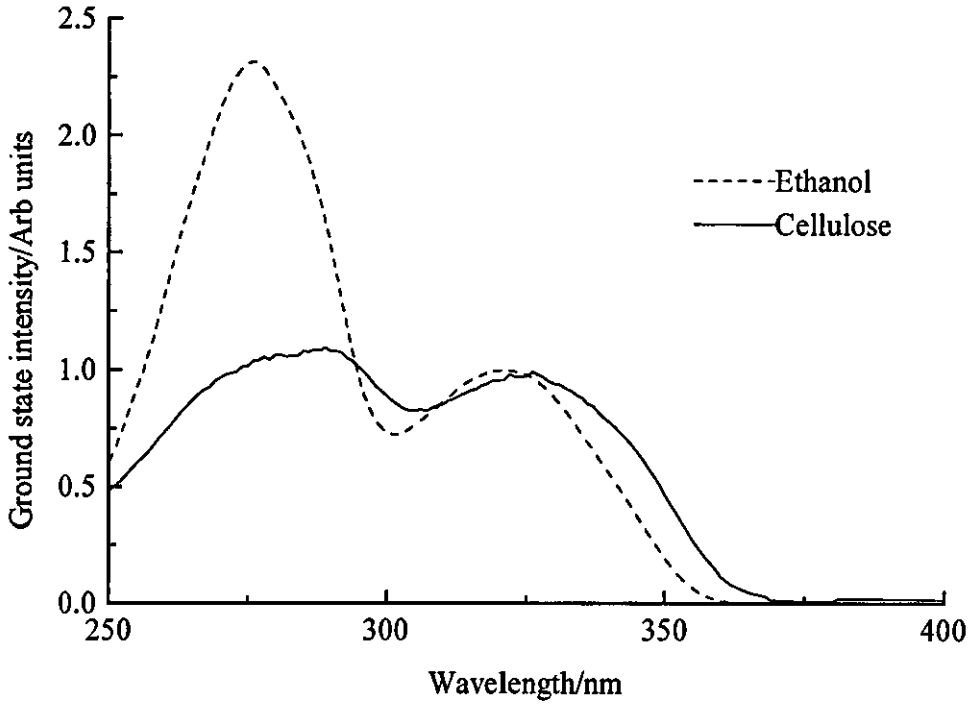


Figure 5.34: Ground state spectra of 6-methylcoumarin adsorbed onto cellulose and in ethanol.

The peak at 324 nm has become the larger of the two peaks in cellulose compared to 6-methylcoumarin in ethanol where the 275 nm peak is the larger peak due to fluorescence by the sample as explained earlier. The broadening of both peaks when 6-methylcoumarin is adsorbed onto cellulose causes the peaks to show an increased overlap and less peak definition. This is caused by the inhomogeneity of the cellulose which results in varying adsorption sites.

5.3.2. Emission Spectroscopy.

5.3.2.1. Fluorescence

6-methyl coumarin is found to fluoresce when excited at 340 nm giving rise to 1 peak with a λ_{max} at 405 nm, as shown in figure 5.35, which is not the same as the literature value of 370 nm in1,4 dioxane.[91]

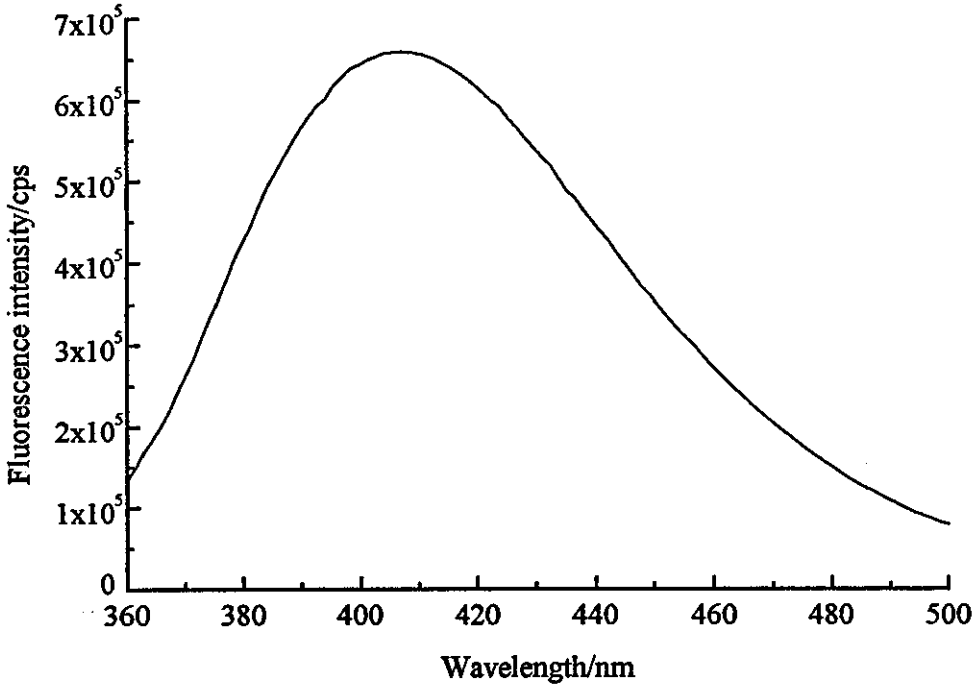


Figure 5.35: Emission spectrum of 6-methylcoumarin in ethanol exciting at 320 nm with excitation slits = 0.5 mm and emission slits = 1 mm.

The energy of the lowest excited singlet state can be calculated from the λ_{max} of fluorescence and the λ_{max} of the ground state absorption and was found using the method described in chapter 3 to be 331 $kJmol^{-1} \pm 5$ $kJmol^{-1}$. The fluorescence was not affected by the presence of oxygen which suggests the singlet excited state is not quenched by oxygen and that the fluorescence lifetime must therefore be very short.

The quantum yield of fluorescence of 6-methylcoumarin was found by comparison with anthracene whose quantum yield is known to be 0.29 in ethanol.[163] This gave a quantum yield of 0.013 for 6-methylcoumarin in ethanol. This is a lot greater than the value of 10^{-4} found by other groups for coumarin,[164,165] the difference therefore being due to the presence of the electron donating effect of the methyl group in 6-methyl coumarin. The fluorescence of 6-methylcoumarin was too fast for the lifetime to be measured directly, however the radiative lifetime was calculated using the method described in chapter 3 to be 4.1×10^{-8} s. The estimated lifetime of the fluorescence found using the measured quantum yield was 5.2×10^{-10} s. The excitation spectra was

the same as the ground state absorption spectra showing no impurities were present to effect the fluorescence emission.

The emission spectrum was unchanged when adsorbed onto cellulose compared to the fluorescence spectrum seen in solution. There is 1 peak with a λ_{max} at 400 nm which is close to the value of 405 nm seen in solution.

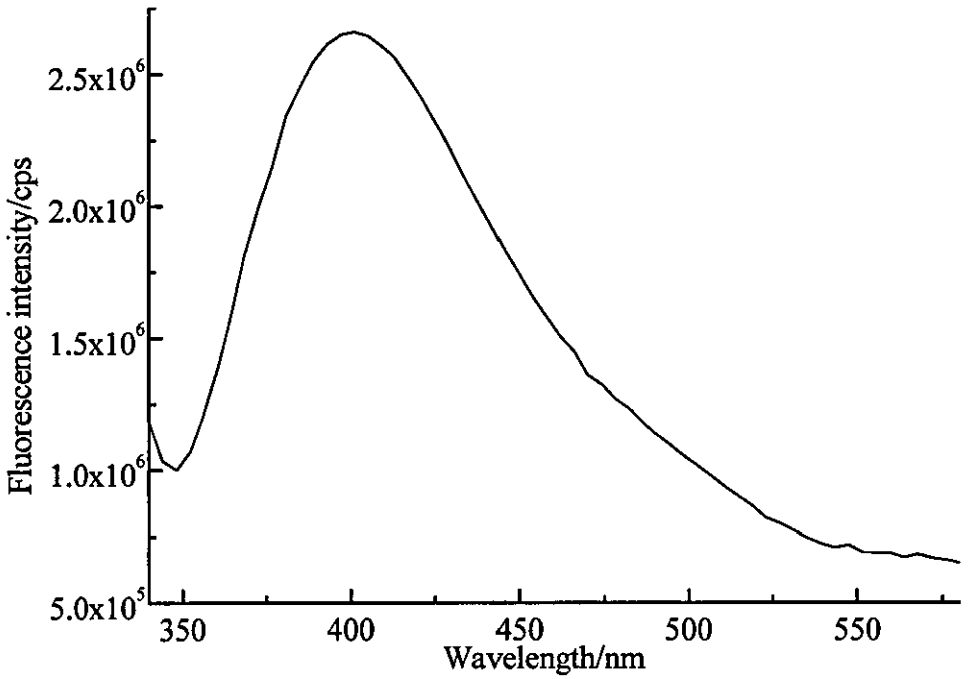


Figure 5.36: Emission spectrum of 6-methylcoumarin 2..3 mg/g adsorbed onto cellulose with excitation 0.3 mm, emission slits 0.6 mm.

The fluorescence in the presence of H.S.A. also had a λ_{max} at 406 nm showing this spectrum is due to the same species, but the fluorescence intensity was reduced by the presence of H.S.A. as shown in figure 5.37

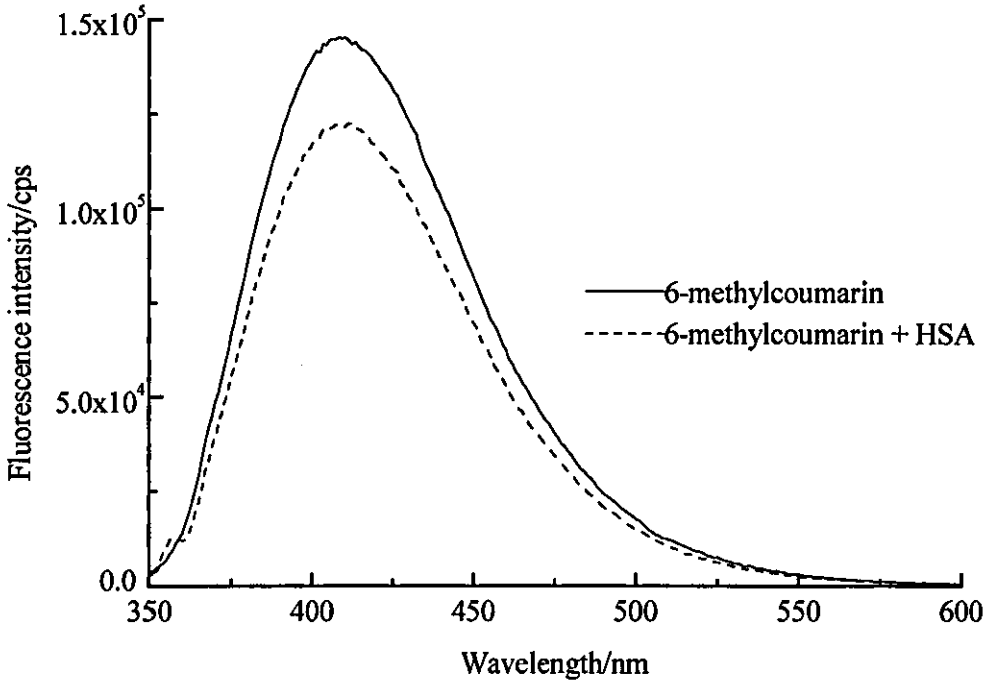


Figure 5.37: Emission spectra of 6-methylcoumarin with and without $2x10^{-6}$ M H.S.A. exciting at 330 nm with excitation slits = 0.4 mm and emission slits = 0.8 mm.

The amount of quenching was measured down to a maximum at which the addition of H.S.A. had no more affect on the fluorescence and the amount of quenching could not be attributed to H.S.A. absorption at 330 nm. The quenching of the fluorescence with addition of H.S.A. is the same effect as seen with TBS and again the quenching of the singlet state can not be explained by an energy transfer mechanism. As with TBS the decrease in the fluorescence is probably due to an increase in the rate of the nonradiative transitions from the singlet state. This may be caused by the formation of a protein-6-methylcoumarin complex on binding and the position in which the photoallergen binds.

5.3.2.2. Phosphorescence

The phosphorescence of 6-methylcoumarin was found to be a structured emission over the range 450 nm to 600 nm with the main peaks at 460 nm and 500 nm in 7:3 isopentane:2-propanol at 77K as seen in figure 5.38.

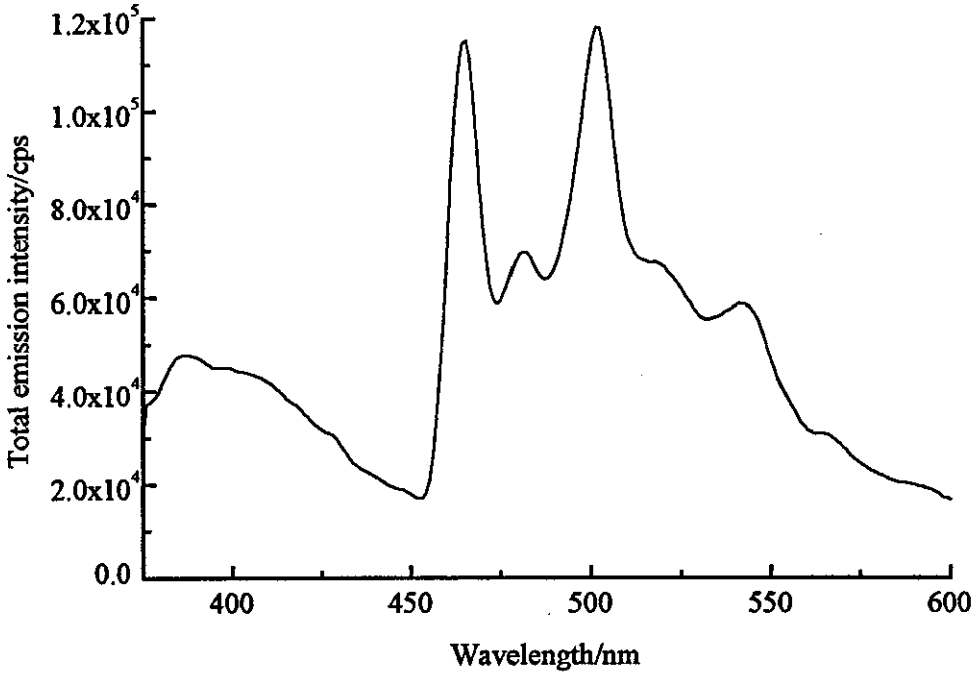


Figure 5.38:Emission spectrum of 6-methylcoumarin in 7:3 isopentane : 2-propanol at 77 K exciting at 320 nm with excitation slits = 0.3 mm and emission slits = 0.6 mm

The peak seen at 460 nm was due to the 0-0 vibronic transition and this was used to calculate the triplet energy for 6-methylcoumarin as being 260.1 $kJmol^{-1} \pm 4$ $kJmol^{-1}$. This is almost the same as the triplet energy of 258 $kJmol^{-1}$ for coumarin[91] suggesting the presence of the methyl group has very little effect on the triplet state energy. The separation between the excited singlet and triplet states was therefore found to be 39 $kJmol^{-1} \pm 9$ $kJmol^{-1}$. There was also an emission seen at 400 nm which was assigned to the fluorescence as it occurs at the same wavelength as the fluorescence seen at room temperature. This means that the intersystem crossing quantum yield must be low for this molecule. An external heavy atom quencher was added to try and promote the intersystem crossing, by quenching the fluorescence and increasing the phosphorescence. The heavy atom containing compound chosen was potassium iodide[166], this was added in increasing quantities to see the effect on the fluorescence and the phosphorescence as shown in figure 5.39

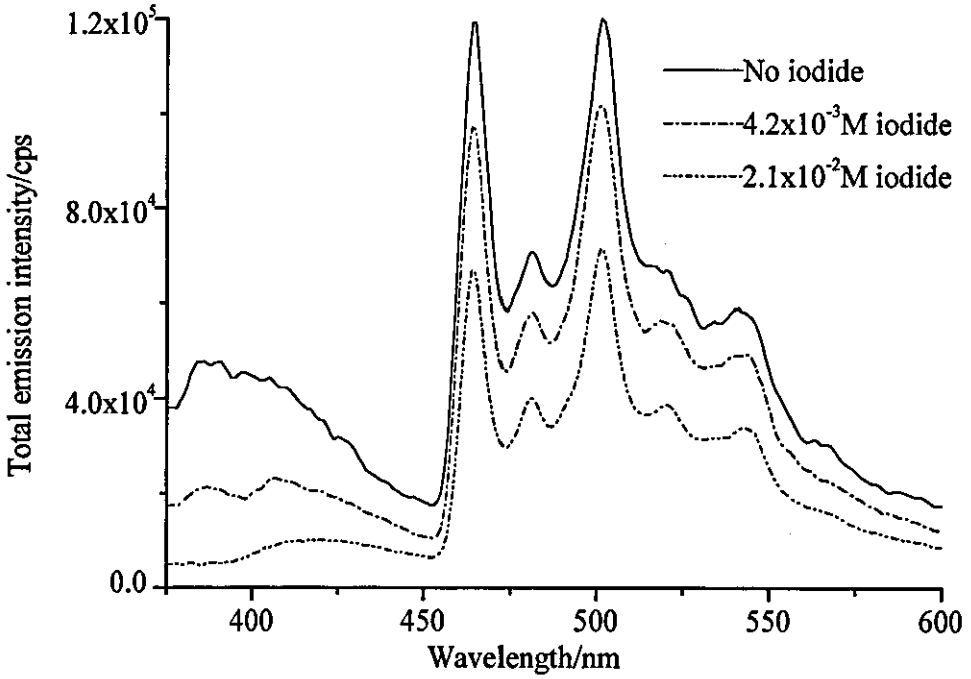


Figure 5.39: Emission spectra of 6-methylcoumarin in 7:3 isopentane:2-propanol at 77K with different concentrations of potassium iodide with excitation slits = 0.3 mm and emission slits = 0.6 mm exciting at 320 nm.

The fluorescence was quenched by the iodide ion at high iodide concentrations the fluorescence disappeared totally. The phosphorescence, however was also reduced by increasing iodide concentrations. This could be due to competition from radiationless processes from the excited triplet state. The same effect was seen by using iodine in ethanol as the quencher.[167] The quantum yield of the phosphorescence was calculated compared to benzophenone whose phosphorescence quantum yield is known to be[154] 0.84 and was found to be 0.075 which is slightly higher than 0.066 quoted by other groups[91] and very similar to 0.055 found for coumarin.[91] The lifetime of the 6-methylcoumarin phosphorescence was calculated using the HY laser in emission only mode to be 0.10 s ± 0.02 s by fitting the emission decay trace at 460 nm to first order kinetics at 77K in 7:3 isopentane:2-propanol. The long phosphorescent lifetime suggests the to triplet state was a $^3(\pi,\pi^*)$ state.

The 6-methylcoumarin showed an increase in the phosphorescence intensity in the presence of H.S.A. compared to the phosphorescence seen without H.S.A.. as shown in figure 5.40.

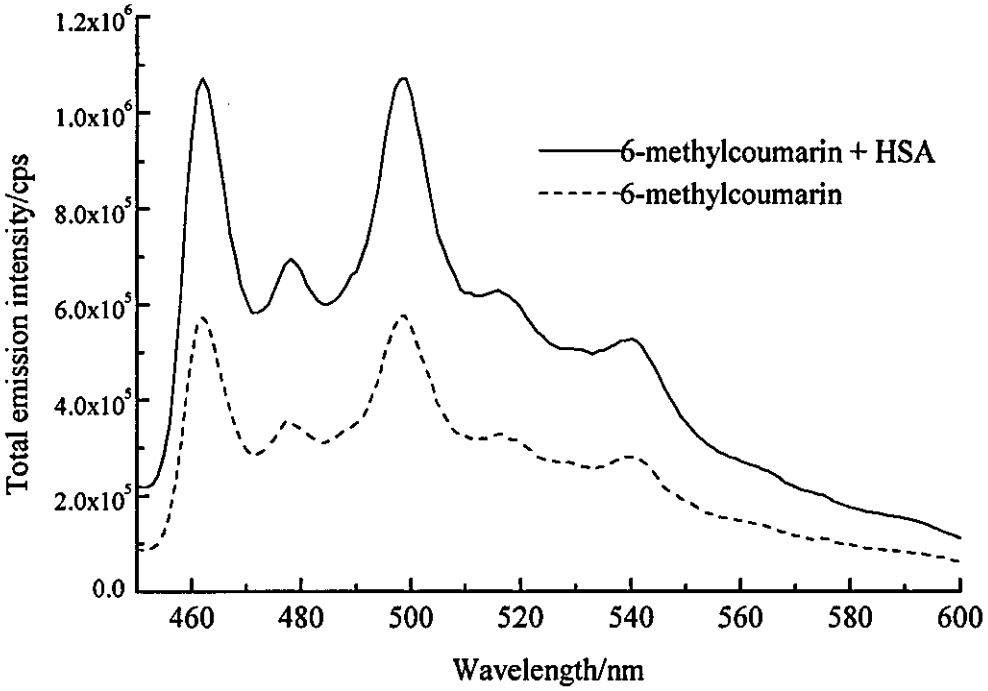


Figure 5.40: Emission spectra of 6-methylcoumarin with and without $1x10^{-6}$ M H.S.A. at 77K exciting at 355 nm, with excitation slits = 0.5 mm, emission slits = 1 mm.

The lifetime in the presence of H.S.A was longer than without H.S.A. increasing to 0.3 s from 0.11s. These results confirm the theory that the H.S.A. increases the nonradiative transitions from the singlet state by forming a complex with the 6-methylcoumarin. In this case H.S.A. appears to be promoting intersystem crossing from the singlet to the triplet state causing the fluorescence to decrease and the phosphorescence to increase. However the fluorescence and phosphorescence can not be compared directly as the temperatures at which the experiments were carried out were different and the rates of the nonradiative transitions may vary with temperature.

5.3.3. Laser flash Photolysis

Degassed and aerated solutions of 6-methylcoumarin were flashed at 355 nm and 266 nm in an attempt to see the transient reported by others. A variety of concentrations of

6-methylcoumarin, solvents, both polar and nonpolar and laser energies were used but no transients were observed. The triplet-triplet absorption has been observed by other research groups[91] and found to have a λ_{max} at 420 nm and a lifetime of 2.3 μs in methanol, but the triplet quantum yield was found to be only 0.04 in benzene which is very similar to coumarin where the triplet species is seen at 400 nm with a lifetime of 3.8 μs in nonpolar solvents and a quantum yield of 0.054.[168] The low quantum yield of 6-methylcoumarin suggests the lack of transient could be due to a lower laser energy or a less sensitive detector than used by other groups. It was therefore decided to use a triplet sensitiser. A suitable sensitiser was found to be benzophenone[169,170] which was known to have a triplet energy of 287 kJmol^{-1} in nonpolar solvents[154] which is higher than the triplet energy of 6-methylcoumarin whose triplet energy is 260 kJmol^{-1} found from phosphorescence data. This produced a spectrum for 6methylcoumarin with a λ_{max} at 430 nm as shown in figure 5.41.

