Application of Fluorescence Microscopy to a Study of Chemical Problems

R. S. Davidson
Department of Chemistry, University of Kent, Canterbury, UK, CT2 7NH

1 Introduction

This review aims to show the reader how the use of fluorescence microscopy using a conventional or modified fluorescence microscope may be used to study chemical problems such as the photodegradation of naturally occurring polymers, photoinduced reactions, the dyeing of fibres and the measurement of T_g (glass transition temperature) values. Hitherto the use of fluorescence microscopy has been the domain of the biologist but now there are opportunities available to the chemist.

Fluorescence may be defined as light which is emitted when an electronically excited state relaxes to an electronic state of lower energy and possessing the same spin state. For organic species in solution and in the solid state, the fluoresence observed is usually associated with relaxation from the first excited singlet state to the ground state.[1] Needless to say, this is only one of the ways that an electronically excited singlet state can relax and these processes are usually shown pictorially by means of a Jablonski diagram (Fig. 1).

A wide range of compounds exhibit fluorescence. One of the features that marks highly fluorescent compounds is that they possess rigid structures. Examples include polycyclic aromatic hydrocarbons (*e.g.* anthracene) and dyes[2] which are based on a fused aromatic ring system (*e.g.* fluorescein **1**, the coumarin derivative 2, Fig. 2).

The rigid structure prevents the electronically excited state deactivating *via* undergoing intramolecular isomerisation (*e.g.* as exemplified by stilbenes which undergo *cis–trans* isomerisation[3]) or conformational changes (*e.g.* twisting about the 1–1′ C–C bond in 1,1′-binaphthyl). In some cases, intramolecular motion can lead to a new emitting species which does not possess an equivalent stable ground state, *i.e.* emission occurs from the almost planar form of 1,1′-binaphthyl **3** (Fig. 3).

As a consequence the fluorescence spectrum of 1,1′-binaphthyl shows a broad structureless band at lower energy than that expected from the primarily excited species, *i.e.* the intramolecular motion has generated a sizeable Stokes shift. Compounds which exhibit such shifts and possess high quantum yields of fluorescence are particularly valuable as fluorescence probes[4] and no more so than when the probes are used in fluorescence microscopy. Many compounds exhibit fluorescence in solution which does not emanate from the initially created excited singlet state but rather may be attributed to the excited state undergoing solvation (thereby giving rise to solvatochromic shifts), deprotonation or protonation, intramolecular charge transfer *etc.* If the fluorescence spectrum of a compound is sensitive to solvent polarity, then such a material may be used to probe the polarity of cell membranes *etc.*[5] and such a process can be visualised by means of fluorescence microscopy. The phenomenon of excited singlet states undergoing protonation and deprotonation is well established[6] and is well illustrated by the hydroxy coumarin **4**[7] (Fig. 4).

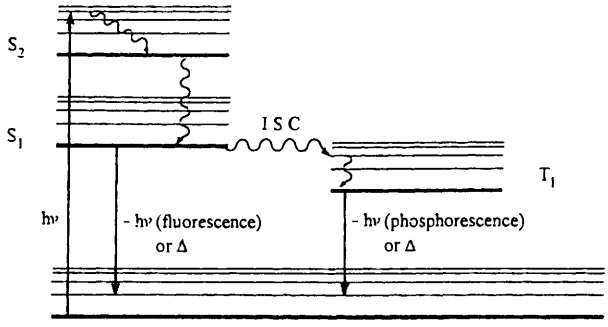
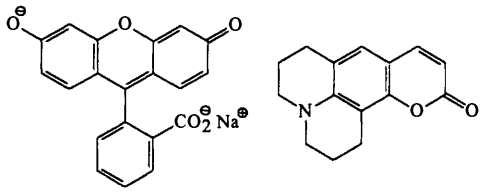
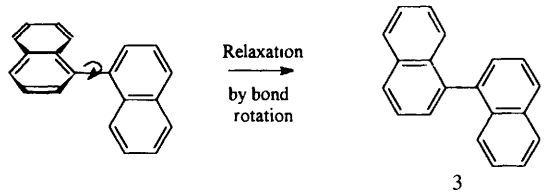


Figure 1 Jablonski diagram (A. Jablonski *Nature*, 1933 **131** 839; *Z. Phys.*, 1935, **94**, 38).