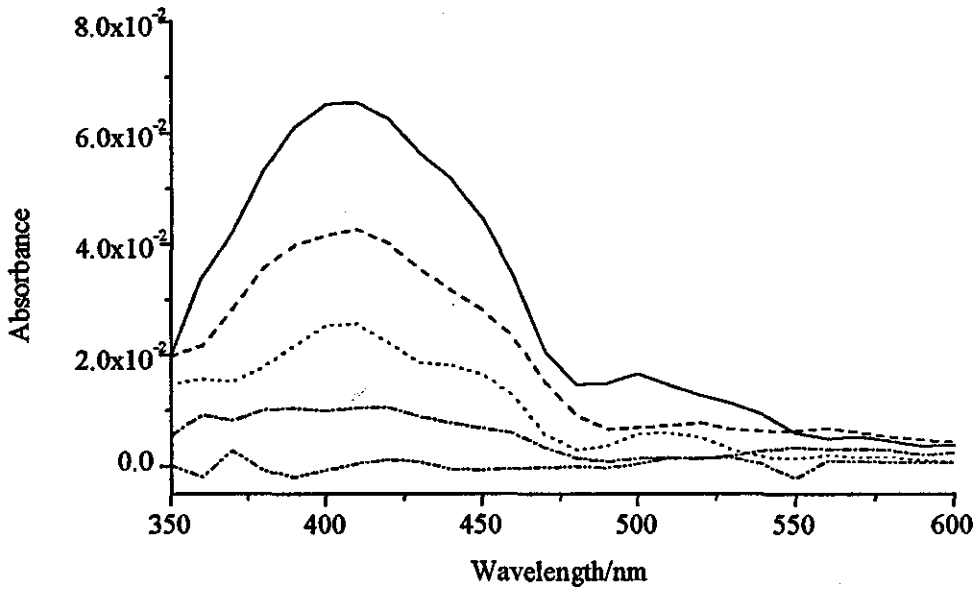


Figure 5.41: Transient absorption spectra of 2.8x10^{-4} M 6-methylcoumarin sensitised with 4.3x10^{-3} M benzophenone in acetonitrile exciting at 355 nm.

It is known that although the benzophenone triplet[171] has an absorption with a λ_{max} at 530 nm there is also some absorption at 430 nm. It was therefore necessary to use a concentration of 6-methylcoumarin where the benzophenone triplet could no longer be seen to interfere at 430 nm. There was also a decrease in the benzophenone transient at

530 nm allowing the position where the benzophenone triplet was fully quenched to be detected. This decrease in the benzophenone triplet lifetime also confirmed that the species being detected from excitation of 6-methylcoumarin was due to a triplet species or formed from it. The lifetime of the transient of 6-methylcoumarin was found from first order kinetic fitting of the transient decay trace at 430 nm to be 2.6 μs ± 0.5 μs. This confirms that the reason the transient could not be seen was not a short triplet lifetime but the low quantum yield of the triplet. The transient decay trace returns to the original absorption showing no long lived species are being detected using laser flash photolysis.

When 6-methylcoumarin was adsorbed onto cellulose a transient was seen with a λ_{max} at 420 nm as shown in figure 5.42. This value agrees with literature values for λ_{max} in solution[91] and values obtained by sensitisation experiments in solution, which gave values for λ_{max} of 420 nm and 430 nm respectively.

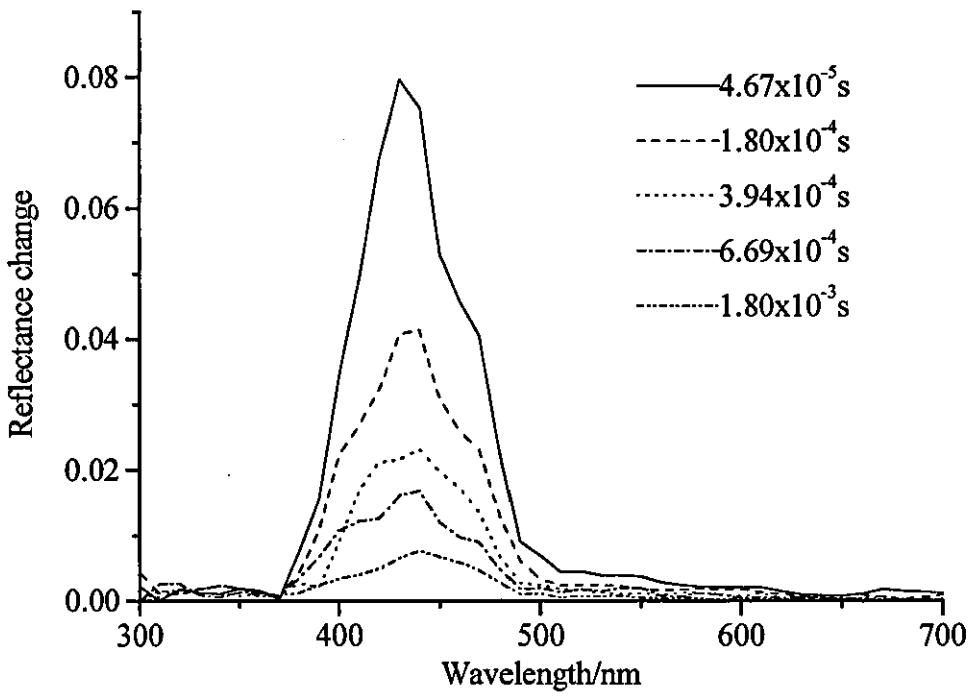


Figure 5.42:Transient difference spectrum of 2.3mg/g 6-methylcoumarin adsorbed onto cellulose

This was identified as being due to the triplet species and the lifetime was found to be 0.04 s which is a lot longer than the 2.3 μs found by Becker et al in methanol[91] and 2.5 μs found by sensitisation in solution. The increase in the lifetime on adsorbing 6-methylcoumarin onto cellulose which agrees with previous compounds and other work carried out on cellulose as explained earlier. The rigidity of the cellulose must also cause an increase in the intersystem crossing, therefore increasing the triplet yield as 6-methylcoumarin has a very low triplet quantum yield in solution and was only seen in this work using a triplet sensitiser, whereas when it is adsorbed onto cellulose the triplet species is easily seen. Cellulose is also known to protect the triplet species from quenchers such as molecular oxygen as explained in chapter 3.

The 6-methylcoumarin and H.S.A were mixed together without a sensitiser and a transient absorption was detected at 420 nm which is the wavelength at which the triplet species was shown to be present in this thesis and by Becker et al[91]. H.S.A. has been shown in the phosphorescence and fluorescence studies to be increasing the intersystem crossing to the triplet state and this experiment now allows the triplet species to be seen directly by laser flash photolysis.

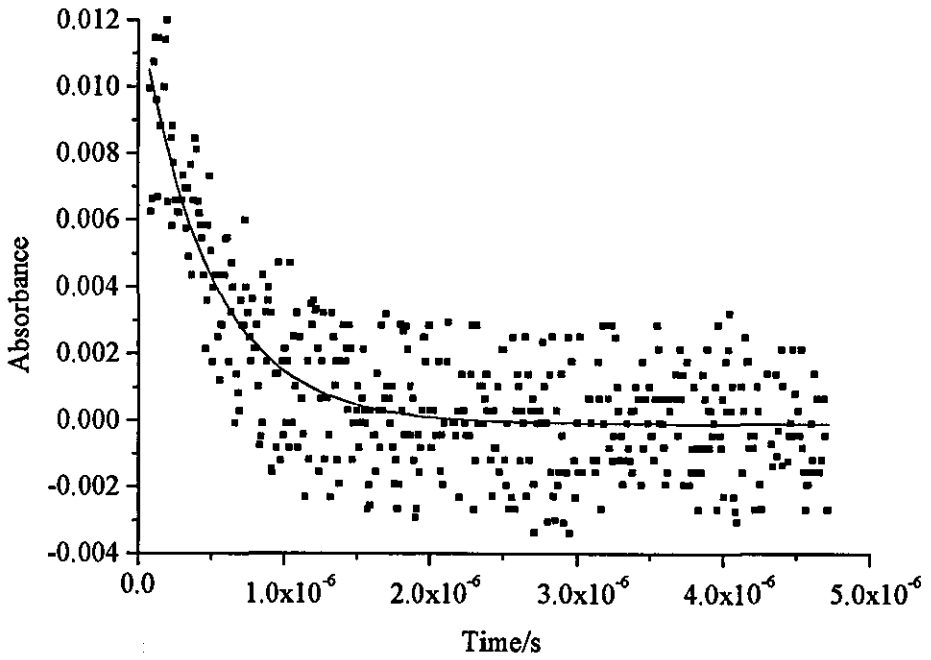


Figure 5.43: Transient decay trace at 420 nm of a degassed solution of 1.7×10^{-3} M of 6-methylcoumarin with 1.1×10^{-4} M H.S.A., exciting at 355 nm.

The lifetime however, was quicker at $4.7x10^{-7}$ s than seen in solution with a sensitiser, which was 2.5 μs. Degassed solutions of 6-methylcoumarin, H.S.A. and a sensitiser were excited with the laser to observe any changes in the transient spectrum. There was no obvious change in the intensity or the lifetime however this was not felt to be an accurate way to measure any differences due to the number of compounds present.

5.3.4. Singlet Oxygen

A singlet oxygen signal for 6- methyl coumarin was detected with excitation at 355 nm but it was very weak signal. The value for the quantum yield was found to be 0.02 compared to 2-acetonaphthone whose quantum yield is 0.79 in acetonitrile. This low value is to be expected due to the low value for the triplet quantum yield of 0.04. This value for the singlet oxygen quantum yield of 6-methylcoumarin is very similar to the singlet oxygen quantum yield of 0.03 in methanol known for coumarin.[172] The low value for the singlet oxygen quantum yield of 6-methylcoumarin may suggest that singlet oxygen production would not be a major pathway in photoallergy, although the amount of singlet oxygen which may be required is not known. However since the triplet yield is also very low and very little other photochemistry has been observed this may actually prove to be a significant process in the 6-methylcoumarin photoallergy mechanism.

5.3.5. Photodegradation of 6-methylcoumarin

There was no sign of the formation of product or a longer lived species at any wavelength for 6-methylcoumarin from the laser flash photolysis work. An aerated solution of 6-methylcoumarin was irradiated for several hours and it was found that a small amount of degradation took place over this time.

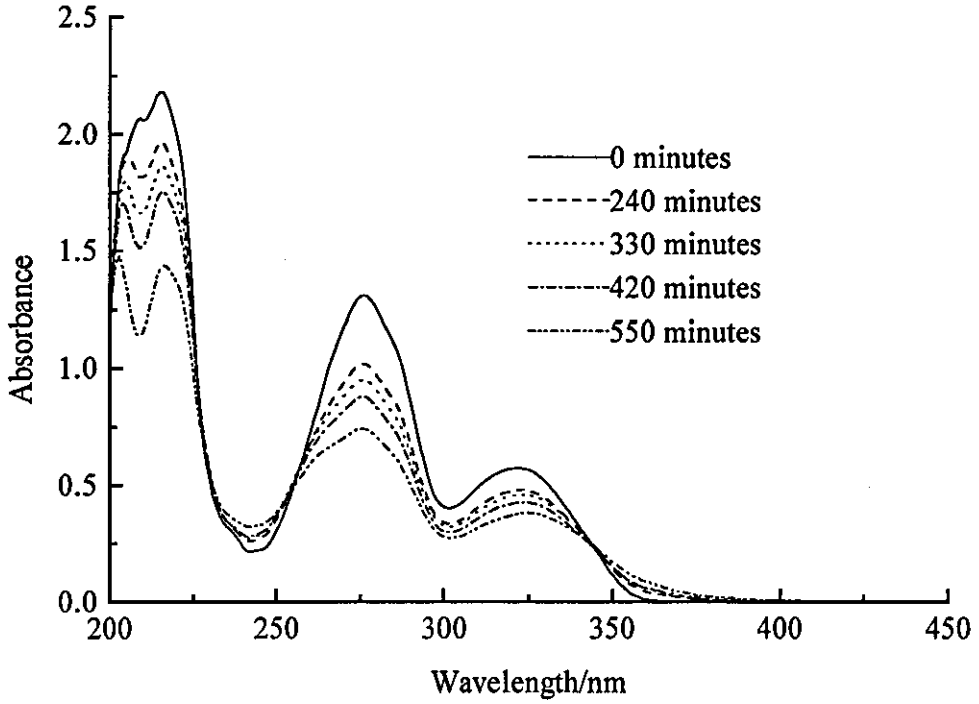


Figure 5.44: 6-Methylcoumarin in ethanol with irradiation using a full arc lamp over time.

This suggests there is some formation of a product from aerated solutions. The amount of degradation in degassed solutions is the same as for aerated solutions which suggests that the product is formed from the singlet state, since it is not affected by the presence of oxygen. The position of the peaks did not change but the amount of absorption decreased at 420 nm and 275 nm. There was an increase in absorption above 330 nm due to the formation of a product. There has been no formation of products seen from the laser flash photolysis work and no transients observed in aqueous solutions. However the presence of some degradation in aerated solutions may suggest the possibility of radical formation from the singlet state at quantum yields too small to be detected by our equipment which then go on to form the photoproducts seen here.

Due to the small amount of degradation over very long periods of time seen in aerated solutions in figure 5.44 it was very hard to tell if there was any difference seen in the presence of H.S.A. A more accurate method of measuring the degradation would be

needed for such small changes in the degradation to be measured with certainty. If the product is formed from the singlet state it would be expected that there would be a drop in the degradation with H.S.A. present.

Summary of the photochemical properties of 6-methylcoumarin

The basic photochemistry of this compound has been studied before and the data found in this thesis confirms those findings.[91] The ground state of 6-methylcoumarin showed peaks at 320 and 275 nm the peak at 320 nm being assigned as a $\pi \rightarrow \pi^*$ transition. As seen with bithionol and fentichlor on adsorbing 6-methylcoumarin onto cellulose the ratio of the peaks altered causing the peak at the higher wavelength to become the larger peak.

The fluorescence spectra was shown to have a maximum at 405 nm which is approximately the same as seen in the literature at 370 nm.[91] The quantum yield of fluorescence was measured to be 0.013 in ethanol. The phosphorescence spectra was found to give a structured phosphorescent emission over the range 450 nm -600 nm, however it also showed the presence of fluorescent emission at 400 nm. The addition of potassium iodide as a heavy atom quencher caused both the fluorescence and the phosphorescence to decrease due to competition from radiationless transitions from the triplet state. The triplet state was assigned as a $^3(\pi,\pi^*)$ from the long phosphorescent lifetime of 0.11 s with a quantum yield of 0.075. The addition of H.S.A. to the 6-methylcoumarin was found to increase the lifetimes and intensities of the phosphorescence and the triplet species seen after laser flash photolysis but to decrease the fluorescence emission. This suggests that the presence of H.S.A. increases the nonradiative transitions from the singlet state by the formation of a protein-6-methylcoumarin complex.

Becker et al[91] have observed the triplet species directly by flash photolysis but in this work it was necessary to use a triplet sensitiser to observe the triplet species due to the low triplet quantum yield. The sensitisation experiments showed the presence of a triplet species confirmed by a decrease in the benzophenone triplet species. The lifetime of the triplet was found to be 2.6 μs which is very similar to the 2.3 μs seen by

Becker et al [91] and to the 3.8 μs observed for coumarin.[168] When the 6-methylcoumarin was adsorbed onto cellulose the triplet species was observed at the same wavelength as seen in the sensitisation experiments in solution but with a longer lifetime. This is probably due to the cellulose protecting the 6-methylcoumarin from quenchers present in fluid solutions. The addition of H.S.A. to a solution of 6-methylcoumarin allowed the triplet species to be observed, probably due to an increase in the intersystem crossing or a decrease in the nonradiative transitions from the triplet state.

Singlet oxygen production was measured and found to give a low quantum yield of 0.02 which is very similar to the coumarin singlet oxygen quantum yield of 0.03. Since 6-methylcoumarin shows very little other photochemistry, the singlet oxygen may be considered a major pathway but since the similar amount of singlet oxygen is recorded for coumarin it seems unlikely.

The photochemistry of 6-methyl coumarin has been shown to be very similar to that of coumarin which has been studied before.[168] However coumarin is not a known photoallergen and this would therefore suggest that the triplet species is not involved in the photoallergy mechanism. The singlet oxygen is also probably not involved in the photoallergy mechanism but may cause phototoxicity as it is seen in both coumarin and 6-methylcoumarin.

There has been no previous steady state irradiation on this compound or on coumarin but 6-methylcoumarin showed a limited amount of degradation occurred in aerated solution showing the formation of a product. This is probably formed from the singlet state as previous work[91] shows the triplet state is quenched by oxygen as a lifetime of 2.3 μs would indicate. This suggests the involvement of another transient which is not affected by oxygen, in addition to the triplet species, which could possibly suggest a radical species. This species must have a very low quantum yield and would therefore need to be investigated using methods such as spin trapping. The photodegradation of coumarin should also be investigated for comparison as it is known not to be a photoallergen.

5.4. Omadine (sodium salt)

Omadine was found to dissolve easily in water therefore most of the experiments were done in water which was usually buffered to pH 7.4, the physiological pH. There have been several studies using E.S.R. [93,95,97,98] on omadine and with laser flash photolysis.[94,98]

5.4.1. Ground state absorption spectra

The ground state absorption spectra of omadine has λ_{max} at 335 nm 280 nm and 240 nm in water as seen in figure 5.45.

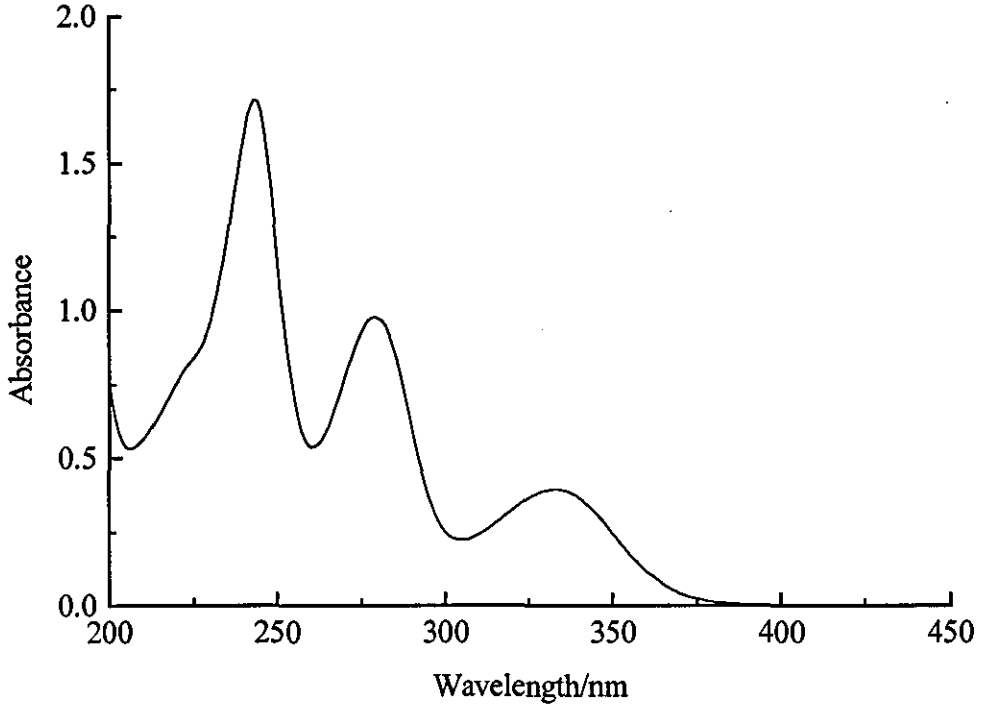


Figure 5.45: Ground state absorption spectra of 1.2×10^{-4} M omadine in water at pH 7

The peak at 335 nm being due to a $\pi \rightarrow \pi^*$ transition. The molar absorption coefficients was calculated to be 2900 $mol^{-1}dm^3cm^{-1} \pm 200$ $mol^{-1}dm^3cm^{-1}$ at 335 nm in water. There are known to be two tautomers of omadine, the thione and thiol forms [92] and as explained in chapter 2 the thione form is highly preferred to the thiol form in the neutral state. Each tautomer can be present in three ionisation states, cationic, anionic

and neutral so experiments were carried out varying the pH of the solution to find the change in the ground state absorption spectrum as shown in figure 5.46.

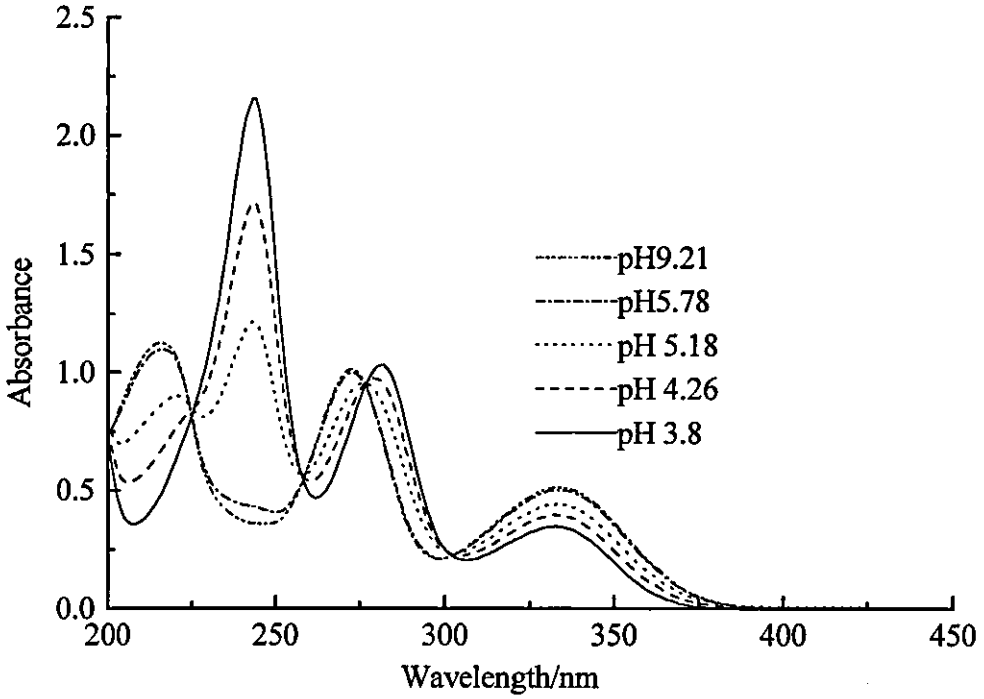
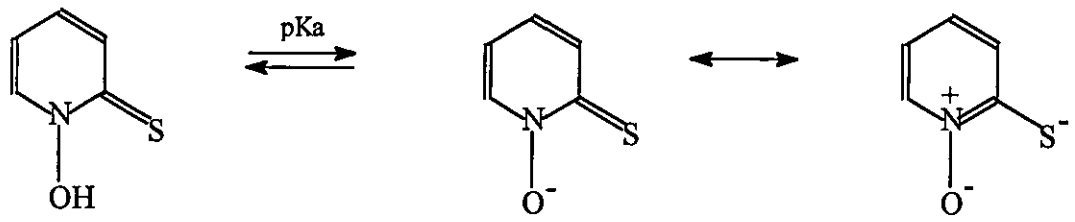


Figure 5.46: Ground state absorption spectra of 1.1×10^{-4} M omadine in water at varying pH.

There are 4 isosbestic points at 227 nm, 261 nm, 278 nm an 305 nm but these points do not move with pH showing that only 1 protonation reaction is occurring from pH 1 to pH 12, which agrees with results found by other groups in aqueous solution.[92] The peak at 243 nm is due to the protonated form of omadine and the peak at 213 nm is due to the non protonated form which has a extinction coefficient at pH 2 of 4800 mol^{-1}dm^{3}cm^{-1} $\pm$ 200 mol^{-1}dm^{3}cm^{-1} at 333 nm. The pKa was calculated using the method described in chapter 3 and was found to be 4.7. This value agrees with the value of 4.67 found by other groups.[92,93] There is only one protonation reaction which is shown below. There may also be some of the thiol tautomer present in the anionic form.[92,93]



The ground state diffuse reflectance (Kubelka Munk) spectrum of omadine has peaks with λ_{max} at 340 nm, 290 nm and 245 nm as shown in figure 5.47 which is approximately the same as seen in solution.

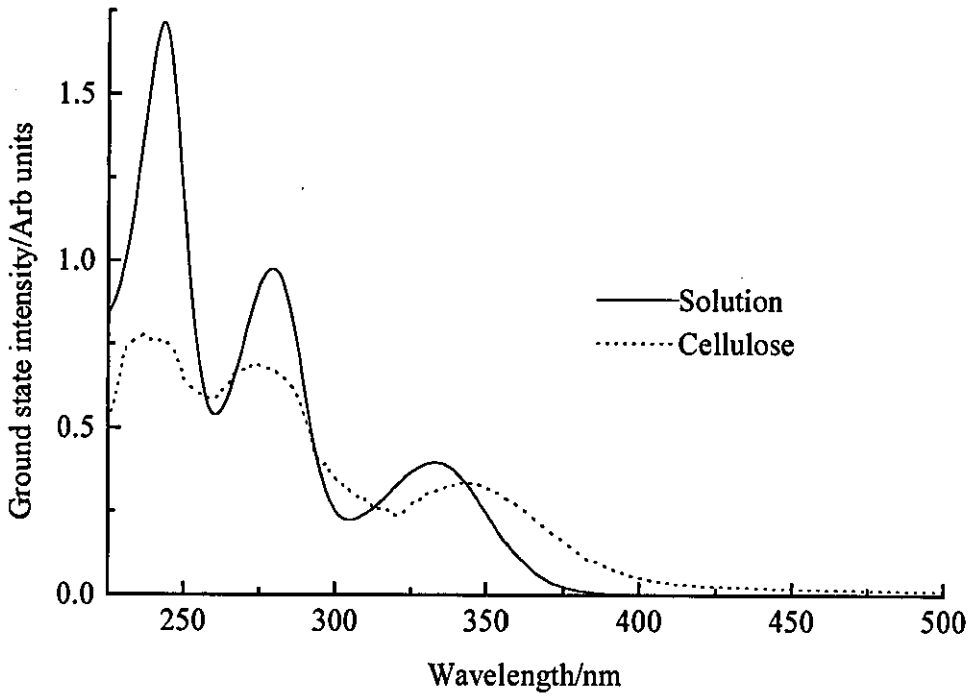


Figure 5.47: Ground state spectra of omadine adsorbed onto cellulose and in water.

The size of the peaks relative to each other has changed when the omadine is adsorbed onto cellulose, compared to omadine in water. The peak at 290 nm is now the same size as the peak at 245 nm, this may however be accounted for by some absorption by the barium sulfate standard used as explained earlier.

5.4.2. Emission spectroscopy

5.4.2.1. Fluorescence

There was no fluorescence detected from omadine on our instrument exciting at any wavelength. Thus the quantum yield of fluorescence is too small for our instrument to be able to detect it.

5.4.2.2. Phosphorescence

The omadine was excited at 350 nm in ethanol at 77K and was found to give a broad structureless phosphorescent emission over the range 440 nm to 700 nm. The phosphorescent emission had a λ_{max} at 500 nm as shown in figure 5.48.

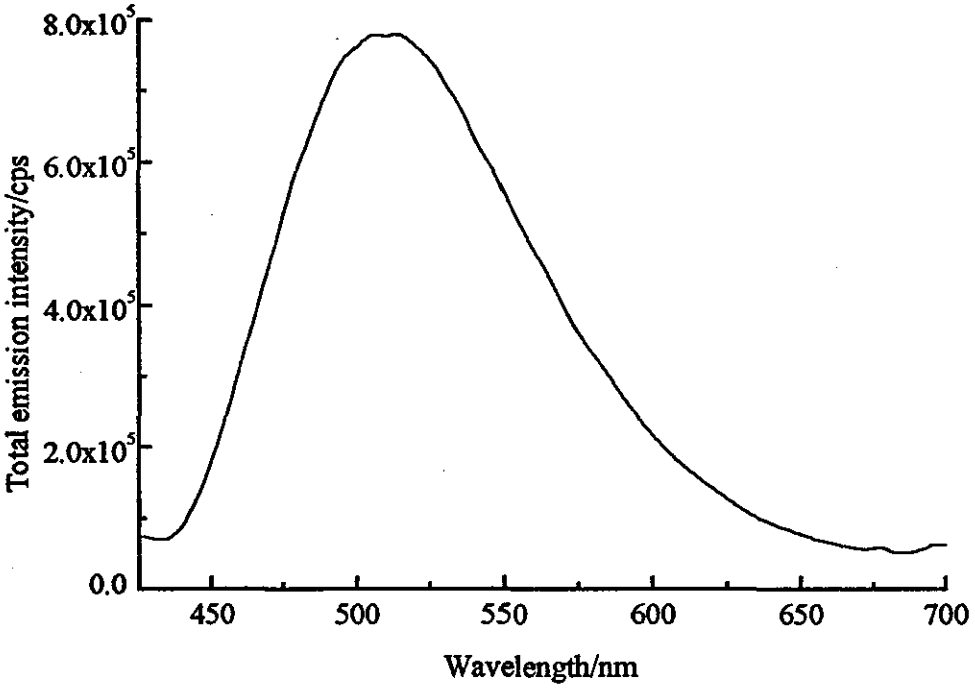


Figure 5.48: Emission spectrum of omadine in 1:1 ethanol : methanol at 77K exciting at 350 nm with slits of excitation = 0.35 mm and emission = 0.7 mm.

The λ_{max} of the emission was used to calculate the lower limit of the lowest excited triplet state and was found to be 239 $kJmol^{-1} \pm 3$ $kJmol^{-1}$. The quantum yield was calculated compared to naphthalene [173] which has a quantum yield of 0.039, exciting at 300 nm. This gave a value for the quantum yield of omadine of 0.04. The phosphorescent lifetime, which was measured on the HY laser in emission only mode,

was found to be $1.83x10^{-3}s \pm 6x10^{-4}$ s in 1:1 ethanol:methanol. The triplet state was assigned as a (π,π^*) state.

The phosphorescence intensity of omadine increases in the presence of H.S.A. The phosphorescent lifetime is longer with H.S.A. than without H.S.A. changing from $1.5x10^{-3}$ s without H.S.A. present to $2.7x10^{-3}$ s with H.S.A present in aqueous media.

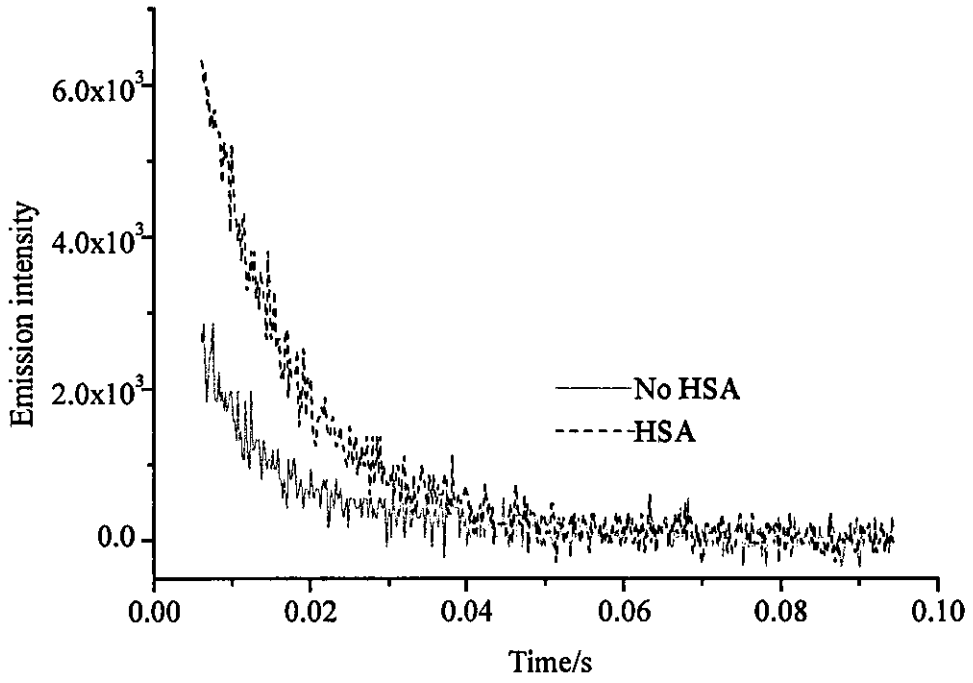


Figure 5.49: Emission decay trace of omadine with and without the presence of H.S.A. exciting at 355 nm at 77 K in phosphate buffer.

This suggests a decrease in the nonradiative transitions from the triplet state or an increase in the nonradiative transitions including intersystem crossing from the singlet state. However as the fluorescence is so low it suggests that the intersystem crossing yield is already very high and therefore the increase in the phosphorescence is probably just due to the phosphorescence competing more favourably with the radiationless transitions.

5.4.3. Laser flash photolysis

The transient absorption spectra were obtained using a laser excitation wavelength of 355 nm. These experiments were carried out at two different pH's. The first pH was

pH 7.4, the physiological pH. This showed the presence of a very weak transient at 460 nm as shown in figure 5.50.

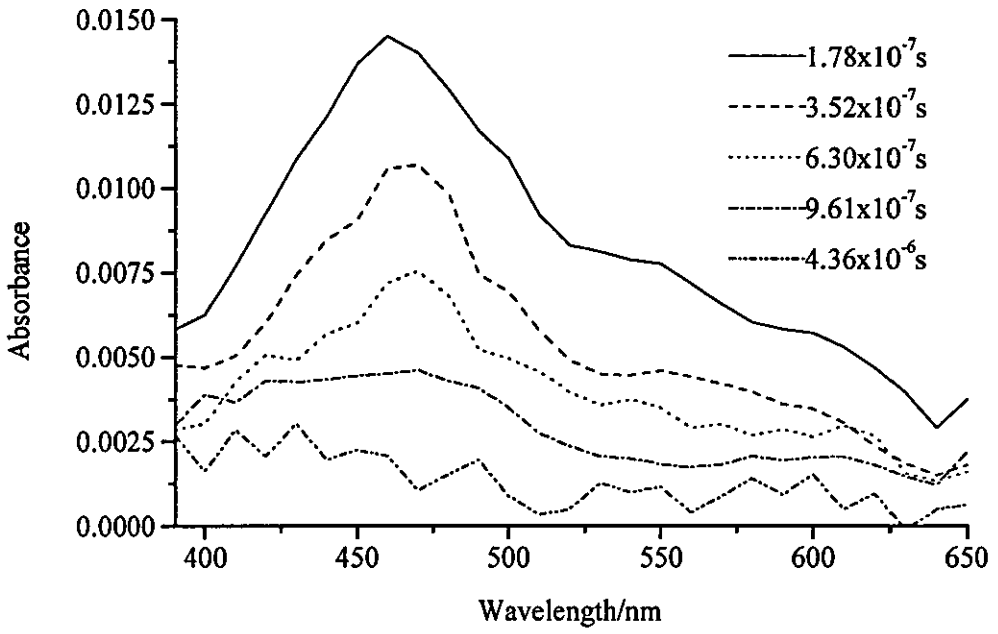


Figure 5.50: Transient absorption spectra of 3.8 $x10^{-4}$ M omadine in water at pH 7 exciting at 355 nm.

The transient was present in both degassed and aerated solutions. The transient was assigned as 2-pyridylthiyl radical as seen by Redmond et al by laser flash photolysis.[98] The lifetime of this radical was found by fitting the transient decay at 460 nm to first order kinetics with a baseline and was found to be 0.6μs which agrees with the literature value of 0.5μs found for the thiyl radical using laser flash photolysis. Previous laser flash photolysis experiments have also shown the cleavage of the N-O bond allowing formation of hydroxyl radicals in aqueous solution at pH 7.4 which are known to be photosensitisers.[94] These hydroxyl radicals could be used to probe this reaction in future experiments. The thiyl radical has also been seen using E.S.R. with the simultaneous production of hydroxyl radical.[93,95,97,98] The transient absorption signal was very weak and therefore prevented radical quenching experiments being carried out. There appeared to be a residual absorption still present after the decay had finished, possibly due to the formation of a product which needs further investigation.

At pH 2 the spectrum was very similar to that seen at pH 7.4. Redmond et al [98] have seen the presence of the triplet at 660 nm in methanol using laser flash photolysis at this pH but this could not be seen in these experiments possibly due to a low triplet quantum yield. The lifetime of the transient at 460 nm was found to be 0.5 μs which is slightly shorter than seen at pH 7.4. From previous studies using laser flash photolysis at pH 2[94,99] it has been shown that the most likely intermediate is the thiyl radical

The transient difference spectrum of omadine adsorbed onto cellulose shows 1 peak with a λ_{max} at 480 nm as shown in figure 5.51 which is red shifted from the λ_{max} of 460 nm observed in pH 7.4 buffer.

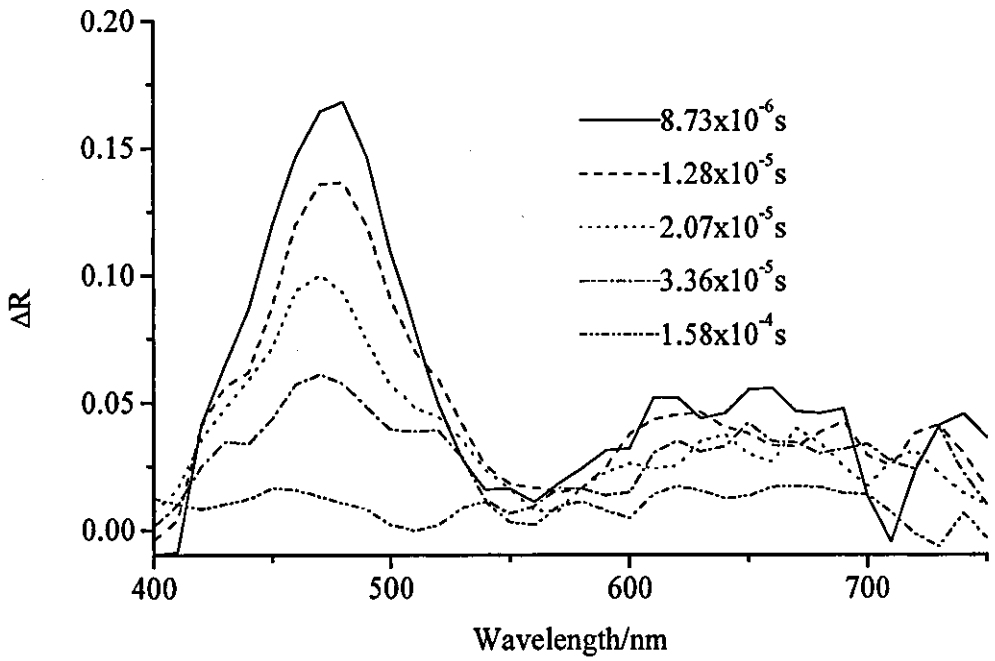


Figure 5.51: Transient difference spectrum of 4mg/g omadine adsorbed onto cellulose exciting at 355 nm

The transient decay traces at 470 nm shows the reflectance does not return to the initial reflectance level. This suggests the formation of a product which could not clearly be seen in solution due to the weak signal. This however would be expected from other work done using E.S.R[93,95,97,98], where it has been shown that the formation of a product was due to the thiyl radicals reacting with each other. The lifetime of omadine on cellulose was found to be 2.11×10^{-5}s which is longer than the 600 ns seen

in solution. This species has previously been identified as a thiyl radical in solution by previous workers by E.S.R. and laser flash photolysis.[93-95,97,98] There is a second peak seen at 650 nm on cellulose which was not seen in aqueous solution but has been seen by Redmond et al at pH 2,[98] which is probably due to the triplet species. This is seen on cellulose due to the increased rigidity and due to it protecting the triplet species from quenchers which may be present in solution.

There appears to be no change in the transient absorption intensity or lifetime with the addition of H.S.A. The peak is due to the thiyl radical and these experiments seem to suggest it is formed from the singlet state rather than the triplet state. This is because the triplet state is affected by the presence of H.S.A. shown by phosphorescence studies whereas the transient absorption is not affected by the presence of H.S.A.. The triplet species is still not seen either due to a very low quantum yield or due to rapid decay of the triplet due to quenchers in solution.

5.4.4. Singlet oxygen

There was no singlet oxygen detected when the compound was excited at 355 nm.

5.4.5. Photodegradation

The product seen at the end of the laser trace shows that the compound degrades to form products. The most likely product is due to the 2-pyridylthiyl radicals reacting together as suggested by several workers on the basis of E.S.R. studies.[93,95,97,98] Attempts to degrade at one wavelength produced no change in the ground state absorption spectra, therefore the quantum yield could not be measured. The rate of degradation was however shown to be slow relative to the other photoallergens with only a small quantum yield of formation of product. The compound was therefore irradiated in front of a full arc lamp as shown in figure 5.52.

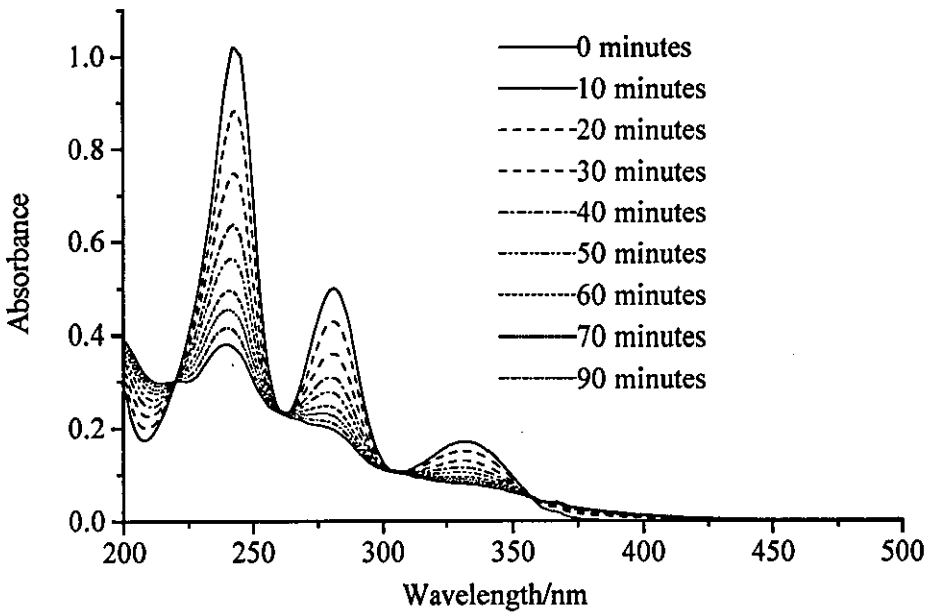


Figure 5.52: 8.5×10^{-5} M omadine irradiated over time using a full arc lamp.

The peaks at 340 nm, 240 nm and 280 nm all decreased in absorption while there was an increase in the absorption above 225 nm. There was very little absorption above 365 nm where the laser flash photolysis work was analysed. The isosbestic points on the degradation spectra are good and it suggests a constant ratio of products with no secondary photochemistry occurring. If time had allowed a fuller investigation of the products, GC-MS would have been carried out. The product is probably formed from the singlet state via the radical as the formation of the product is seen the same amount in both degassed and aerated solutions whereas from previous work the triplet species has been shown to be quenched by oxygen as a lifetime of 0.5 μs would suggest.

There is no change in the degradation of omadine with the addition of H.S.A.. This is further confirmation of the product being formed from the radical species as that is also not affected by the presence of H.S.A from the laser flash photolysis experiments. The triplet state however is affected by the presence of H.S.A and it might therefore, be expected that if the product is formed directly or indirectly from the triplet state it would also be affected by the presence of H.S.A. These results also suggests that the singlet state is not affected by the presence of H.S.A. and therefore the increase in the phosphorescence is not due to increased intersystem crossing.

Summary of the photochemical properties of omadine

This thesis has shown the ground state spectra of omadine has peaks at 280 nm, 340 nm and 335 nm the latter of which was assigned as a $\pi \rightarrow \pi^*$ transition. The ground state spectrum was found to change with pH due to the deprotonation of the hydroxyl group giving a pKa of 4.7 which confirms the findings of other groups.[93]

It has been shown here that this compound shows no fluorescence at room temperature but has a phosphorescent quantum yield of 0.04 at 77 K. The phosphorescent lifetime was found to be 1.83×10^{-3} s and the triplet state was assigned as a $^3(\pi,\pi^*)$ state. The addition of H.S.A. to the omadine in aqueous solution shows an increase in the phosphorescence intensity and lifetime due to the phosphorescence competing more favourably with the radiationless transitions due to the formation of the protein-omadine complex.

Previous work has shown the presence of a thiyl radical at pH 7.4 after laser excitation [94,98] and with E.S.R. [93,95,97] and the presence of both the radical and triplet species at pH 2. This work found the radical at both pH's with differing lifetimes of 600 ns at pH 7.4 and 450 ns at pH 2 but there was no sign of the triplet species at either wavelength. With adsorption of the omadine onto cellulose the lifetime of the transient at 460 nm increased and an additional transient species was observed at 650 nm after laser excitation at 266 nm. This species was assigned as a triplet species due to the triplet species having been seen at 660 nm at pH 2 by other researchers.[98] The addition of H.S.A. to the omadine in aqueous solution had no effect on the lifetime or amount of the transient seen compared to that observed in solution. If the thiyl radical is formed from the singlet state this may suggest that H.S.A. has no effect on the singlet state which would suggest that there was no change in the rate of intersystem crossing.

There has been no previous study of the degradation of the compound which was not very large in aerated solution. The ground state absorption spectra showed good isosbestic points upon degradation showing a constant ratio of products with no secondary photochemistry. The product is probably due to the thiyl radical reacting with itself and this could be further investigated using GC-MS.

5.5. Ketoprofen

Ketoprofen is a substituted benzophenone derivative it is therefore expected in view of the structurally similarity it could also be photochemically similar to benzophenone. The photochemistry of benzophenone has been studied in detail before, whereas only E.S.R studies have been carried out on irradiated ketoprofen.[102,103]

5.5.1. Ground state absorption spectra

The ground state absorption spectra were recorded using ethanol as solvent, they gave showed peaks at 255 nm and 205 nm as shown in figure 5.53.