Fluorescein 1 A coumarin laser dye 2

Figure 2 Some fluorescent dyes.



Twisted S_1 state of 1,1-binaphthyl

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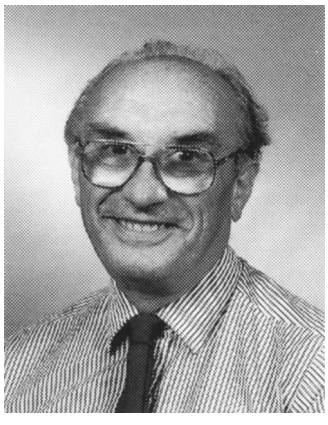
Almost planar relaxed S_1 state of 1,1-binaphthyl

Figure 3 Relaxation of the initially created excited singlet state of 1,1′-binaphthyl by bond rotation. (MFM Post, J. Langelaar and J. D. Van Voorst, *Chem. Phys. Lett.*, 1975, **32**, 59).

R. S. Davidson gained an ARIC from Leeds College of Technology in 1958 and then carried out research for a PhD degree under the supervision of Professor B. Lythgoe FRS at the University of Leeds. This work led to a successful synthesis of tachysterol$_3$ and the award of the J. B. Cohen prize from the University of Leeds. Having spent a year working with Professor R. B. Woodward (Harvard University) on a synthesis of vitamin B he took up the post of lecturer in organic chemistry at the University of Leicester (1964) where he pursued his interests in photochemistry. Particular interests at this time were photoinduced electron-transfer reactions and photo-oxidation reactions. He was awarded a DSc degree from the University of Leeds in 1978. He moved to the City University, London, in 1979 to take up the chair of organic chemistry. It was whilst in this post that his interest in microscopy and the photodegradation of natural polymers was awakened. In 1990 he moved to the University of Kent where he is currently Professor of Applied Chemistry. Most of his research is concerned with radiation curing (photoinitiated polymerisation processes) and he is also investigating ways to prevent the photoyellowing of papers made from high yield pulps. He had the title of Emeritus Professor of Organic Chemistry conferred upon him by City University in 1993. He has over 200 publications in the field of photochemistry.



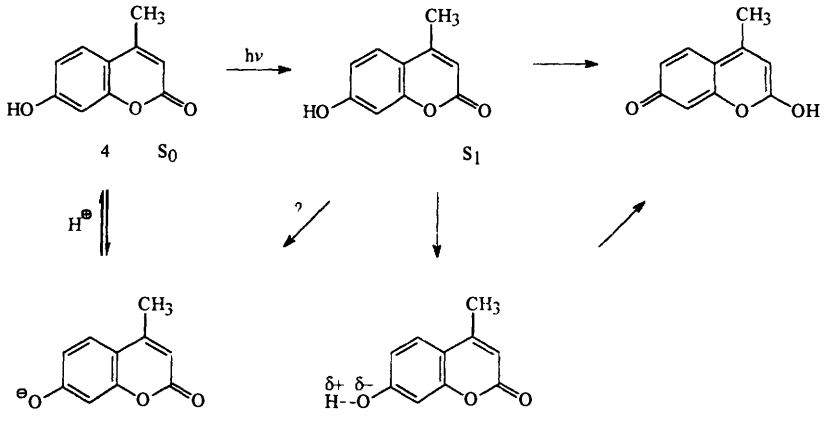


Figure 4 Some protonation and deprotonation reactions exhibited by **4**

Fluorescent species which are sensitive to pH have found application in determining the pH of intracellular material [8] A topic which is currently attracting much attention is that of intramolecular charge transfer, and fluorescence spectroscopy has been used extensively to study the dynamics of the process [9] Perhaps the most frequently studied compound which exhibits this phenomenon is 4-dimethylaminobenzonitrile This compound exhibits a broad structureless emission band which shifts to lower energy and exhibits a decrease in quantum yield as the solvent polarity increased Such behaviour is the hallmark of an excited state which possesses a considerable amount of charge transfer and consequently has a high dipole moment Many experimental results support the view that excitation of the aminobenzonitrile leads to rotation about the C–N single bond with concomitant electron transfer from the amino to the cyanobenzene moiety (Fig 5) thereby leading to a twisted intramolecular charge transfer (TICT)