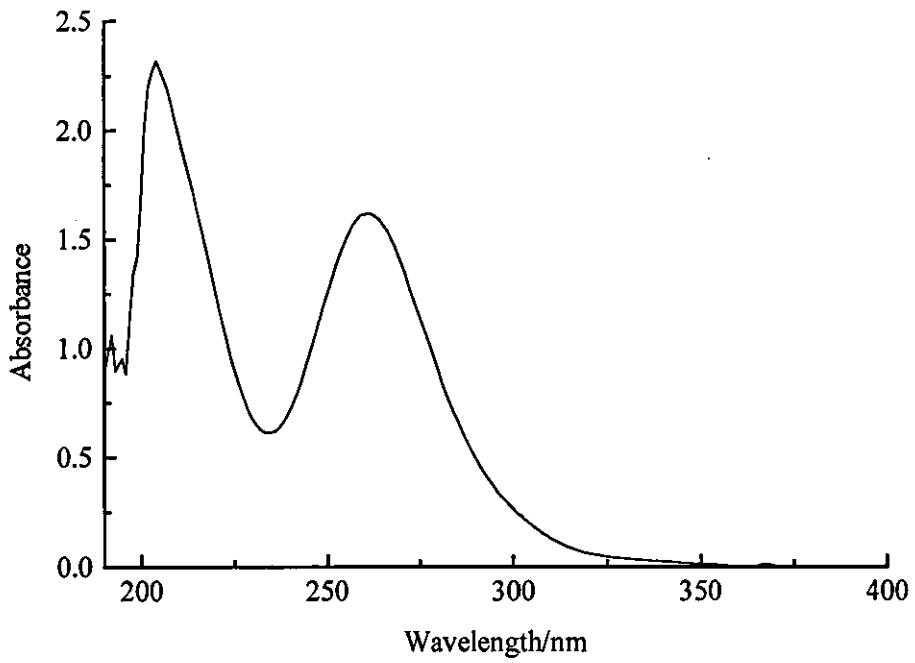


Figure 5.53: Ground state absorption spectra of $1.5x10^{-4}$ M ketoprofen in ethanol

Benzophenone has an absorbance at 340 nm due to a $n\rightarrow\pi^*$ transition[174] due to the lone pair on the oxygen of the carbonyl group. The spectra for ketoprofen does not show a peak at this wavelength in the above spectra. However at high concentrations a peak appears at 335 nm as shown in figure 5.54 which was assigned as a $n\rightarrow\pi^*$ transition.

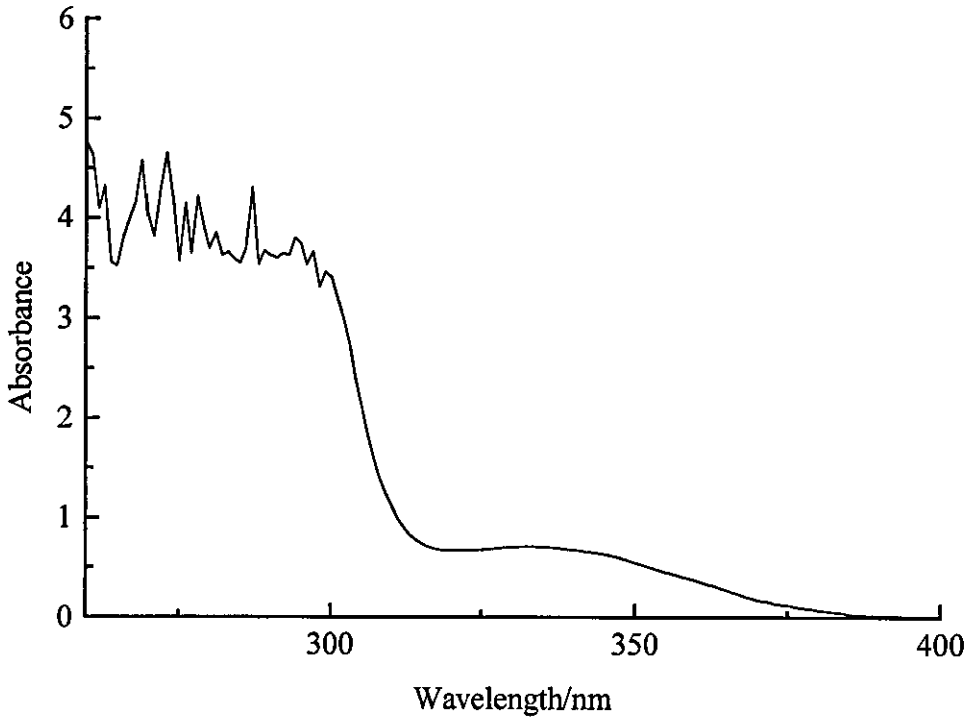


Figure 5.54:Ground state absorption spectra of $1x10^{-3}$ M ketoprofen in ethanol.

The peak at 255nm is therefore assigned as a $\pi \rightarrow \pi^*$ transition. The molar absorption coefficient was calculated to be 700 $mol^{-1}dm^3cm^{-1} \pm 50$ $mol^{-1}dm^3cm^{-1}$ in 10%ethanol : 90% water solution at 335 nm and 14700 $mol^{-1}dm^3cm^{-1} \pm 350$ $mol^{-1}dm^3cm^{-1}$ in 10% ethanol : 90 % water solution at 255 nm. The low value for the molar absorption coefficient at 335 nm being typical of a $n \rightarrow \pi^*$ transition of a carbonyl compound.

The ground state diffuse reflectance (Kubelka Munk) spectrum of ketoprofen adsorbed onto cellulose shows a peak with a λ_{max} at 266 nm as shown in figure 5.55.

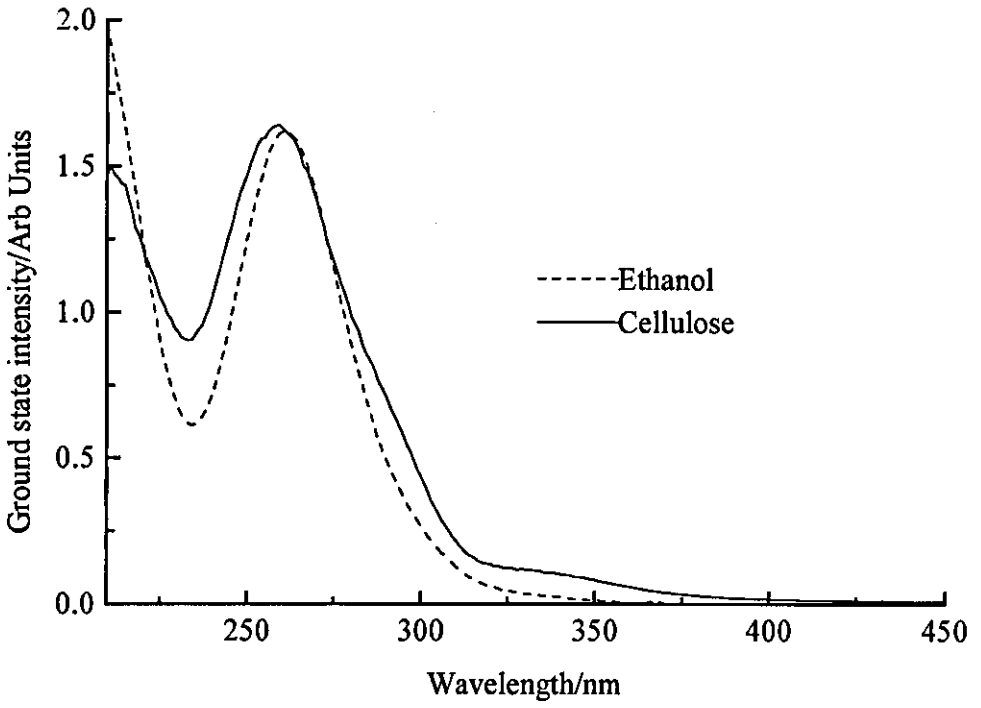


Figure 5.55: Ground state spectra of ketoprofen adsorbed onto cellulose and in ethanol.

The peak at 335 nm is also noticeable on cellulose whereas it was only detectable in solution at high concentrations. The peak at 255 nm is slightly broader when ketoprofen is adsorbed onto cellulose than in ethanol due to the non homogeneous nature of the cellulose as explained in chapter 2. The ratio of the peaks has again altered with adsorption of the ketoprofen onto cellulose due to some absorption of the barium sulfate below 300 nm as explained earlier.

5.5.2. Emission spectroscopy

5.5.2.1. Fluorescence

No fluorescence was detected from ketoprofen using our instruments probably due to the quantum yield of fluorescence being too small for the detector. This agrees with values found for benzophenone fluorescence[175] where the quantum yield of fluorescence is known to be $4x10^{-6}$.

5.5.2.2. Phosphorescence

The ketoprofen phosphorescence was measured in ethanol at 77 K, exciting at 266 nm and produced a structured emission over the range 400 nm to 600 nm.

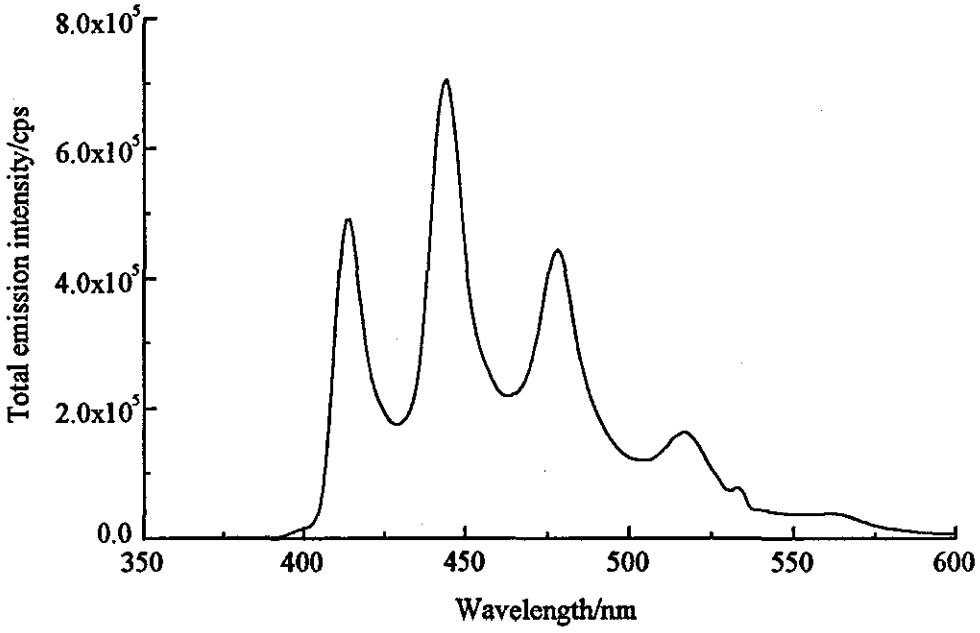


Figure 5.56: Emission spectra of M ketoprofen in ethanol at 77K exciting at 266 nm with excitation slits = 0.2 mm, emission = 0.4 mm.

The structure shows three peaks with λ_{max} occurring at 445 nm, 415 nm and 475 nm. The peak at 415 nm was taken to be due to the 0-0 vibronic level of the lowest excited triplet state and was used to calculate the lowest triplet energy of ketoprofen. The triplet energy was found to be 288 $kJmol^{-1} \pm 5kJmol^{-1}$ which is very similar to the triplet energy value of 289 $kJmol^{-1}$ found for benzophenone[176] in polar solvents. The phosphorescence of ketoprofen is the same shape as the phosphorescence for benzophenone in solution, therefore the quantum yield of phosphorescence was found by comparison with benzophenone. The value for the quantum yield of phosphorescence was found to be 0.95 which is higher than the value of 0.84 used for benzophenone.[154] This is probably caused by the electron withdrawing nature of the carboxylic acid group. The high value obtained for the phosphorescence quantum yield of ketoprofen suggests that like benzophenone[171] the quantum yield of intersystem

crossing from the singlet state to the triplet state must be ~ 1. The lifetime of the phosphorescence was found to be $5.7x10^{-3}$ s $\pm$ $2x10^{-4}$ s which is approximately the same as the phosphorescent lifetime of benzophenone[154] of $6x10^{-3}$ s. The triplet state was assigned as a $^3(n,\pi)$ state due to the millisecond phosphorescent lifetime and the high quantum yield of the phosphorescence.

The ketoprofen showed a phosphorescence peak in the same place with H.S.A. present as without H.S.A. present when excited at 266 nm. The shape of the phosphorescence was less well defined than seen for ethanol. This was because the experiments were carried out in aqueous solutions which do not form good glasses at 77 K as explained earlier. The intensity of this phosphorescence however, was smaller in the presence of H.S.A. than without H.S.A. and could not be explained by any absorption from the H.S.A. at 266 nm.

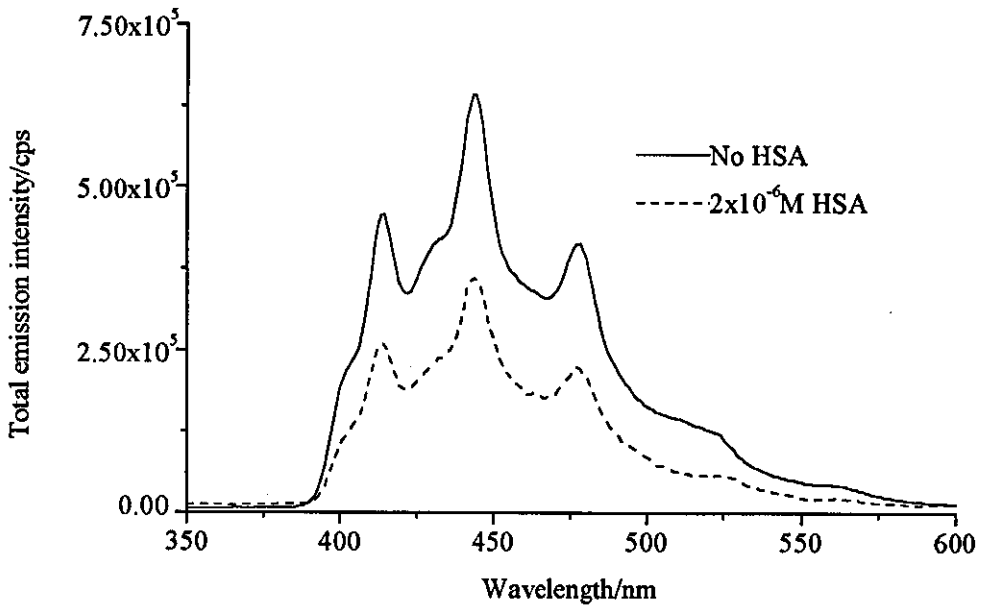


Figure 5.57:Emission spectra of ketoprofen with and without the presence of $2x10^{-6}$ M H.S.A. at 77 K exciting at 266 nm with excitation slits = 0.3 mm, emission slits = 0.6 mm.

The lifetime of the ketoprofen phosphorescence was reduced in the presence of H.S.A. from $9.4x10^{-4}$ s without H.S.A. to $8.6x10^{-4}$ s with H.S.A present. This decrease has not

been seen with the other compounds. It appears in this case that the addition of H.S.A. is increasing the rate of the non radiative transitions from the triplet state or decreasing the intersystem crossing from the singlet state to the triplet state. It can be seen here that the change in the lifetime with the addition of H.S.A. is not as significant as the change in the intensity which may be explained using the equation

$$\phi_P = \frac{k_P k_{ISC}}{\Sigma k_j \Sigma k_i}$$

where, ϕ_P = quantum yield of phosphorescence

k_P = Rate coefficient of phosphorescence

k_{ISC} = Rate coefficient of intersystem crossing from the singlet state

Σk_j = Rate coefficient of all decay pathways from the triplet state

Σk_i = Rate coefficient of all decay pathways from the singlet state.

Where it can be seen that although the quantum yield of phosphorescence is dependent on the rate of phosphorescence it also depends on the rate of intersystem crossing and other decay routes from both the singlet and triplet states. It is not known which of these rates is altering and therefore it can not be predicted how the rate of phosphorescence will alter in relation to the quantum yield of phosphorescence.

5.5.3. Laser Flash Photolysis

The ketoprofen does not absorb light at 355 nm therefore it was necessary to use the fourth laser harmonic at 266 nm to excite the sample. On excitation there was a very weak transient seen with a λ_{max} at 530 nm, as shown in figure 5.58. This is the same λ_{max} as for the benzophenone triplet species.[171]

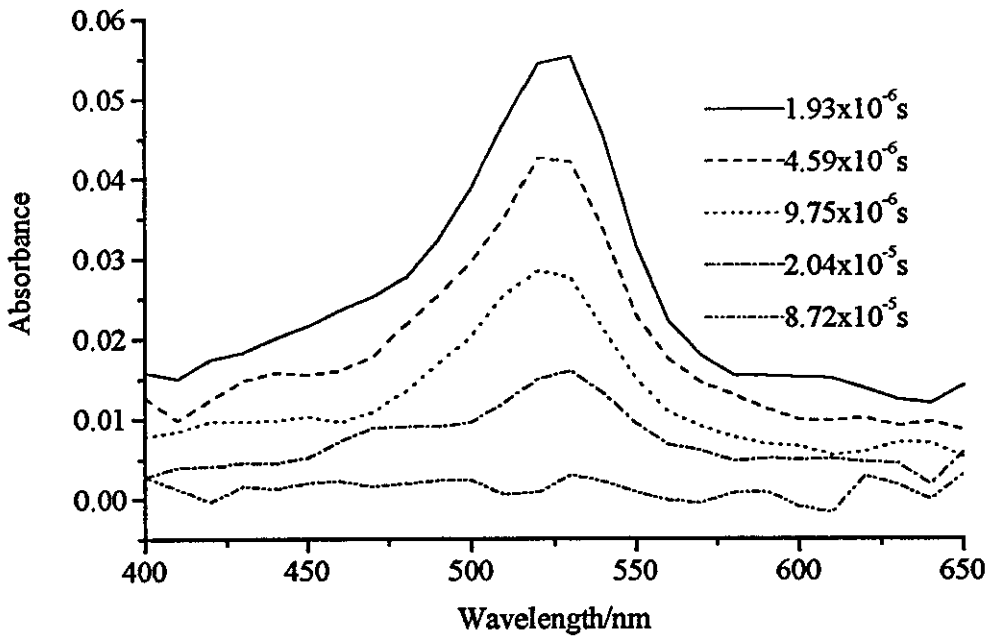


Figure 5.58: Transient absorption spectra of $4x10^{-5}$ M ketoprofen in acetonitrile exciting at 266 nm

The transient species could not be seen in the presence of oxygen which suggests this transient is a triplet species. This was confirmed by using naphthalene as a triplet quencher, which has a triplet energy[175] of 253 $kJmol^{-1}$ which is lower than the triplet energy of ketoprofen found from phosphorescence. Naphthalene quenched the ketoprofen triplet with a k_q of $7.9x10^9$ $M^{-1}s^{-1}$ as shown in figure 5.59. There was also a corresponding increase observed in the naphthalene triplet at 415 nm, which confirms the species seen is due to the triplet.

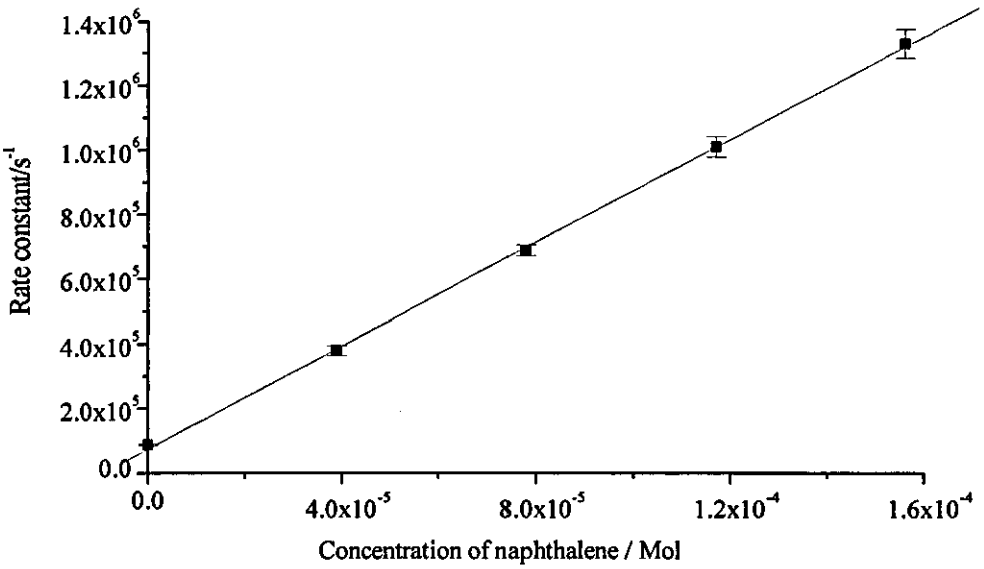


Figure 5.59: A graph showing the quenching of the ketoprofen triplet with increasing concentration of naphthalene.

The lifetime of the transient was found by fitting the decay trace obtained at 530 nm to first order kinetics which gave a lifetime of 1.17×10^{-5}s $\pm$ 5×10^{-7} s. This value is longer than the 7×10^{-6}s in nonpolar solvents seen for benzophenone.[177] The difference may well be from the carboxylic acid group present on the ketoprofen.

Benzophenone photochemistry shows the presence of the ketyl radical at 545 nm in alcoholic solutions due to hydrogen abstraction from the solvent by the carbonyl group[178]. The ketyl radical is formed via the triplet state by the mechanism shown below.

$$Ph_2CO \xrightarrow{h\nu} {}^1(Ph_2CO)^* \longrightarrow {}^3(Ph_2CO)^* \xrightarrow{EtOH} Ph_2\dot{C}OH + CH_3\dot{C}HOH$$

It was therefore decided to excite the ketoprofen in alcoholic solutions to see if the ketyl radical could also be seen in ketoprofen.

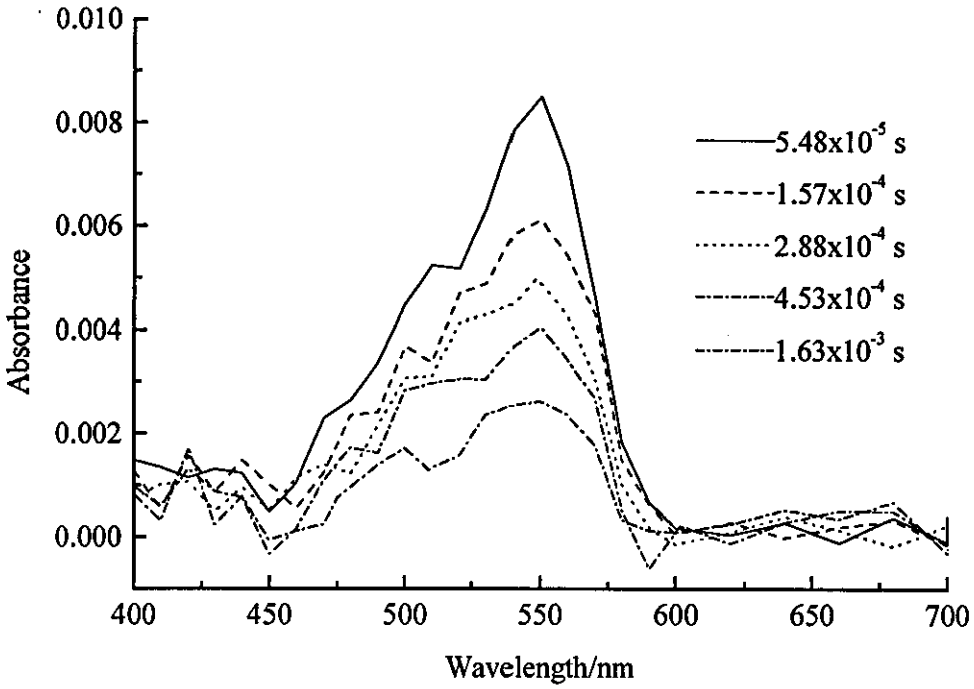


Figure 5.60: Transient absorption spectrum of 4.2 x10^{-5} M ketoprofen in ethanol exciting at 266 nm

Figure 5.60 shows there was a change in the transient absorption spectrum with a second peak becoming apparent at around 550 nm which agrees with the value of 545 nm seen in alcoholic solutions of benzophenone.[178] The kinetic traces showed non-exponential behaviour which probably shows the presence of the ketyl radical. The photochemistry so far has been comparable with benzophenone and shows the carboxylic acid group has very little effect on the photochemistry seen here.

The transient seen when ketoprofen was adsorbed onto cellulose was at 540 nm as shown in figure 5.61 which is slightly higher than the 530 nm observed in solution.

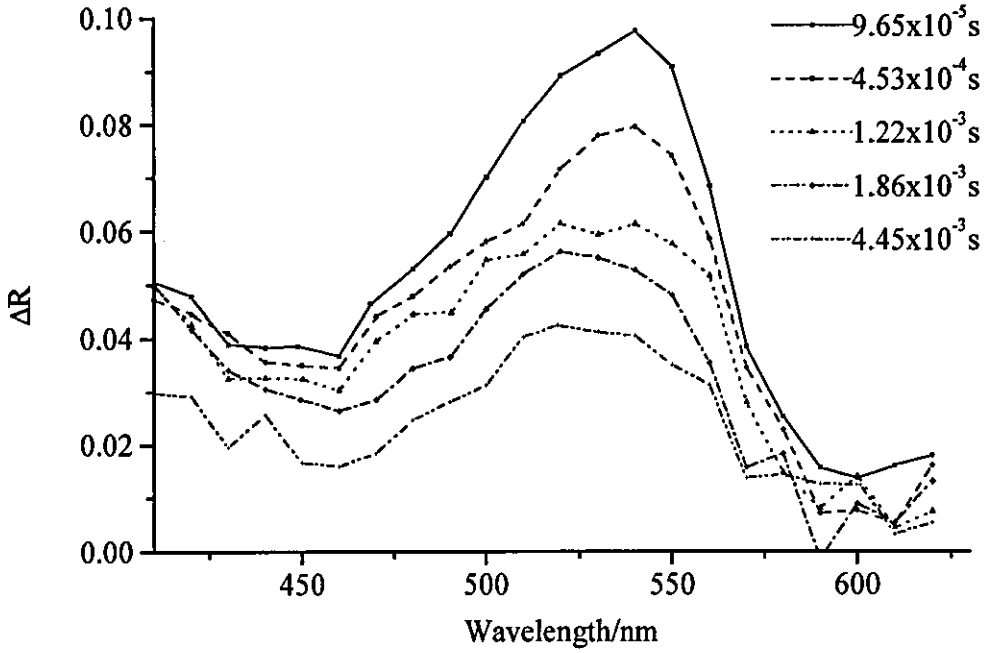


Figure 5.61: Transient difference spectrum of 4mg/g ketoprofen adsorbed onto cellulose exciting at 266 nm.

This transient was identified as the same triplet species seen in solution due to the peak being observed at a similar wavelength to that seen in solution. The lifetime of this transient was found to be $6.25x10^{-4}$s on cellulose which as expected is longer than the $1.2x10^{-5}$ s found in solution.

There was no change in the intensity of the transient absorption for ketoprofen in the presence of H.S.A,. in degassed aqueous solutions but the signal was very weak and therefore it is hard to make an accurate assessment either way. The lifetime is a more accurate assessment of the effect of the H.S.A. on the triplet species. It was found that with H.S.A. present the lifetime of $1x10^{-5}$ s was shorter than the lifetime of $1.5x10^{-5}$ s without H.S.A. This transient is due to the triplet species and therefore this agrees with the decrease seen in the phosphorescence lifetime with the addition of H.S.A.

5.5.4. Singlet oxygen

Singlet oxygen was detected exciting at 266 nm using the set up described in chapter 3. The standard used for calculation of the singlet oxygen quantum yield was benzophenone whose quantum yield in acetonitrile[179] is known to be 0.37. The value

for the singlet oxygen quantum yield of ketoprofen was found in this work to be 0.25. This value is large enough for this to play a part in the photoallergy mechanism however benzophenone has a similar singlet oxygen quantum yield but is not considered to be a photoallergen.

5.5.5. Degradation studies

There was no sign of a product being formed from the laser flash photolysis studies but the formation of products may have a low quantum yield. The ketoprofen when degraded in front of a full arc lamp showed a very small amount of degradation in aerated solutions.

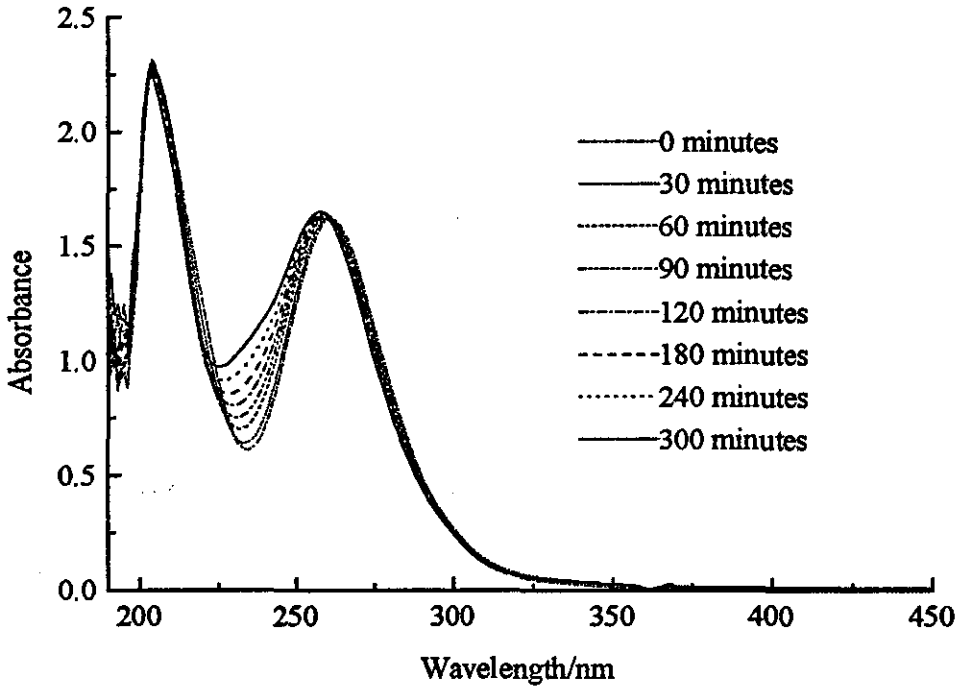
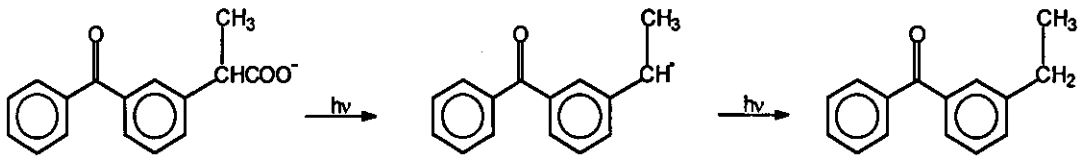


Figure 5.62: An aerated solution of $7x10^{-5}$ M Ketoprofen irradiated over time with a full arc lamp

The ground state spectrum showed a shift to the blue region from 255 nm to 250 nm. This reaction probably comes from the singlet state as it is seen in aerated solution where the triplet species has been shown to be quenched by oxygen. This is possibly due to the reaction shown below which has been suggested by E.S.R. of neutral aqueous solutions of ketoprofen carried out by Bosca et al .[102] There have been no radical species seen in these laser flash photolysis experiments however, this may just

mean these radicals have very low quantum yields and can not be detected by our instrumentation.



A degassed solution of ketoprofen showed more degradation than the aerated solution which suggests there is some formation of the product from the triplet state. This would probably occur via the ketyl radical species and would therefore only occur in hydrogen containing solvents.

There was very little difference in the degradation observed with or without the presence of H.S.A. in aerated solutions. This suggests the product is not formed from the triplet state as this is reduced by the presence of H.S.A.. However in degassed solutions where more degradation is seen, probably due to the triplet state, the presence of H.S.A. decreases the amount of degradation as expected. This shows the difference in the degradation between degassed and aerated solutions is due to the triplet forming a product in the degassed solutions, probably via the ketyl radical.

Summary of the photochemical properties of Ketoprofen

Ketoprofen has only been photochemically studied using E.S.R and E.P.R before so all the photochemistry reported here has not been studied before. The ground state absorption spectrum shows these peaks at 255 nm and 205 nm. At 335 nm which was assigned as a $n \rightarrow \pi^*$ transition, from it's very low molar absorption coefficient, due to the carbonyl group. This peak at 335 nm was also noticeable when the ketoprofen was adsorbed onto cellulose. The peak at 255 nm was assigned as a $\pi \rightarrow \pi^*$ transition due to its large molar absorption coefficient.

Emission spectra recorded at room temperature and 77 K showed that while there was no fluorescence observed, the quantum yield of phosphorescence was 0.95 with a lifetime of 5.7×10^{-3} s. The triplet state was therefore assigned as a $^3(n,\pi^*)$ state due to the millisecond lifetime of the phosphorescence. The high triplet quantum yield suggests that like benzophenone[171] the intersystem crossing quantum yield must be

approximately one. The addition of H.S.A. to the aqueous solutions of ketoprofen gave a decrease in the phosphorescence intensity and lifetime.

Laser excitation of the ketoprofen produces a triplet species at 530 nm with a lifetime of $1.2x10^{-5}$ s in acetonitrile, which was approximately the same as the triplet species seen for benzophenone in acetonitrile. The ketoprofen transient was confirmed as a triplet by oxygen quenching and triplet quenching using naphthalene. The triplet species was also seen when ketoprofen was adsorbed onto cellulose but with a longer lifetime due to the non homogeneous nature of the cellulose. In alcoholic solutions there was a difference in the spectrum and the kinetics which was probably due to the formation of the ketyl radical of ketoprofen. This is the same effect as seen in benzophenone in alcoholic solutions.[182]The singlet oxygen quantum yield was high at 0.25 however, it is very similar to the value of 0.37 seen for benzophenone which is not a known photoallergen.[183]

Irradiation of the ketoprofen in alcoholic solutions showed degradation in degassed solutions which was probably formed from the ketyl radical especially as very little product was seen in acetonitrile solutions. In aerated solutions the amount of degradation was reduced but there was still some occurring. An explanation for the degradation in aerated solutions is a radical reaction involving the carboxylic acid group as seen by Bosca et al.[102]

The photochemistry of ketoprofen has been found to be very similar to the benzophenone photochemistry suggesting the additional carboxylic acid group is not involved in the photochemistry reported here. This would therefore suggest that the photochemistry seen is not responsible for photoallergy as benzophenone is not a known photoallergen. The photoallergy reaction may therefore possibly be connected to the radical reaction which occurs with the carboxylic acid group which has been seen previously by E.S.R.[102] and probably seen in this work as the degradation observed in aerated solution. This radical would need to be studied in more detail using a more sensitive technique such as spin trapping due to the small quantum yield of this species.

5.6. Chlorpromazine and Promethazine

These compounds have a very similar structure and are therefore expected to have a similar photochemistry. There have been many studies carried out on chlorpromazine [106,107,109,110] but none carried out on promethazine.

5.6.1. Ground state absorption spectra

The ground state absorption spectra of chlorpromazine and promethazine were very similar both showing several peaks, with λ_{max} at 310 nm, 260 nm and 210nm for chlorpromazine and peaks at 300 nm, 255 nm and 210 nm for promethazine as shown in figure 5.63.

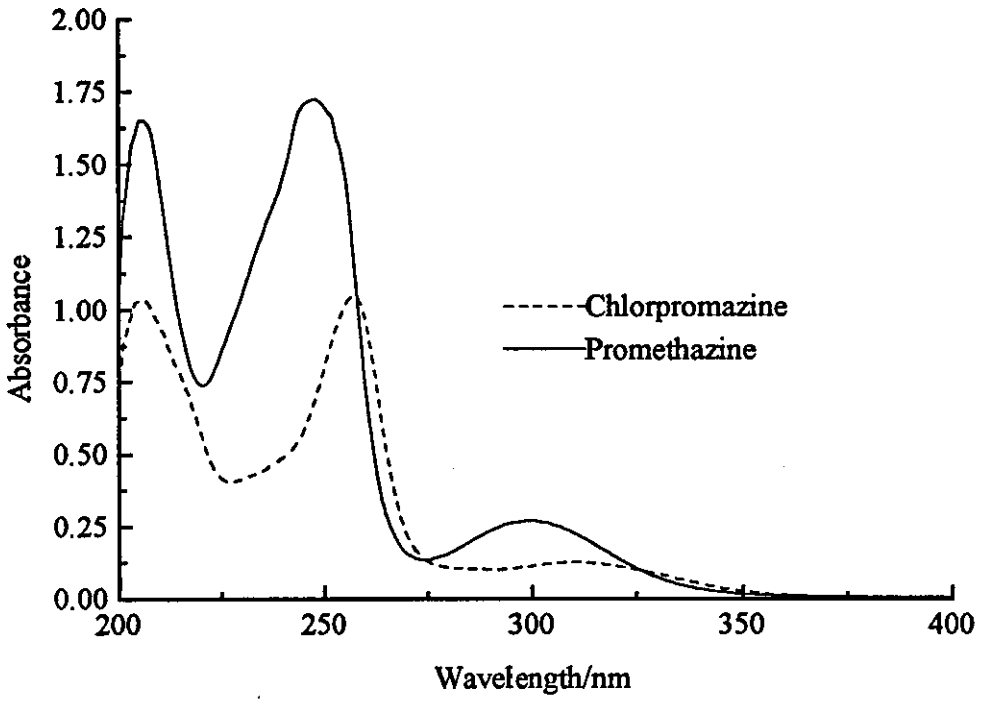


Figure 5.63: Ground state absorption spectra $8x10^{-5}$M chlorpromazine and $1.2x10^{-4}$ M promethazine in 10% ethanol 90% water solution.

The molar absorption coefficient of chlorpromazine at 310 nm was found to be 4000 mol^{-1} dm^3cm^{-1} ± 150 mol^{-1} dm^3cm^{-1} and for promethazine at 300 nm to be 4100 $mol^{-1}dm^3cm^{-1}$ ± 200 mol^{-1} dm^3cm^{-1} These transitions are both assigned as $\pi \rightarrow \pi^*$ transitions. The presence of the chlorine on the chlorpromazine is thought to act as an electron withdrawing group causing a red shift in the absorption maximum for chlorpromazine.

The effect of pH on the ground state absorption spectra was studied for promethazine in 10 % ethanol 90 % water solution and it was found that the ground state absorption spectra changed with pH showing a blue shift in the λ_{max} as shown in figure 5.64.

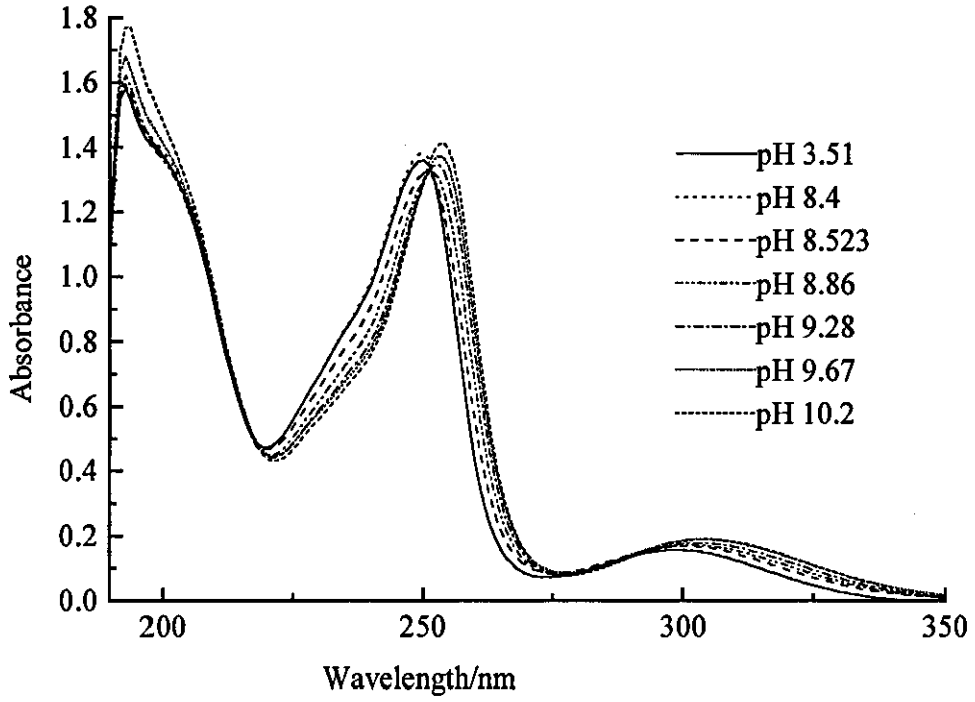


Figure 5.64: Ground state absorption spectra of 5.1×10^{-5} M promethazine at various pH's

The 255 nm peak was found to move to 248 nm changing position at about pH 8. The peak at 305 nm moves to 295 nm at lower pH's. Chlorpromazine was not studied due to solubility problems at the higher pH's. This reaction is probably due to the protonation of the tertiary amine group and the effect should be the same in chlorpromazine.

The ground state diffuse reflectance (Kubelka Munk) spectra of these compounds adsorbed onto cellulose and in ethanol are shown in figure 5.65 and figure 5.66.

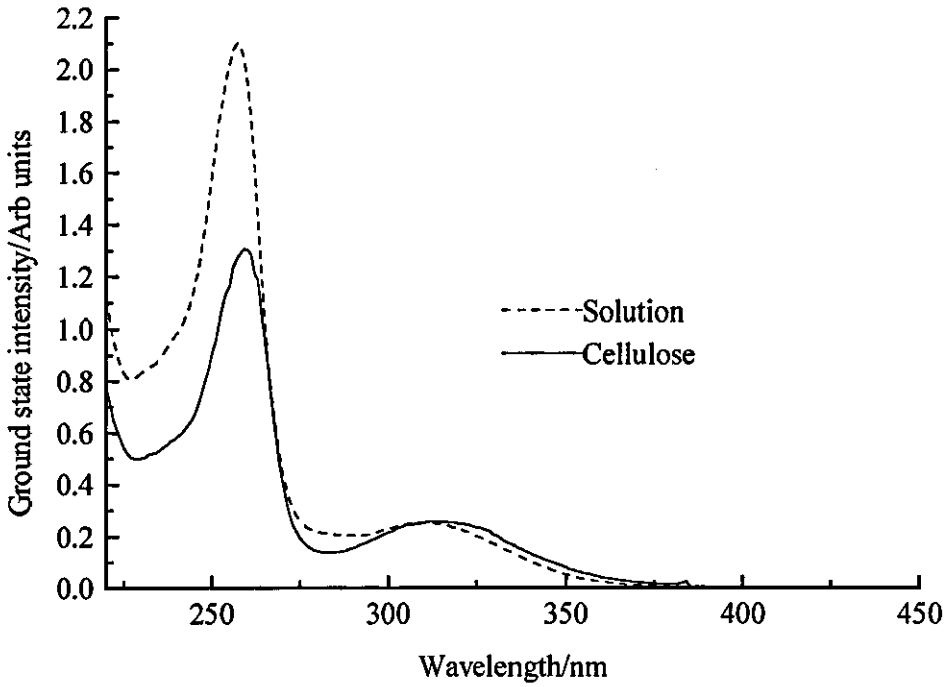


Figure 5.65: Ground state spectra of chlorpromazine adsorbed onto cellulose and in ethanol

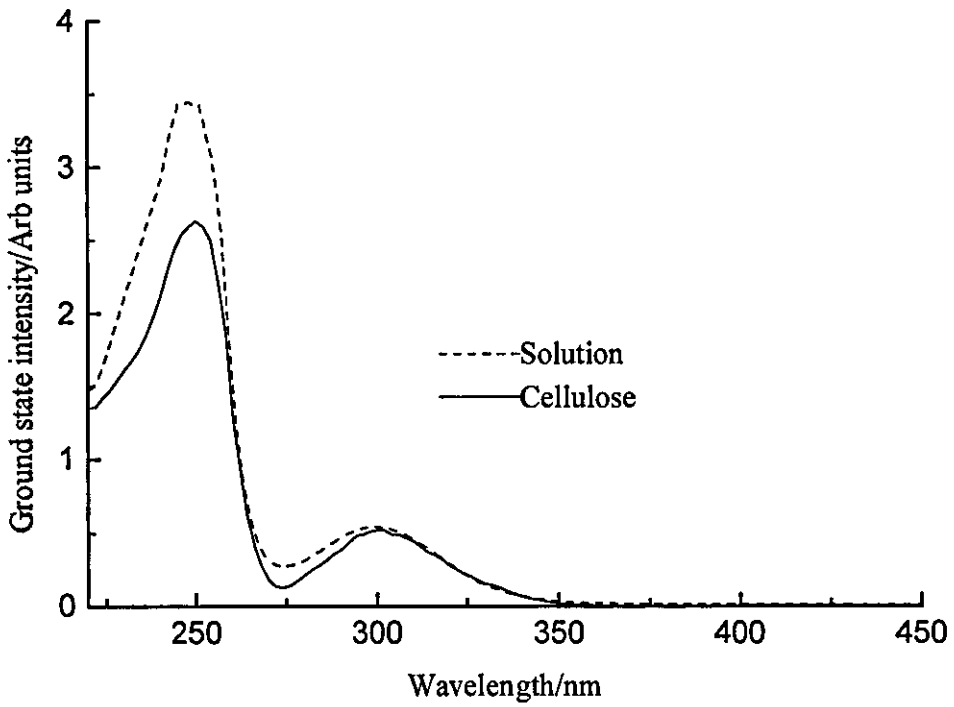


Figure 5.66: Ground state spectra of promethazine adsorbed onto cellulose and in ethanol.

There is a slight red shift in the position of the λ_{max} of the peaks when these compounds are adsorbed onto cellulose. The peaks at 255 nm for promethazine and chlorpromazine are slightly reduced in relation to the peak at 310 nm when adsorbed onto cellulose compared to the spectra obtained in ethanol. This maybe due to the effect of some absorption by the barium sulfate at wavelengths below 300 nm as explained earlier.

5.6.2. Emission spectroscopy

5.6.2.1. Fluorescence

Chlorpromazine and promethazine both showed one fluorescence peak with a λ_{max} at 450 nm for chlorpromazine and 440 nm for promethazine from this and the ground state absorption spectra, the energy of the lowest excited singlet state was found, using the method described in chapter 3, to be 314 $kJmol^{-1} \pm 4$ $kJ\,mol^{-1}$ for chlorpromazine and 323.0 $kJmol^{-1} \pm 4$ $kJmol^{-1}$ for promethazine. The fluorescence was quite weak and broad as shown in figure 5.67.