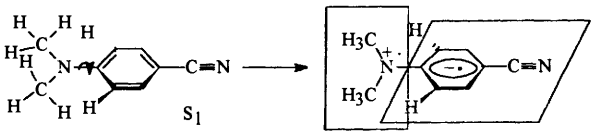


Figure 5 Formation of a twisted intramolecular charge transfer complex

Many compounds have been found to behave in a similar fashion to the aminobenzonitrile and compounds of particular value in fluoresence analysis (*e g* end group determination of peptides using the Edman degradation)[10] and fluorescence microscopy are sulfonated amino naphthalenes such as 1-dimethylaminonaphthalene-5-sulfonic acid [4] Since the formation of a TICT complex involves a change in molecular conformation, it is not surprising to find that the efficiency of the process is dependent upon solvent viscosity Thus such compounds have found use as probes for monitoring the change in viscosity which occurs when materials such as acrylates undergo polymerisation [11] The occurrence of charge transfer in excited states is not limited to examples where the donor is directly linked to the acceptor group Thus many examples are known of compounds which exhibit excited state charge transfer in which the donor and acceptor groups are separated by a rigid spacer group or a flexible chain [12] Compounds having a donor and acceptor group separated by rigid spacers (*e g* **5**, Fig 6) of varying dimensions have been used to show that electron transfer can occur over large distances, *e g* 25 Å For compounds having donor and acceptor

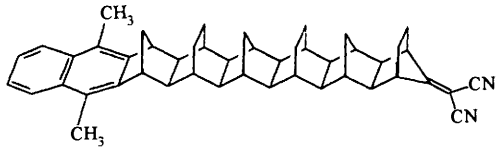


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Figure 6 A rigid compound which exhibits long distance electron transfer (N Paddon Row and J W Verhoeven, *New J Chem* , 1991, **15** 107, J W Verhoeven, *Pure Appl Chem* , 1990, **62**, 1585

groups linked by a flexible chain, the efficiency of excited complex formation can in part or wholly be dominated by the conformational flexibility of the chain For compounds such as **6** (Fig 7) it is

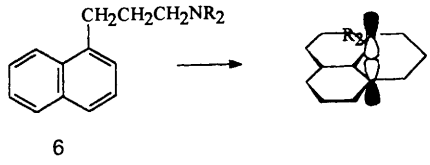

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Figure 7 Formation of an intramolecular excited charge transfer complex

necessary for the donor group (the amine) to interact with the π-electron system of the aromatic hydrocarbon acceptor group [12]

Another requirement for compounds such as **7** to exhibit excited charge transfer complex formation is that the amino group is able to donate an electron This is clearly impossible when the amino group is protonated or if the lone pair of electrons is part of a dative bond This simple fact has been used to design compounds which will act as molecular switches and signalling devices Compound **7** (Fig 8)

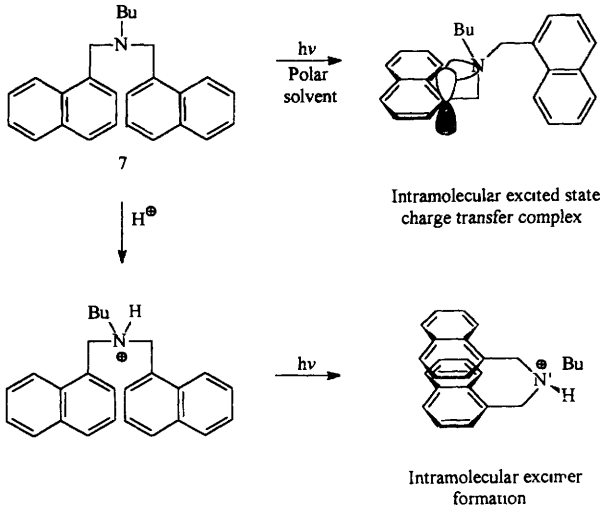
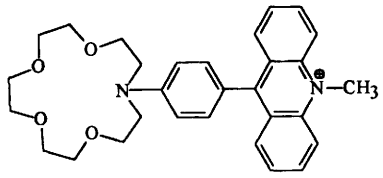