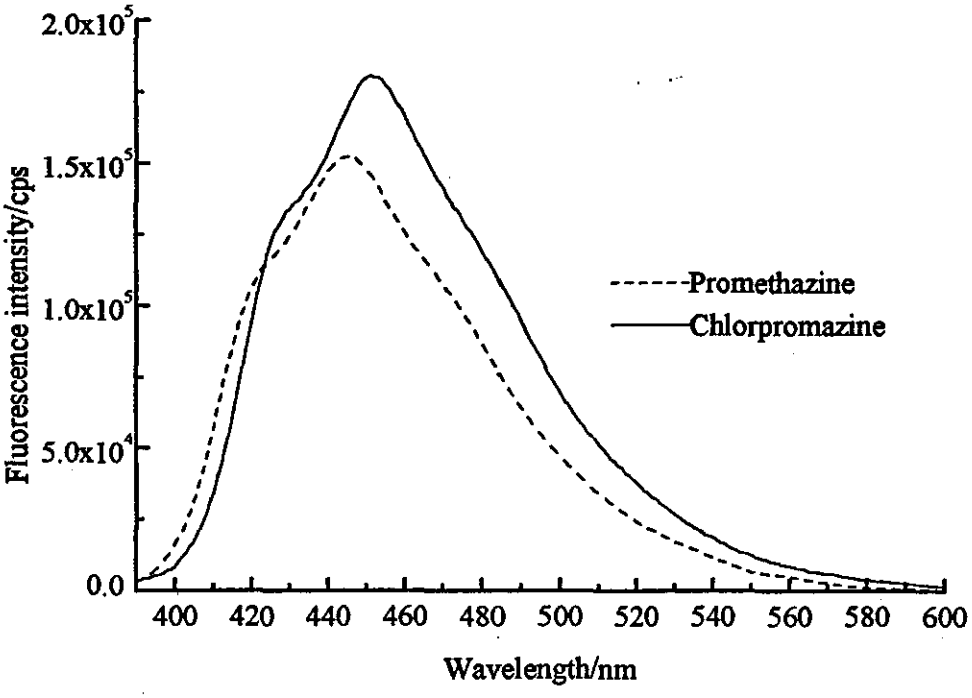


Figure 5.67: Emission spectra of promethazine and chlorpromazine in ethanol using excitation slits = 0.5 mm, emission slits = 1 mm, exciting at 266 nm.

The quantum yield of fluorescence was calculated compared to the fluorescent standard 9,10-diphenylanthracene, whose fluorescence quantum yield is known to be 0.9 [180] and it was found that the quantum yield for chlorpromazine was 0.004 and for promethazine it was 0.012. This difference in the quantum yields could be due to the extra chlorine on the chlorpromazine causing an increase in the rate of intersystem crossing due to the internal heavy atom effect. The presence of oxygen had no effect on the fluorescence suggesting the lifetime for the fluorescence is very short. The radiative lifetime for the fluorescence was found using the method described in chapter 3 to be ~ $2.5x10^{-8}$ s for chlorpromazine and promethazine. The estimated lifetime was calculated using the fluorescence quantum yields to be around ~ 9.8 $x10^{-11}$s and ~ $2.94x10^{-10}$ s for chlorpromazine and promethazine respectively. The excitation spectra were the same as the ground state absorption spectra showing there were no impurities present which could effect the fluorescence.

The effect of pH on the fluorescence spectra was investigated and again there were problems with the solubility of chlorpromazine at high pH's but the fluorescence of promethazine was shown to change with pH. There is only a slight blue shift with increasing pH but a large drop in the fluorescence quantum yield.

The fluorescence of chlorpromazine and promethazine was also seen on cellulose figure 5.68 and was found to be very similar to that seen in solution. The chlorpromazine and promethazine emission were in the same positions as seen in the solution, but show a slight broadening. This shows the adsorption of chlorpromazine and promethazine onto cellulose has very little effect on the fluorescence.

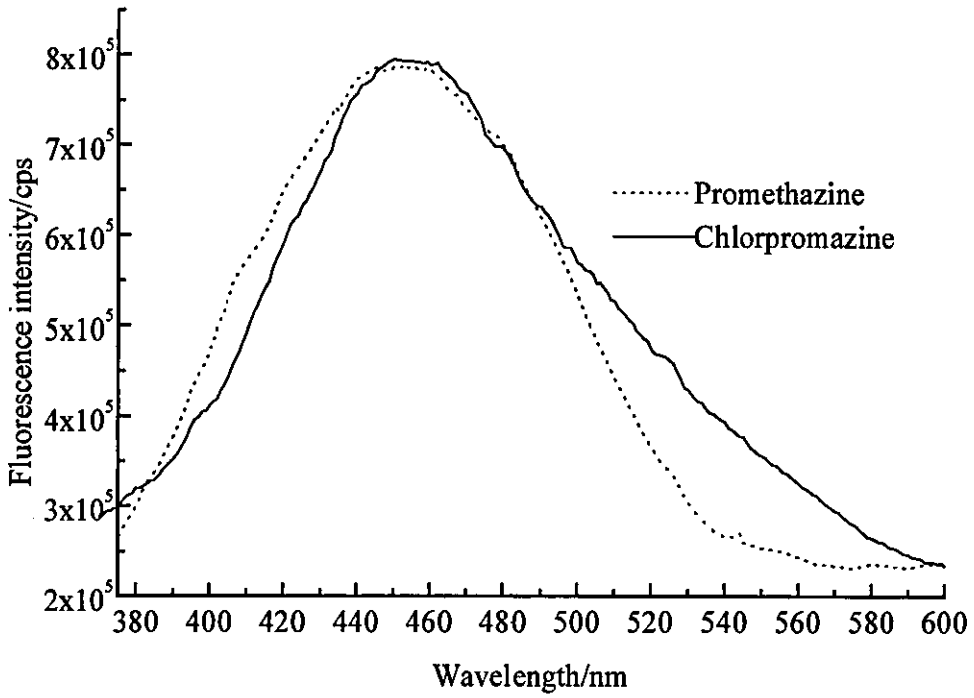


Figure 5.68: Emission spectra of 5mg/g chlorpromazine and 6mg/g promethazine adsorbed onto cellulose exciting at 310 nm with excitation slits = 0.3 mm, emission slits = 0.6 mm.

The fluorescence observed in the presence of H.S.A. was the same as the fluorescence of the compounds on their own. There was no change in the position of the peaks or the intensity which suggests that the singlet state is not affected by the presence of H.S.A. It could be suggested that there is no binding is occurring for these compounds. However by exciting the tryptophan residue of H.S.A. it can be seen that the presence of these compounds reduces the tryptophan fluorescence. Very low concentrations of chlorpromazine and promethazine were used in these experiments and therefore any changes in the fluorescence were probably caused by the formation of a protein-photoallergen complex rather than due to the photoallergen absorbing some of the light. These experiments therefore indicate that binding is occurring between the photoallergens and the protein.

5.6.2.2. Phosphorescence

The phosphorescence was found at 475 nm for chlorpromazine and 480 nm for promethazine in ethanol at 77 K. They were shown to be structureless spectra as shown in figure 5.68.

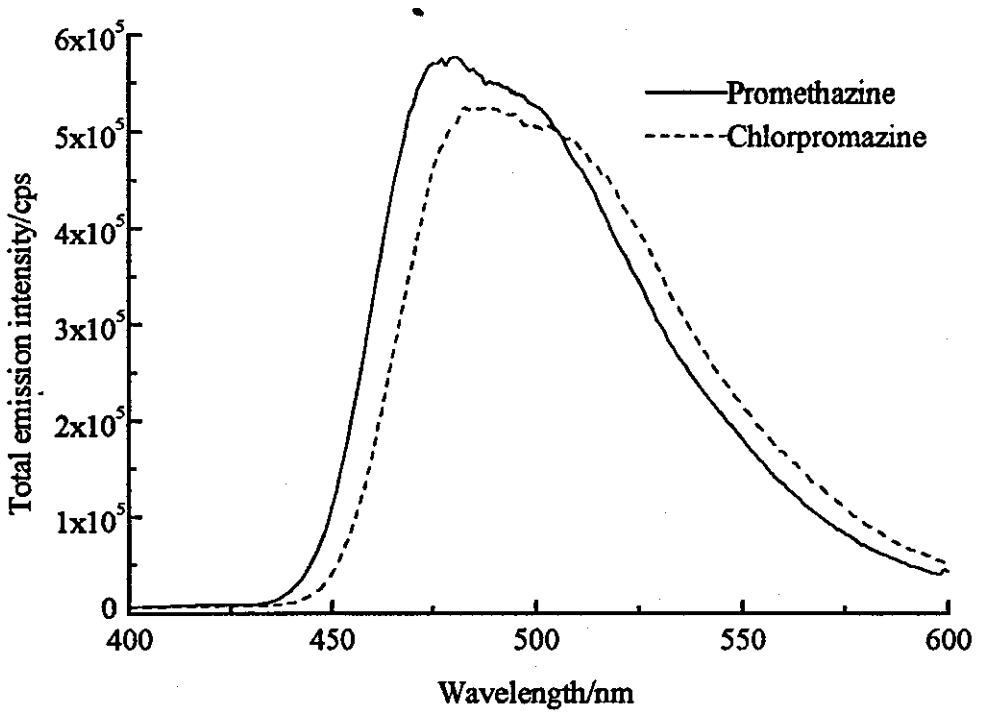


Figure 5.68:Emission spectra of chlorpromazine and promethazine in 1:1 ethanol : methanol at 77 K with excitation slits = 0.2mm and emission slits = 0.4 mm

The triplet energy was found using the method described in chapter 3 from the λ_{max} to be 249 kJmol^{-1} ± 3 kJmol^{-1} for chlorpromazine and 252 kJmol^{-1} ± 4 kJmol^{-1} for promethazine which means the energy difference between the singlet and the triplet state is 65 kJmol^{-1} ± 7 kJmol^{-1} for chlorpromazine and 71 kJmol^{-1} ± 8 kJmol^{-1} for promethazine. These values for the energy gap between the singlet excited state and the triplet excited state indicate that the singlet to triplet transition is a $\pi \rightarrow \pi^*$ transition. The quantum yield of the phosphorescence was measured compared to naphthalene which phosphoresces in the same region. The phosphorescence quantum yield of chlorpromazine was found to be 0.63 and for promethazine to be 0.79. These values are very similar however, it would be expected that the phosphorescence quantum yield of chlorpromazine would be larger than the phosphorescence quantum

yield of promethazine due to the increased intersystem crossing to the triplet state from the extra chlorine. However chlorine is not a very heavy atom and therefore the rate of intersystem crossing may be too fast to be perturbed by the chlorine. The phosphorescent lifetimes were found to be 0.068 s ± 0.009 s for promethazine and 0.073 s ± 0.009 s for chlorpromazine.

There was no change in the phosphorescence spectrum or lifetime of chlorpromazine with or without the presence of H.S.A.. However for promethazine there was a very slight increase in the intensity and lifetime of the phosphorescence.

5.6.3. Laser Flash Photolysis

Laser flash photolysis was carried out at both 266 nm and 355 nm for chlorpromazine and at 266 nm only for promethazine. The transient absorption spectra for both compounds show the presence of one peak as seen in figure 5.69 and 5.70

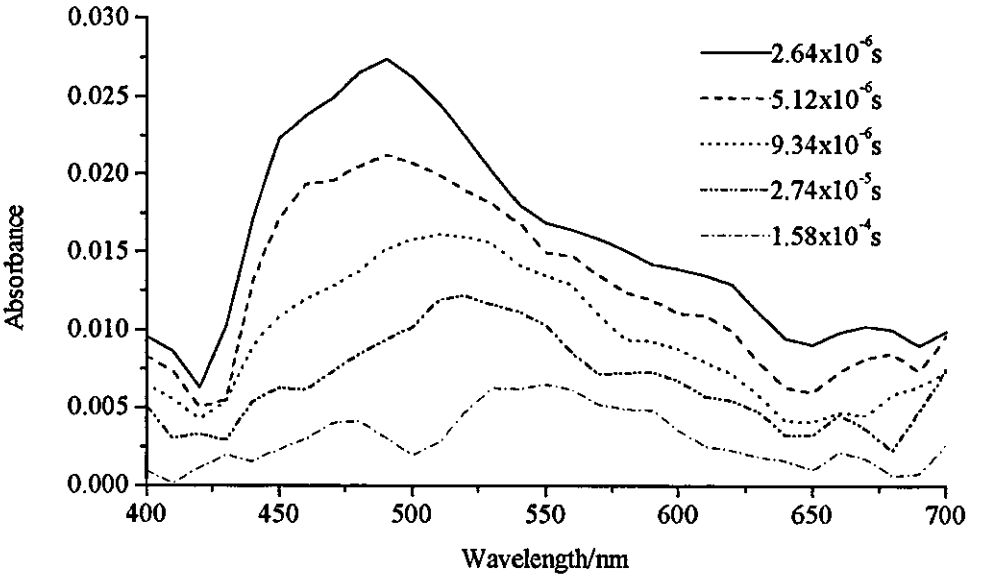


Figure 5.69: Transient absorption spectrum of 1.7×10^{-4} M chlorpromazine in 10% ethanol exciting at 266 nm.

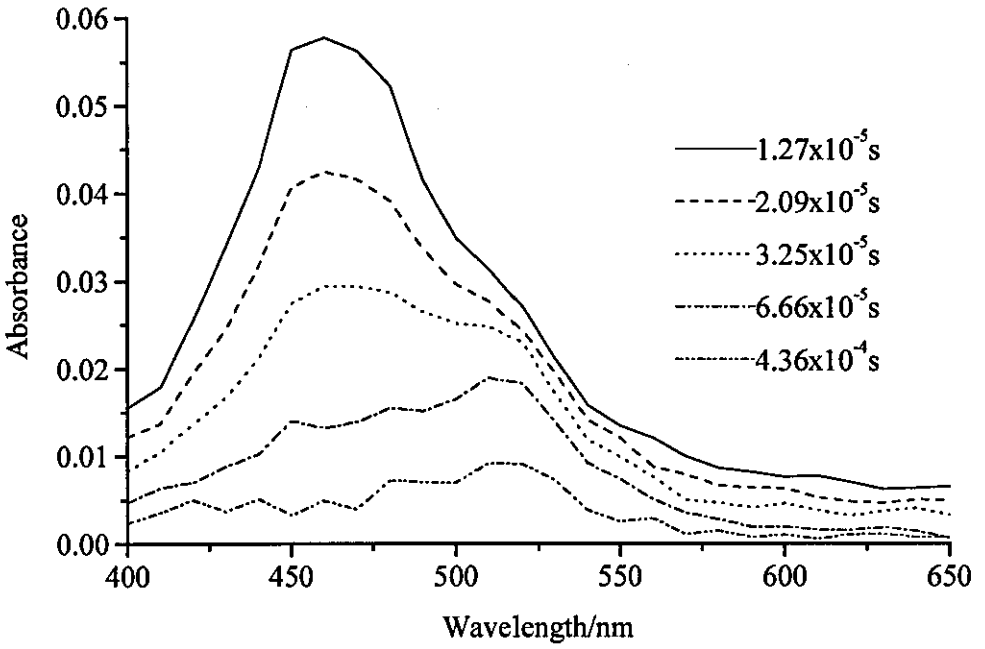


Figure 5.70: Transient absorption spectrum of 1.8×10^{-4} M promethazine in 10 % ethanol solution exciting at 266 nm.

However on closer inspection it can be seen that for both compounds there are actually two peaks in the spectra. The first peak is around 460 nm is seen at short times and at longer times a second peak appears at around 510 nm for promethazine and 525 nm for chlorpromazine. These two peaks are more noticeable for promethazine where there are peaks at 460 nm and at 510 nm. Previous laser flash photolysis [106,107] data showed that on laser excitation of chlorpromazine the peak seen at 525 nm is due to the cation radical, which is also detected by E.S.R.[109] which is unaffected by oxygen, and the peak at 460 nm is due to a triplet species only, which is quenched by oxygen. The peak at 510 nm can be seen in both degassed and aerated solution but appears reduced in aerated solution as shown in figure 5.71.

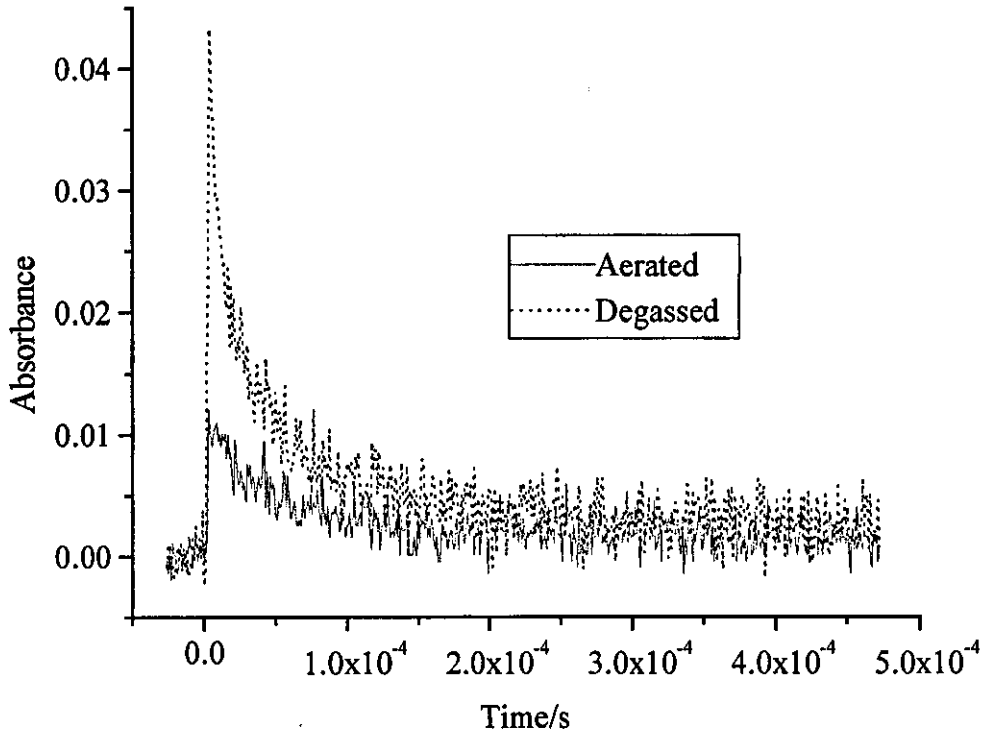


Figure 5.71: Transient absorption traces at 510 nm of chlorpromazine in ethanol, exciting at 266 nm degassed and aerated.

The peak at 460 nm reduced in aerated solutions for both chlorpromazine and promethazine showing it is probably partly due to a triplet species or a species formed directly from the triplet. The peak at 525 nm for chlorpromazine and 510 nm for promethazine is probably due to the cation radical species found by other researchers by laser excitation[106,107,110] and E.S.R.[109] The decrease in the peak at 460 nm in aerated solution is due to the triplet species being quenched by oxygen. The cation radical which also shows transient absorption at this wavelength is not affected by the presence of oxygen as the lifetime of the cation radical seen at 525 nm in aerated and degassed solution is the same. Previous reports using laser flash photolysis [106,107,110] have speculated on whether the cation radical is formed from the triplet or the singlet state as explained in chapter 2. This thesis has shown that the radical is probably formed via direct photoionisation of the singlet state, as the triplet state is shown to be quenched by oxygen whereas the cation radical is not quenched by oxygen. The transient at 460 nm was confirmed as a triplet species using a triplet quencher.

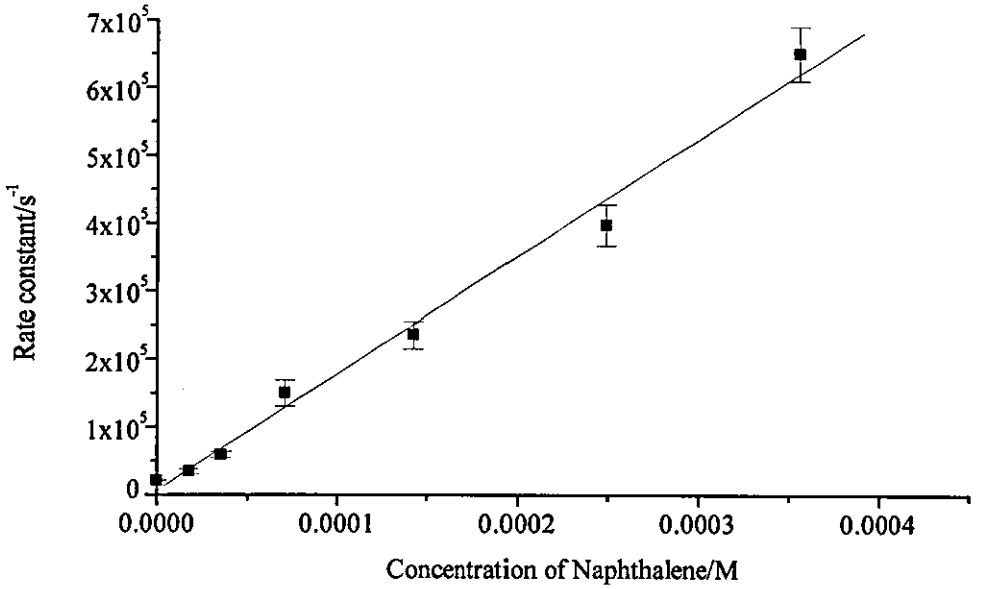


Figure 5.72: A graph of the promethazine transient at 460 nm being quenched by naphthalene.

The quenching constant for the promethazine triplet in the presence of naphthalene was 1.52×10^9 $M^{-1}S^{-1}$. The chlorpromazine triplet was seen to be fully quenched by the naphthalene but the quick lifetime of the triplet did not allow more detailed studies to be carried out. The naphthalene triplet was also seen to increase as the chlorpromazine and promethazine triplets decreased. There have been previous laser flash photolysis studies [106,107,110] carried out on chlorpromazine which have identified the species at 460 nm as due to the triplet species, this work has confirmed that it is a triplet and also shown that the species at 460 nm for promethazine is also a triplet species. The kinetic traces of these compounds show the presence of two species and therefore needed to be fitted for two exponentials.

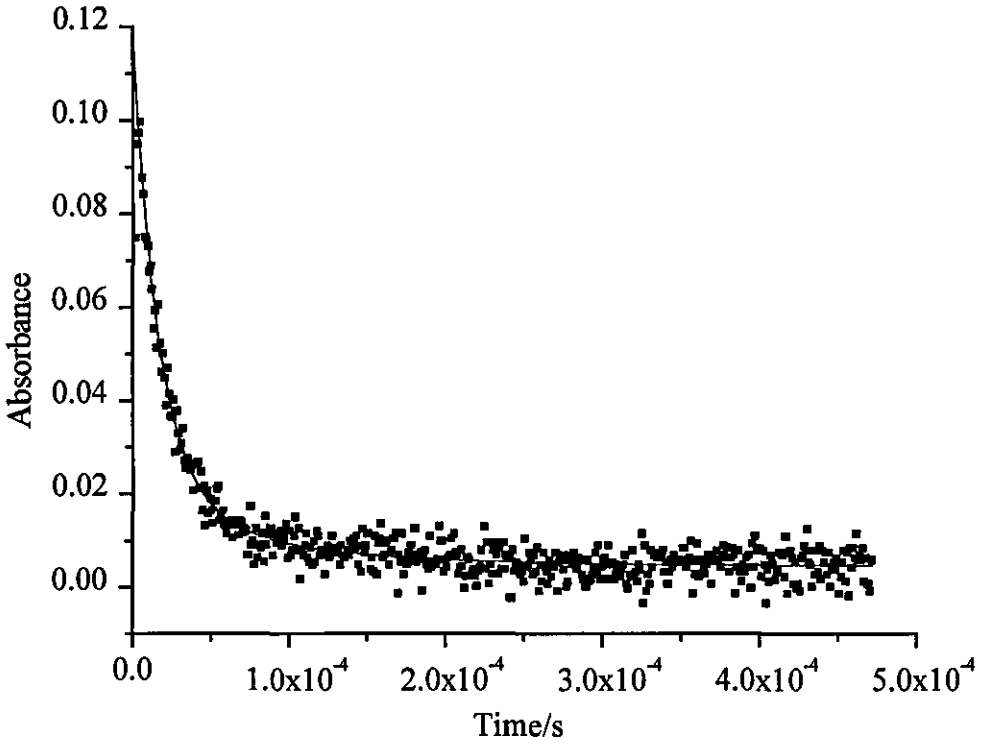


Figure 5.73: Transient decay trace of 1.8×10^{-4} M promethazine at 510 nm fitted to biexponential kinetics

The traces were also fitted at wavelengths above 510 nm for first order kinetics as only the cation radical is present at these wavelengths. The decay traces also showed the presence of a permanent change of absorption at these wavelengths which was taken to be due to formation of a product. The permanence of the product was checked by recording the absorption spectra before flashing, after flashing and 24 hours after flashing.

The promethazine shows a lot longer initial part of the curve which suggests that the triplet is longer lived than the chlorpromazine triplet. It is known that the promazine triplet is longer lived than the chlorpromazine triplet therefore it is likely that promethazine will also be longer, due to the lack of the chlorine and the addition of a methyl group. The lifetime of the triplet species for promethazine was found to be 2.1×10^{-5} s $\pm$ 2×10^{-6} s which is longer than the promazine triplet which has a lifetime of 3.1×10^{-6} s, showing the presence of the methyl group actually increases the triplet

lifetime. The lifetime of the triplet for chlorpromazine could not be found accurately on this laser but an estimate of the lifetime would be in the region of 100 ns which agrees with the values found by Navaratam et al[107] but is longer than the values of Chignell et al[106] of 23 ns in nitrogen saturated solutions. It seems likely that the longer lifetime is correct as the triplet has been shown both here and by previous workers[106,107] to be quenched by oxygen and the shorter lifetime would probably not be fully quenched by the concentration of oxygen present in aerated solutions. More accurate measurements of the triplet lifetime of chlorpromazine would require the use of a picosecond laser. The lifetime of the cation radical of promethazine was found to be 9.4×10^{-5} s $\pm$ 4×10^{-5}s and for chlorpromazine the lifetime of the cation radical was found to be 5.6×10^{-5} s $\pm$ 3×10^{-6} s.

Navaratnam et al [107] with chlorpromazine has shown a peak at 510 nm due to the promazine radical in 2-propanol, although it was not seen in aqueous solutions, formed by the loss of the chlorine radical. The promazine radical was thought to be formed via the triplet state as explained in chapter 2. However this work is in agreement with Chignell et al [106,110] who could not observe this species with this type of experiment due to the presence of the triplet and the cation radical at the same wavelengths. The peaks identified earlier as the cation radical and triplet are not due to the promazine radical, as they are at the same wavelengths as those seen by other workers [106,107,110] with chlorpromazine and promazine and in this work for promethazine.

The transient difference spectra shows peaks with λ_{max} at 510 nm and 480 nm for chlorpromazine and promethazine adsorbed onto cellulose respectively. The promethazine again shows the presence of two peaks when adsorbed onto cellulose. However the chlorpromazine shows the presence of only 1 peak which is assigned to the cation radical species as shown in figures 5.74 and 5.75

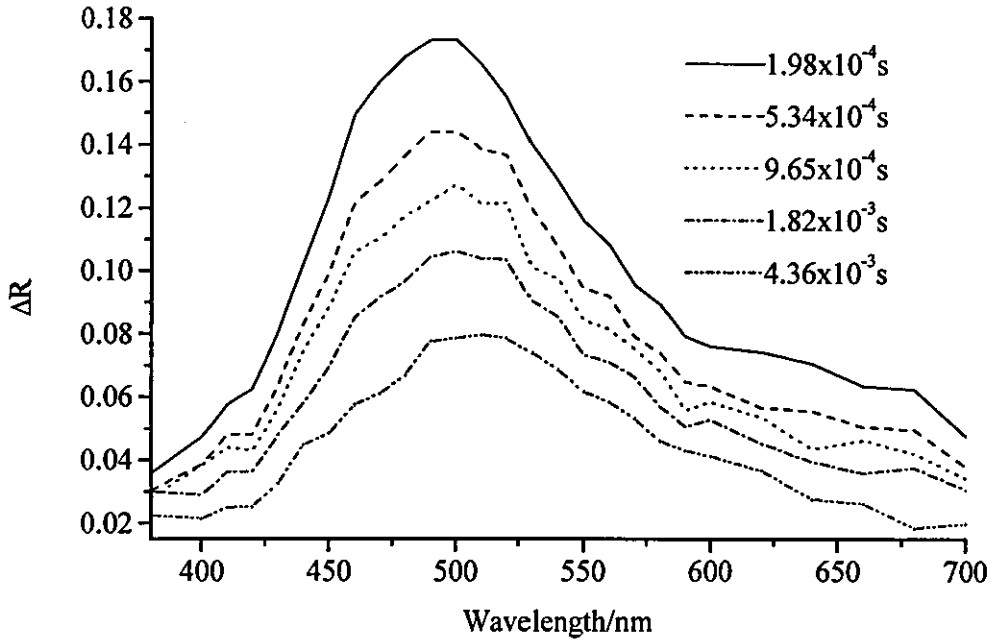


Figure 5.74: Transient difference spectrum of 5mg/g chlorpromazine adsorbed onto cellulose, exciting at 266 nm.

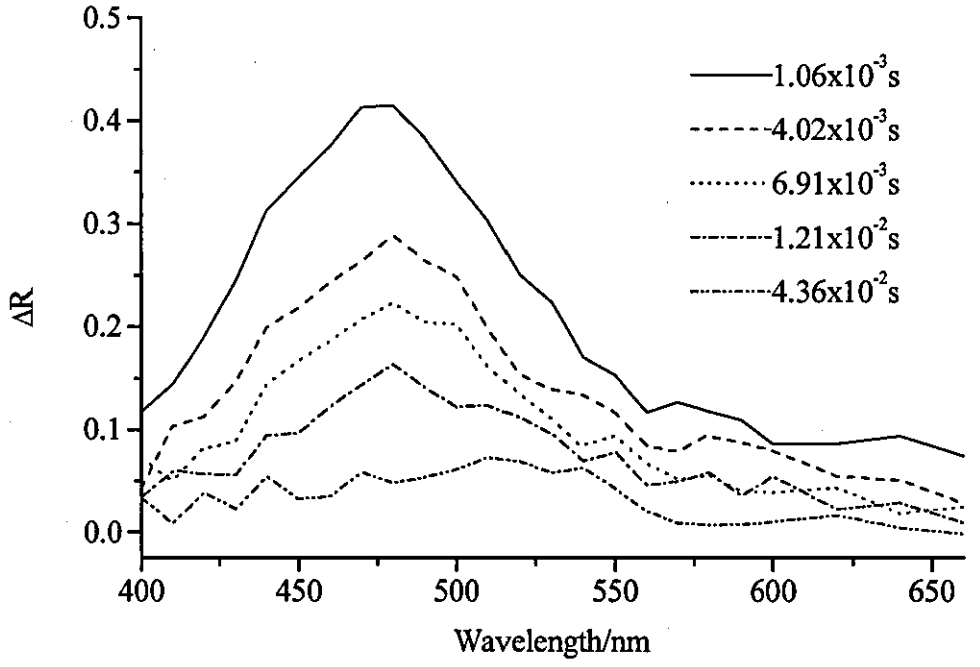


Figure 5.75: Transient difference spectrum of promethazine 6mg/g adsorbed onto cellulose, exciting at 266 nm

The transient decay traces for chlorpromazine however, still show the presence of a small very short lived species which is probably due to the triplet species. The trace was analysed using biexponential kinetics and found to give a lifetime for the cation radical species at 510 nm of $2.2x10^{-3}$ s. For promethazine the kinetic decay trace clearly showed the presence of two species and was therefore fitted to biexponential kinetics. The promethazine triplet was found to have a lifetime of $2.0x10^{-3}$ s and the cation radical which was located at 510 nm had a lifetime of $9.2x10^{-3}$ s. All the lifetimes of these species were found to be longer than seen in solution due to the cellulose protecting the excited species from quenchers as explained in chapter 2.

There is no change in the transient absorption spectrum or lifetime on the addition of H.S.A. to chlorpromazine. The H.S.A. appears to have no affect on the photochemistry of these compounds. Although fluorescence studies show these compounds appear to be binding, it may be the position of the binding is such that it does not effect the photochemistry.

5.6.4. Singlet oxygen

Both these compounds show large singlet oxygen quantum yields. The quantum yields were found compared to 2-acetonaphthone in acetonitrile and were found to be 0.25 for promethazine and 0.20 for chlorpromazine which is very similar to the literature value for chlorpromazine of 0.27 in benzene [181] when excited at 308 nm. These values were found using laser excitation at 266 nm because the concentration required to excite these compounds at 355 nm caused quenching of the singlet oxygen by the compound. This quenching could be either physical or chemical or both and if it is chemical quenching it could a be significant process in the photoallergy mechanism. The quenching constant was found by taking the value for the rate constant at various different concentrations of the compound when exciting at 266 nm. The quenching constant was found from figures 5.76 and 5.77 to be $4.27x10^{6}$ $s^{-1}M^{-1}$ for chlorpromazine and $3.93x10^{6}$ $s^{-1}M^{-1}$ for promethazine.

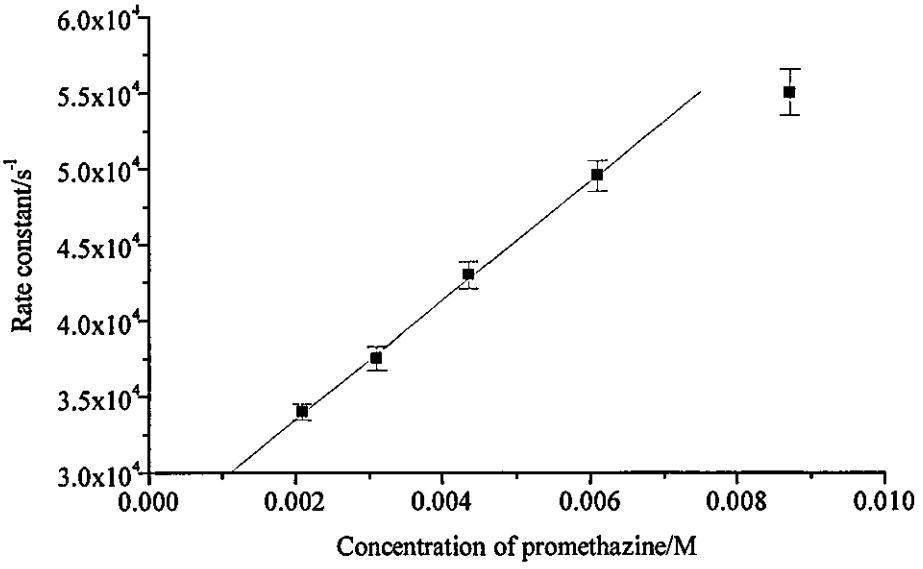


Figure 5.76: Quenching of the singlet oxygen by promethazine in acetonitrile exciting at 355 nm

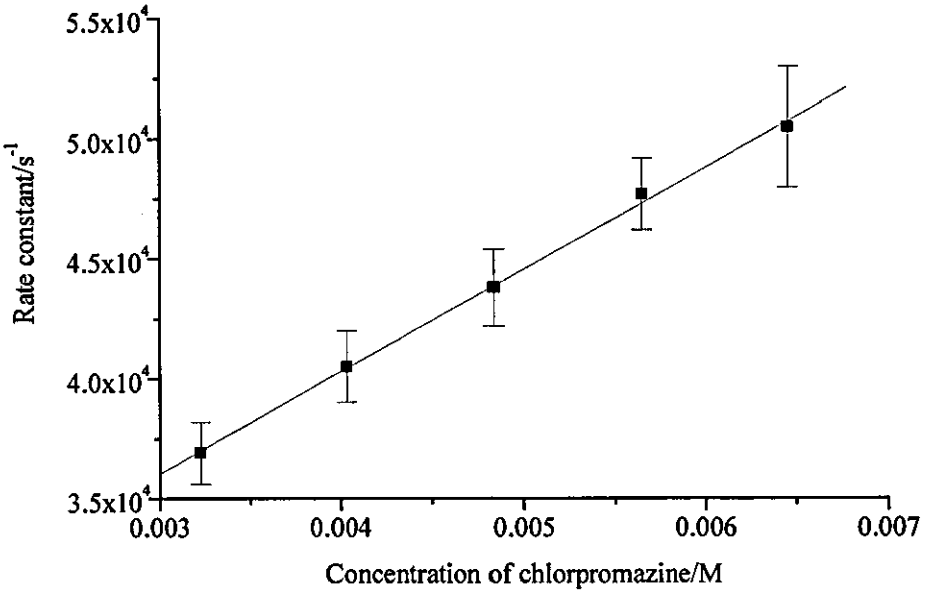


Figure 5.77: Quenching of the singlet oxygen with chlorpromazine in acetonitrile exciting at 355 nm

Promethazine showed a decrease in the singlet oxygen quantum yield when measured in D_2O in the presence of H.S.A. compared to without H.S.A. As no change was seen in the triplet yield of promethazine with the addition of H.S.A. this change is probably due to the H.S.A. protecting the triplet from molecular oxygen due to the position of the binding.

5.6.4. Photodegradation

The samples were degraded under a full arc lamp as shown in figures 5.78 and 5.79.

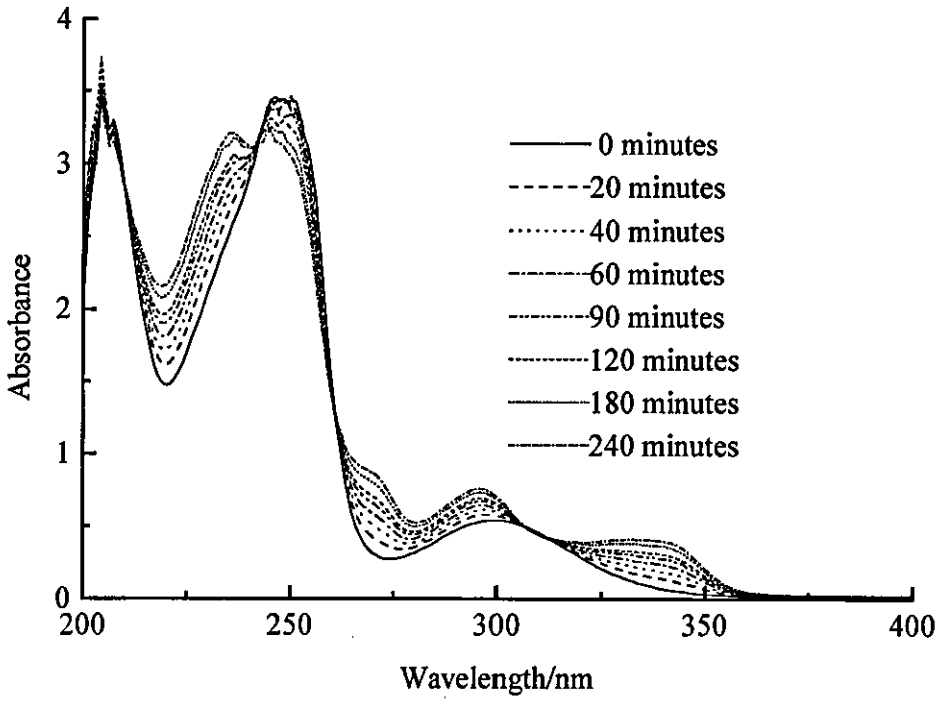


Figure 5.78: Irradiation of promethazine using a full arclamp over time.

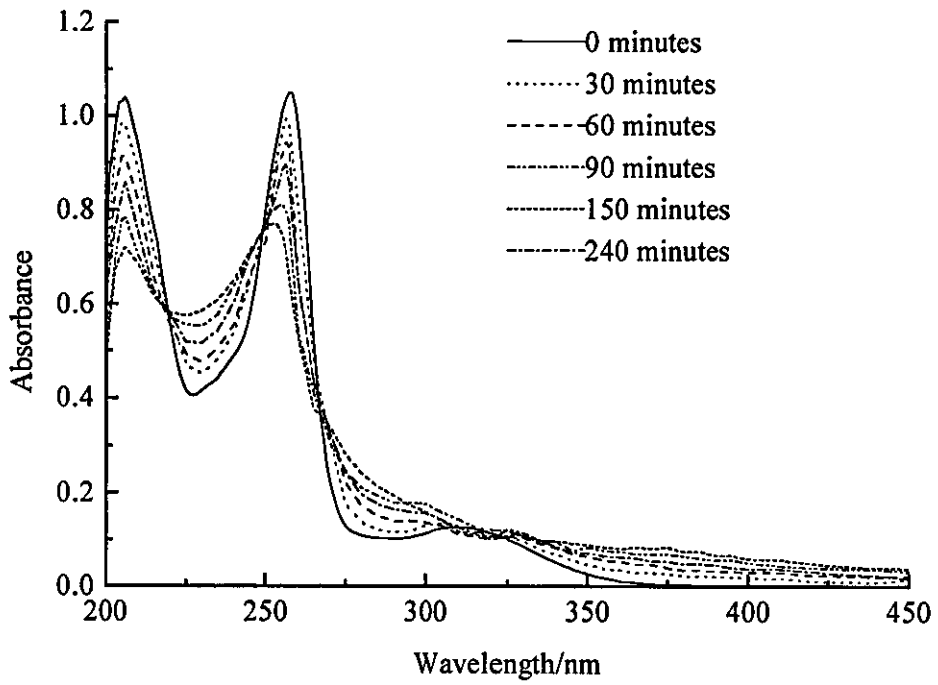


Figure 5.79 Irradiation of chlorpromazine using a full arclamp over time.

The promethazine absorbance increases to give peaks at 335 nm at 295 nm 270 nm and 230 nm but decreases at 250 nm therefore more peaks are present after degradation than before. Chlorpromazine shows a similar trend but shows a product absorbing above 325 nm. The peak above 325 nm seen in chlorpromazine could be due to the loss of the chlorine in a free radical process which has been reported in E.S.R. studies carried out by other research groups.[109,107] As the degradation takes place in aerated solutions it is probably partly due to the singlet state which is not quenched by oxygen. The triplet is quenched by oxygen, however the cation radical is not quenched by oxygen and therefore, one can conclude the cation radical is probably formed from the singlet state. The cation radical is likely to be involved in the formation of the product. The radical formed from loss of the chlorine has been suggested by Navaratnam et al [107] to form from the triplet state as explained in chapter 2, however this work can not confirm or deny this result.

There was no change in the degradation of either compound on addition of H.S.A. which is not unexpected as there has been no change observed to either the triplet or the singlet state on the addition of H.S.A.

Summary of the photochemical properties of chlorpromazine and promethazine.

Chlorpromazine has been studied by laser flash photolysis previously but there have been no previous photochemical studies carried out on promethazine. The ground state spectra showed 3 peaks for each compound the lowest energy transition being due to a $\pi \rightarrow \pi^*$ transition at 300 nm for promethazine and 310 nm for chlorpromazine. The ground state absorption spectra of promethazine was found to alter with pH which was due to the protonation of the amine group. The adsorption of both compounds onto cellulose caused a slight decrease in the absorption at lower wavelengths as seen in several other compounds.

These compounds showed fluorescence emission at 450 nm and 440 nm for chlorpromazine and promethazine respectively, at room temperature in ethanol, giving fluorescence quantum yields of 0.004 and 0.012 respectively. The phosphorescence

emission measured at 77 K showed an emission at 475 nm and 480 nm for promethazine and chlorpromazine respectively with quantum yields of 0.79 and 0.63 respectively. The lifetime of the phosphorescent emission was found to be 0.073 s and 0.068 s for chlorpromazine and promethazine respectively. The energy gap between the triplet and singlet excited states was found to be 65 $kJmol^{-1}$ and 71 $kJmol^{-1}$ for chlorpromazine and promethazine respectively indicating the transition from the ground state to the triplet state is a $\pi \rightarrow \pi^*$ transition. The presence of H.S.A. seemed to have no effect on the photochemical properties of these compounds.

The laser flash photolysis of these compounds showed the presence of a triplet species at 460 nm and a cation radical species at 510 nm for promethazine and 525 nm for chlorpromazine. The chlorpromazine results were the same as recorded by other research groups.[106,107,110] The triplet species was confirmed by oxygen quenching and triplet quenching using naphthalene. The cation radical was shown to come from the singlet state since neither the lifetime nor the amount of the cation radical were affected by oxygen and the triplet state was found to be quenched by oxygen. This was different to the results found by Chignell et al by laser flash photolysis [106] who suggest the cation radical came from the triplet state. The lifetime of the promethazine triplet was found to be 2.1×10^{-5} s and an estimate of the lifetime of the chlorpromazine triplet was found to be100 ns. This chlorpromazine lifetime is the same as found by Navaratnam et al[107] but longer than found by Chignell et al.[106] However since both groups, as well as the results found here, show quenching of the triplet species with oxygen the longer lifetime seems more likely. The cation radical species had lifetimes of 9.4×10^{-5} s and 5.6×10^{-5} s for promethazine and chlorpromazine respectively. There was no absorption change seen for the promazine radical due to loss of the chlorine with chlorpromazine which had been observed by Navaratnam et al[107] The effect of adsorbing chlorpromazine and promethazine onto cellulose was minimal except for an increase in the transient lifetimes due to the cellulose protecting the transient species from quenchers as explained in chapter 3.

The singlet oxygen quantum yields of these compounds were 0.20 for chlorpromazine and 0.25 for promethazine which are high enough to be involved in reaction. These

compounds also showed quenching of the singlet oxygen which could be either physical, chemical or both. The quenching constants for the quenching of the singlet oxygen was 4.27×10^6 $S^{-1}M^{-1}$ and 3.93×10^6 $S^{-1}M^{-1}$ for chlorpromazine and promethazine respectively. The chemical quenching of the singlet oxygen by these compounds may play an important part in the photoallergy process.

The degradation of these compounds under the full arclamp showed the formation of products probably by the hydrogen abstraction by the cation radicals. There was a difference in the spectra after irradiation between chlorpromazine and promethazine seen as a increase in the absorption above 325 nm. This may well be due to the loss of the chlorine to form a promazine radical and by hydrogen abstraction of a hydrogen atom from the solvent to form a product. This promazine radical has been seen in E.S.R. studies and with laser flash photolysis in alcohol previously[107,109] and the transient difference spectra here have shown no differences between the two compounds suggesting the formation of the promazine radical is in very small yields.

Chapter 6
Conclusions and Further Work

6. Conclusions and Further Work.

6.1 Summary and comparison of photoallergens

The aim of this PhD was to find a physical model of the properties of a photoallergen. This would also allow photoallergens to be identified from their photochemistry. The previous work carried out on these compounds falls into 2 categories. The first of these is from biological work carried out *invivo* to identify which compounds are photoallergens. The second type, studies the photochemistry of these compounds mostly using techniques such as E.S.R. and some laser flash photolysis. These studies have not connected the photochemistry observed to the mechanism of photoallergy. Past workers[39,40] have suggested that photoallergy occurs only if the compound has previously photobound to a protein in the skin, usually assumed to be H.S.A. It was therefore thought that a reactive species would need to bind to the protein. This species has been assumed in many cases to be a radical species. Although it can be seen that the compounds must bind to the skin by the fact that they can remain in the skin for several months there is no clear evidence to show this binding must be photochemical binding rather than dark binding. If the binding were dark binding rather than photochemical binding, the need for a reactive species would be removed and the photochemistry would be for the photoallergen protein complex and not the compound alone.