Figure 8 A compound which exhibits both excimer and exciplex formation

exhibits intramolecular excited charge transfer complex formation in neutral solution, *e g* ethanol, but in the presence of acid the formation of an excited charge transfer process is switched off and is replaced by intramolecular excimer formation [13] Since the two types of complex emit at different wavelengths they can be readily distinguished Compounds have been designed which exhibit intramolecular excited charge transfer complex formation and which have been used as molecular switches in which the on–off process is regulated by pH [14] In many cases, intramolecular electron transfer leads to fluorescence quenching and consequently, if in these compounds the donor is an amino group, protonation or involvement of



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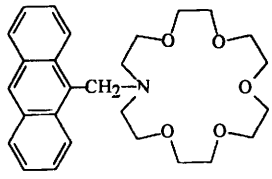

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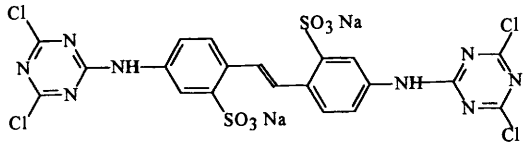
Figure 9 Compounds which exhibit an increase in fluorescence yield when the lone pair electrons on nitrogen are involved in bonding

the nitrogen lone pair in complexation leads to enhancement of the fluorescence quantum yield Protonation of the nitrogen atom in **8** (Fig 9) leads to a spectacular increase in quantum yield[15] and in the case of compound **9** (Fig 9) complexation with potassium led to a 47-fold increase in fluorescence intensity [16]

A process which is somewhat similar to quenching of fluorescence by intramolecular complex formation is that of quenching *via* the heavy atom effect [17] Quenching by halogen atoms increases in efficiency as the atomic mass of the halogen atom is increased and consequently bromine is a more efficient quencher than chlorine For quenching to be observed the halogen atom may be directly attached to the fluorogenic chromophore, or it may be linked to the chromophore *via* a flexible chain[18] or alternatively may be present in solution as part of the solvent Where the atom is present in the molecule, its removal *via* ground- or excited-state reactions will lead to an increase in fluorescence yield An example which illustrates such a process is afforded by reactive dyes **10**, **11** and **12** (Fig 10) These dyes become covalently attached to wool *via* nucleophilic displacement of a halogen group by the ε-amino group of lysyl groups present in the wool It was found that this process in itself did not remove all the halogen atoms and that further reaction with external reagents such as water or an amine was necessary for the dyes to exhibit their maximum fluorescence intensity [19]



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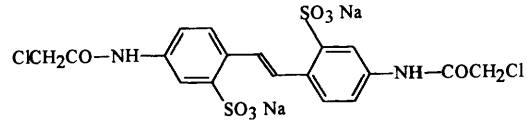

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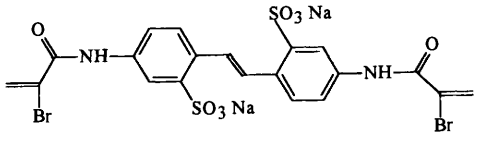


Figure 10 Reactive dyes which are non fluorescent but which become fluorescent upon removal of the halogen atoms *via* nucleophilic displacement

2 Fluorescence Spectrometers and Microscopes

For the routine recording of fluorescence spectra a spectrometer which contains the basic elements shown in Fig 11 can be used It is a relatively simple task to replace the sample accessory (3) by one which will accommodate solid samples [20] In order to decrease the influence of scattered light upon the emission spectrum it is best to avoid the right angle configuration and to have a system whereby the angle of the sample with respect to the excitation beam can be varied In this way the angle can be varied so as to find the one which gives the least disturbed and highest intensity signal With a spectrometer such as the one shown in Fig 11, the monochromators are equipped with entrance and exit slits and by appropriate choice of slit widths maximum spectral resolution can be achieved If the fluorescence spectrum of the sample does not exhibit a Stokes shift,