In the cases of T_4CS, fentichlor and bithionol which have been studied by E.S.R.[59,60,67-71] the radicals found due to dehalogenation were assumed to be responsible as no other reactive species were detected with the methods employed. Chlorpromazine was studied using laser flash photolysis and E.S.R. and was shown to form a cation radical[106-110] after excitation, however this radical was not believed to be responsible for the photoallergy due to the biphotonic nature of the excitation needed at 355 nm, which would not be reproduced in sunlight. A previous thesis written by another Loughborough student on T_4CS found radicals due to dehalogenation and has assumed these to be responsible for the photoallergy.[56] However no conclusive proof can be shown in support of this mechanism.

This thesis has tackled the problem from another angle by studying several different photoallergens in an attempt to find a common factor in the photochemistry of all the photoallergens and therefore be able to define photoallergy.

From the previous chapter it can be seen there is no obvious connection between all the photoallergens and therefore the definition of photoallergy is probably very complex involving several different factors. The relative strength of the photoallergens may also have an effect on the mechanism of the photoallergy. The relative strengths of the photoallergens however are hard to distinguish, as no clear order of photoallergy has been found, due to the difficulty in differentiating between photoallergy and phototoxicity.[37,88] From different papers an approximate ranking can be found with chlorpromazine and promethazine as the strongest photoallergens, followed by 6-methylcoumarin, bithionol and fentichlor lower down and TBS as the weakest photoallergen. Ketoprofen and omadine were not included in these ranking tables as very little information on their photoallergic strength is available, although ketoprofen has been shown to be a weak photoallergen.[37,88]

The comparison of the results found in the previous chapter may therefore be useful in suggesting some possibilities for the connection between photoallergy and the photochemistry of these compounds.

All the photoallergens were white compounds and therefore have no absorption above 400 nm. The ground state of the compounds in general showed peaks at around 300 nm due to $\pi \rightarrow \pi^*$ transitions as shown in Table 10. However, sunlight is more intense above 320 nm and although it can emit light down to 290 nm the intensity of the radiation is lower at these wavelengths.[87] The radiation from the sunlight only penetrates to the dermis, where photoallergy is believed to occur, at wavelengths below 320 nm. Ketoprofen has a low value for the $\pi \rightarrow \pi^*$ transition but has a higher $n \rightarrow \pi^*$ transition and TBS has a $n \rightarrow \pi^*$ transition around 380 nm. This shows not all the photoallergens show the same transition as their lowest excited singlet state.

Compound	λ_{max}	Transition
Bithionol	305	$\pi \rightarrow \pi^*$
Fentichlor	305	$\pi \rightarrow \pi^*$
TBS	360	$\pi \rightarrow \pi^*$
Omadine	335	$\pi \rightarrow \pi^*$
6-methylcoumarin	320	$\pi \rightarrow \pi^*$
Chlorpromazine	310	$\pi \rightarrow \pi^*$
Promethazine	300	$\pi \rightarrow \pi^*$
Ketoprofen	340 255	$n \rightarrow \pi^*$ $\pi \rightarrow \pi^*$

Table 10: Ground state parameters of the photoallergens in aqueous or 10% ethanol:90% water solvents.

The fluorescence emission was detected to be present in some compounds but not in others. The quantum yields of fluorescence where they were able to be measured were low for all compounds as seen in table 11.

Compound	Wavelength/nm	Quantum yield of fluorescence	Energy of lowest excited singlet state
Bithionol	400	$<10^{-4}$	299
Fentichlor	400	$<10^{-4}$	278
TBS	440	0.0649	299
Omadine	–	–	–
6-methylcoumarin	406	0.00125	299
Chlorpromazine	450	0.00379	266
Promethazine	450	0.0118	286
Ketoprofen	–	–	–

Table 11 : Fluorescence parameters of the photoallergens

The singlet energies for the transition from the ground state to the lowest excited singlet state are all between 265 nm and 300, where they were able to be measured.

The λ_{max} of the fluorescence was seen to vary from 400 nm to 450 nm and it may therefore be characteristic for the photoallergens to fluoresce in this region with low fluorescence quantum yields. However T_4CS, which is known to be a strong photoallergen although fluorescing in the same region at 440 nm, has a high quantum yield[56] of 0.34 which would seem to dispel this theory. The fluorescence quantum yield however could vary with photoallergic capability, with the stronger photoallergens having a higher quantum yield than the weaker photoallergens. On closer inspection, TBS has the highest fluorescence quantum yield of the photoallergens measured in this thesis, which seems to dismiss this theory. If however the changes in the results seen for the fluorescence in the presence of H.S.A. are taken into account, the fluorescence quantum yields could show a different trend. The quantum yield of TBS fluorescence decreases with the addition of H.S.A., whereas the fluorescence quantum yields of compounds such as chlorpromazine and promethazine do not change. This change in the fluorescence quantum yields could cause the quantum yield to increase with the photoallergic strength, but this relationship would need further study.

The triplet species of these compounds were studied at 77 K using phosphorescence. The energy of the lowest energy triplet state were all found to be around 239 to 288 $kJmol^{-1}$. The highest is ketoprofen which is thought to be a $n\rightarrow\pi^*$ transition. Both ketoprofen and 6-methylcoumarin phosphorescence spectra are structured so the energy measured is for the average energy of the lowest excited triplet state rather than the lower limit of the lowest excited triplet state and the values for these compounds will be slightly higher than the other compounds. There would seem to be a general rule that the triplet energies are all relatively close. It was found in general that the quantum yield of phosphorescence were high, except 6-methylcoumarin and omadine. The measured value for the quantum yield of phosphorescence for 6-methylcoumarin may be low due to the large amount of fluorescence seen at this temperature.

Compound	ϕ_P	τ_P/S	Type of Transition	E_T/kJmol^{-1}	$E_{S1\rightarrow T1}$ /kJmol^{-1}
Bithionol	0.34	$6.3x10^{-3}$	$1\rightarrow\pi^*$	268	73
Fentichlor	0.56	$8.5x10^{-3}$	$1\rightarrow\pi^*$	265	57
TBS	0.59	$8.8x10^{-3}$	$\pi\rightarrow\pi^*$ $n\rightarrow\pi^*$	260	39
Omadine	0.037	$1.8x10^{-3}$	$\pi\rightarrow\pi^*$	239	–
6-methyl coumarin	0.075	0.11	$\pi\rightarrow\pi^*$	260	39
Chlorpromazine	0.67	0.073	$\pi\rightarrow\pi^*$	249	65
Promethazine	0.79	0.068	$\pi\rightarrow\pi^*$	252	71
Ketoprofen	0.951	$5.7x10^{-3}$	$n\rightarrow\pi^*$	288	–

Table 12: Phosphorescence parameters of the photoallergens at 77K.

The lifetime of the phosphorescence was in the millisecond range except for 6-methylcoumarin, promethazine and chlorpromazine. These are thought to be the stronger photoallergens, therefore it may be postulated that the lifetime could be longer in stronger photoallergens. 6-methylcoumarin however, is a longer lived than any other, but is not the strongest photoallergen. As with the fluorescence the addition of H.S.A. changes the phosphorescence of the compounds in different ways as seen in table 16. These changes in lifetime and quantum yields may cause the phosphorescence quantum yields and lifetimes to follow the order of the photoallergic strength. This would need further study of this relationship to confirm this result.

The triplet -triplet absorption results obtained from laser flash photolysis experiments varied considerably, and in three cases the triplet species was only seen with the presence of a sensitiser. This was found in all three cases to be due to the triplet species having a low triplet quantum yield rather than a short lifetime.

Compound	τ_T/s	λ_{max}
Bithionol	$7x10^{-3}$	370
Fentichlor	$6x10^{-5}$	370
TBS	$8.5x10^{-6}$	550
Omadine	_	660
6-methylcoumarin	$2.5x10^{-6}$	430
Chlorpromazine	$2.3x10^{-8}$	460
Promethazine	$2.1x10^{-5}$	460
Ketoprofen	$1.2x10^{-5}$	530

Table 13: Triplet- triplet absorption parameters obtained using laser flash photolysis.

The lifetimes of the triplet species also varied immensely. There was no obvious variation with photoallergic capability, although the stronger photoallergens chlorpromazine, promethazine and T_4CS appear to show the largest triplet quantum yields. Also T_4CS has also been shown to have a lot larger triplet quantum yield than TBS, where T_4CS is a stronger photoallergen. There was no correlation in the position of the triplet which varied from bithionol at 370 nm to omadine at 660 nm. This triplet species is probably not directly responsible for the photoallergy as for both ketoprofen and 6-methylcoumarin the triplet species seen were very similar to their related compounds benzophenone and coumarin, which are not photoallergens.

The easily observable radical species which have long thought to be responsible for photoallergy[39,40] were seen in all but one of the photoallergens upon laser excitation. All past papers have assumed radicals are responsible for photoallergy, mainly because it was believed that a relatively stable reactive species was needed to bind with the protein. Little actual evidence however, has been produced in defence of photobinding rather than dark binding.

Compound	λ_{max} of radical	$\tau_{radical}$/s
Bithionol	370	0.12
Fentichlor	370	0.1
TBS	400	$3x10^{-6}$
Omadine	460	$6x10^{-6}$
6-methylcoumarin	–	–
Chlorpromazine	525	$5.6x10^{-5}$
Promethazine	510	$9.4x10^{-5}$
Ketoprofen	520	

Table 14: Photochemical parameters of the radical species produced after laser excitation of the photoallergens.

The radicals seen after laser excitation were not the same type for all the compounds. The halogen containing compounds all appeared to lose the halogen to form a phenyl type radical and with bithionol, fentichlor and TBS this was the only radical seen. Even with these compounds, the lifetimes varied from 0.05 s to $3x10^{-6}$ s. The difference in the lifetimes has been said to change the way these compounds bind. If the radical is long lived it is thought to photobind to multiple sites in a random manner, whereas if the radical is short lived it will dark bind to 1 major site before photobinding to that site.[53] The difference in the radical lifetime therefore may not affect whether the photoallergic mechanism goes via the radical species but may effect the mechanism by which a radical is involved. With this suggestion the lifetime cannot be used to characterise a photoallergen.

In chlorpromazine, the phenyl radical due to dehalogenation was not seen in this work but has been reported using laser flash photolysis by Navaratnum et al.[107] With both chlorpromazine and promethazine the radical seen after laser excitation was a cation radical with a lifetime of ~10^{-5}s. However in other work on these compounds using both E.S.R. and laser flash photolysis (ns and ps) it has been suggested that this radical was not responsible for photoallergy due to the biphotonic nature of the excitation.[108] At wavelengths above 310 nm the biphotonic process is thought to be dominant and below 310 nm the monophotonic process is thought to dominate.[108] As the intensity of sunlight is greater at wavelengths above 320 nm the biphotonic nature of the excitation

is not likely to be reproduced by sunlight. In order to rule these radicals out, further work is needed to measure the true dependence of the nature of the excitation on the excitation wavelength, and whether sunlight at wavelengths below 310 nm is strong enough to cause the photoallergic reaction to occur. The phenyl radical formed by loss of the chlorine with chlorpromazine cannot be responsible for photoallergy as the photoallergic strength is thought to be similar for both promethazine and chlorpromazine. This suggests a similar photochemistry is responsible for the photoallergy in both of these compounds. This would seem to weaken the argument that the radical caused by the loss of a halogen in TBS, fentichlor and bithionol is responsible for the photoallergy. It is possible however, that different radicals could be responsible for the allergy in different compounds.

The radical seen for ketoprofen was a ketyl radical. This is the same as seen with benzophenone in alcoholic solutions and therefore is probably not responsible for photoallergy as benzophenone is not a known photoallergen. There was no sign of the 3-ethylbenzophenone radical seen by others using ESR[102] in this work which suggests it is formed in very low quantum yield and it could be this radical which is responsible for photoallergy in ketoprofen. With omadine a 2-pyridylthiyl radical was detected using laser flash photolysis and this could be responsible for the photoallergy but this would confirm the theory that there is no one type of radical responsible for photoallergy. The radicals seen were all found in aerated solutions and all thought to be formed from the singlet state.

The products formed from the irradiation of the photoallergens may be the species which are responsible for causing the allergic reaction. The presence of products was investigated by irradiating the photoallergens with an arc lamp and recording the change in their ground state absorption spectra. The data gathered from irradiation of the compounds in aerated solutions showed the formation of products for all the photoallergens. This data was important in cases like 6-methylcoumarin and ketoprofen where no transients were seen in aerated solutions using laser flash photolysis but a product was seen with steady state irradiation in aqueous solutions. This suggests the excited state has either a small quantum yield or a short lifetime and is probably formed from the singlet state as in the case of ketoprofen the triplet state is

quenched by oxygen. This is where the use of E.S.R is useful as past reports[102] have shown the presence of radical species for ketoprofen due to loss of the carboxylic acid group. All the absorption spectra taken after irradiation showed an increase in absorption above 350 nm. Further investigation of these products using methods such as GC-MS which were employed on bithionol and fentichlor would reveal the nature of these products.

Singlet oxygen has previously been known to cause skin irritations and is thought to be phototoxic. It has not however been shown to be connected to photoallergy.[45]

Compound	Quantum yield of singlet oxygen
Bithionol	0.025
Fentichlor	0
TBS	0.036
Omadine	–
6-methylcoumarin	0.015
Chlorpromazine	0.20
Promethazine	0.25
Ketoprofen	0.25

Table 15: Singlet oxygen parameters of the photoallergens

From the studies of singlet oxygen in this thesis there were only 3 compounds which showed a high singlet oxygen quantum yield. These were ketoprofen, chlorpromazine and promethazine. All the other compounds except omadine showed some singlet oxygen production. It is not known how much would be required to cause photoallergy and therefore the amount produced may be enough for the singlet oxygen to be involved in the photoallergy mechanism. In the case of 6-methylcoumarin where there is very little other photochemistry observed, the low singlet oxygen quantum yield maybe the most important process. In all the above cases where the fluorescence was measured it was found not to be quenched by oxygen and therefore the singlet oxygen is probably formed from the quenching of the triplet state. Therefore in all these cases there is a correlation between the amount of triplet seen and the quantum

yield of singlet oxygen except ketoprofen. This may suggest that the singlet state is contributing towards the singlet oxygen quantum yield or the triplet is being quenched by other factors on laser excitation. It is clear that singlet oxygen could play a part in photoallergy and there may therefore be two types of photoallergy, one with singlet oxygen involved and one with a radical mechanism.

Cellulose appears to have very little affect on the photochemistry of these compounds. In all cases there is an increase in the lifetime of the excited species seen after laser excitation. It also has the effect of increasing the quantum yield of some of the excited species therefore allowing the species to be detected using laser flash photolysis. For example in the case of 6-methylcoumarin and omadine. The cellulose results should give an indication of the effect of adsorbing the photoallergen onto a surface. It appears there is very little difference apart from the above and that on a surface the effect of oxygen is no longer important which maybe the same in skin.

Photoallergy is thought to depend on the ability of the radicals to bind with H.S.A. This binding could either be photochemical or dark binding or both[48,55] It is not clear which is necessary for photoallergy. It was also felt that dark binding to H.S.A. may effect the photochemistry of the photoallergens. This was investigated by adding H.S.A. to the photoallergens and recording any changes in the fluorescence, phosphorescence and excited states from laser excitation. The addition of H.S.A. to the photoallergens was found to have a varied effect on the H.S.A.. As stated earlier these experiments were all carried out in water as H.S.A. is denatured by organic solvents.[128] It may not be correct to carry out these experiments in aqueous solutions as it is not known whether photoallergy occurs in an aqueous or non aqueous part of the skin.

Compounds	Fluorescence	Phosphorescence	Triplet	Radical	Singlet oxygen
Bithionol	↑	↓	_	↑	_
Fentichlor	↑	↓	_	↑	_
TBS	↓	↑	_	↓	↓
Omadine	-	↑	_	_	_
6-methyl coumarin	↓	↑	↑	_	_
Chlorpromazine	X	X	X	X	↓
Promethazine	X	X	X	X	↓
Ketoprofen	-	↓	Shorter lifetime	-	_

Table 16: H.S.A parameters of the photoallergens where, ↑ = increase in quantum yield and lifetime, ↓ = decrease in quantum yield and lifetime, - = not measured and X = no change.

The addition of H.S.A. to the photoallergens in solution shows no trends across all the allergens studied. The singlet oxygen quantum yield decreased in the presence of H.S.A. for all the compounds in which it was measured. This was probably due to the triplet species being protected from oxygen and most of the singlet oxygen is produced from the quenching of the triplet state. The effect of H.S.A. on the photochemistry of these compounds varied with different compounds. This variation may be due to the position of binding on H.S.A. and may therefore not tell us which photochemical properties are involved in photoallergy. On binding, the H.S.A. and compound probably form a complex or a series of complexes whose photochemistry may be different to the parent compounds.

Dark binding of fentichlor and bithionol by quenching of the tryptophan signal on H.S.A. showed that dark binding occurs between fentichlor or bithionol and H.S.A.. These experiments showed the possible presence of one class of binding site to which fentichlor and bithionol bind initially and the possible presence of many weaker sites to which they bind randomly. The use of fluorescent probes to investigate the dark binding showed that both fentichlor and bithionol bind to site 1 (Lys199-Glu292) and

site 2 (Pro384-Phe488)[147] on H.S.A. Binding constants for bithionol were found to be 1-10x10^5 $mol^{-1}l$ and for fentichlor to be 6-8x10^5 $mol^{-1}l$. Fentichlor and bithionol probably bind to other sites, or affect the fluorescence of other sites on the H.S.A. and therefore site 1 and site 2 are probably only minor sites as the strength of binding was not large. There is less specificity of binding than seen with T_4CS which showed 2 major binding sites.[56]

6.2. Conclusions

This thesis describes much basic photochemistry of several photoallergens and has explained any differences in that photochemistry when on surfaces and in the presence of H.S.A. Overall it has shown there is not one photochemical property which can be held responsible for photoallergy but that it is probably due to a combination of properties. The ground state absorption spectra were all below 400 nm and the fluorescence energies and phosphorescence energies were similar for all the compounds. Although radicals were found in 6 of the 8 compounds and the other two were suspected to form radicals it is still not known whether these are the radicals causing photoallergy, the quantum yield required to cause photoallergy and how the photochemical binding of the compounds to H.S.A. effects the photoallergy mechanism. There maybe minor radicals which have not been observed in this thesis which cause the allergic response. It is strongly suspected from these results however that the mechanism is not the same for all compounds. It may be possible that some photoallergens cause photoallergy via a singlet oxygen mechanism whereas others proceed via a radical mechanism. Although it is clear that the compounds must bind with the skin, it is not clear if this binding must be dark binding or photochemical binding and more information is needed about the type and position of binding of these compounds before the cause of photoallergy can be identified.

6.3. Further work

It is still believed that the radical species are involved in photoallergy mechanisms. It is therefore, necessary to identify which radicals are responsible and how differences in lifetime affect the photoallergic response. The radicals could be studied in several different ways.

1) Some compounds showed no radical species using laser flash photolysis, which may be due to low yields, therefore these compounds could be studied using sensitisation or radical trapping experiments.
2) The opposite approach could be applied to these experiments so that the radical intermediates being responsible for photoallergy could be studied using a model compound with high radical quantum yields.
3) The examination of compounds of similar structure with similar radical intermediates but different photoallergic strengths may help to explain the photoallergy mechanism. Examples of this are chlorpromazine and promethazine, which have shown very similar photochemistry, bithionol and fentichlor , and TBS and T_4CS. The difference in the photoallergic potential is biggest in TBS and T_4CS and these may be the best choice for studying the differences.

Singlet oxygen has long being held responsible for the phototoxic effects seen from these compounds, and further investigation of the singlet oxygen production would help to clarify its position, if any, in the photoallergy mechanism. Singlet oxygen may also be involved in radical formation in some compounds, for example by the ground state reacting with the singlet oxygen to produce an intermediate, which may undergo further reactions not available to the parent compound. Therefore it is necessary to determine the quantum yields of singlet oxygen, the rate constants for the reaction of singlet oxygen with various compounds and the relative extents of physical and chemical quenching. The difference in the singlet oxygen quantum yield with H.S.A. present was found in two compounds to be reduced, due to the H.S.A. protecting the species from molecular oxygen, therefore further studies of this effect may give some idea of how important oxygen is in the role of photoallergy and whether the presence of singlet oxygen can be held responsible for photoallergy.

The binding to H.S.A. of these compounds has been shown by this work to be important in the photoallergic mechanism. Therefore further study of both the dark and photochemical binding of these compounds is necessary. It would be particularly interesting to see whether there is any correlation between the position of binding, on the H.S.A. and how the photochemical properties are affected by the H.S.A. and whether this affects the strength of the photoallergic reaction. Further information is

also required to determine whether the photochemical binding and dark binding occur in the same place and whether they occur to the same extent. T_4CS must dark bind to H.S.A. before it can photochemically bind, whereas fentichlor and bithionol have shown no need to dark bind first. This was related to the lifetime of the radicals involved. Radicals with long lifetimes do not need to dark bind first, whereas with shorter lifetimes they do. This relationship needs to be investigated further and with other compounds.

The use of solvents in this thesis has been varied to find the basic photochemistry whereas when studying in H.S.A. it was necessary to use aqueous solutions or less than 10% ethanol : 90% water solutions. Binding of the compounds to H.S.A. is known to be to the hydrophobic part of H.S.A., however it is not known whether the photochemistry occurs in the aqueous part or the hydrophobic part of the protein. Many of the compounds are not very soluble in water and therefore it is likely that they occur in hydrophobic regions in which case the solvent should be altered accordingly.

The photochemical compounds have generally been studied in solution, and on cellulose to see the effect of adsorbing them onto a surface There have been some experiments with H.S.A. to see the effect that binding to a protein has on the photochemistry. The next step would be to adsorb the compounds onto skin to see how their photochemistry varied. The technique to study compounds on skin would initially be carried out using a model compound with well known photochemistry. Further investigation of how the compounds bind, where they bind and to exactly which protein or amino acid would need to be carried out. The skin is not a homogeneous surface and may therefore still not be good for many basic studies of a photoallergen. With further understanding of the biology of the skin and the way in which the compounds bind to it, it may be possible to find a better model for studying the photoallergens than that used in this thesis, such as the use of micelles. In the last 20 years there have been extensive photochemical studies carried out in aqueous micellar solutions. It has been found in many cases to dramatically change the nature and/or rate of reaction compared to homogeneous media.[182-186]This is due to their ability to solubilise hydrophobic molecules into a bulk aqueous solution. The change in

the products seen and/or the rates of reaction, compared to homogeneous media can help to identify the mechanism of the reaction.

The photoallergens are known to protein bind into a hydrophobic environment and it has been shown[187,188] that micelles are a good model for this biological environment. Chlorpromazine has been studied in micelles using irradiated samples with liquid chromatography and for this allergen it was found that the dechlorination reaction, which was not seen in aerated solutions, now becomes important.[187]

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