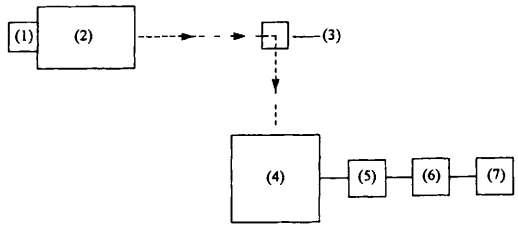


Figure 11 Layout of the components of a spectrofluorimeter
(1) Light source, usually Xe or Hg/Xe arc lamp
(2) Excitation monochromator
(3) Sample shown as a quartz cuvette in which the emission is observed at right angles to the exciting beam
(4) Emission monochromator
(5) Photomultiplier tube
(6) Amplifier
(7) Data recording device

great care has to be taken to ensure that scattered exciting light does not affect the spectrum and that the emission spectrum is not perturbed by some of the fluorescence being absorbed by the sample (inner filter effect) When weakly emitting samples are being examined, better sensitivity can be achieved by operating the detection system in the photon counting mode [21] With such a spectrometer it is possible to use the exciting light as a means of bringing about photochemical reactions in the sample and if those reactions cause a change in fluorescence intensity or spectral shift, the course of the reaction can in principle be monitored in real time This can be difficult to achieve in practice since usually it is necessary to employ large slit widths for the excitation monochromator which can lead to difficulties in recording the spectra However, it is a relatively simple job to record spectra after set illumination times by changing the slit widths In this way the photodimerisation of styrylpyridinium groups (Fig 12) appended to a poly(vinyl alcohol) (PVA) was monitored [22] The spectra in Fig 13 show that the excimer emission exhibited by the films of modified PVA decreases

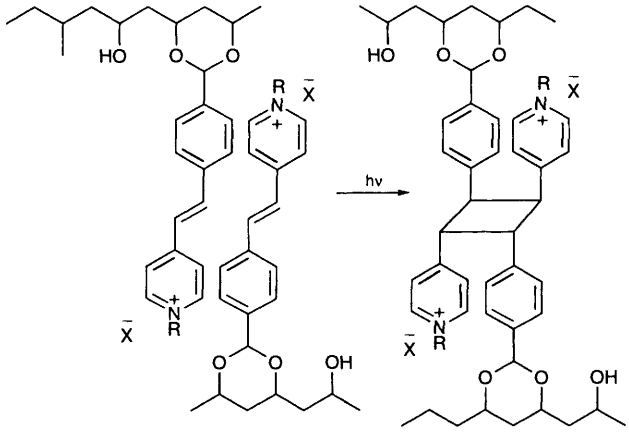


Figure 12 The photodimerisation of a styrylpyridinium salt

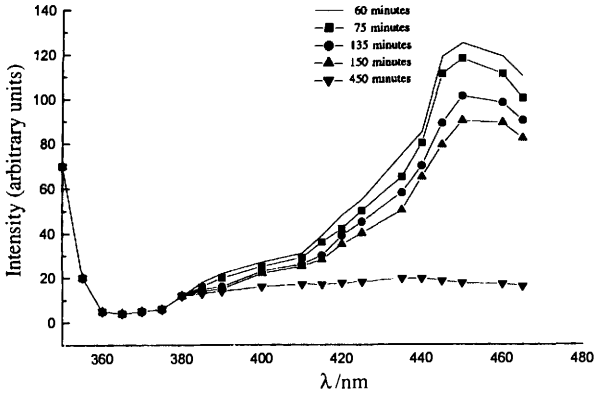


Figure 13 Fluorescence spectra recorded during irradiation of styrylpyridinium groups pendant to a poly(vinyl alcohol) chain.

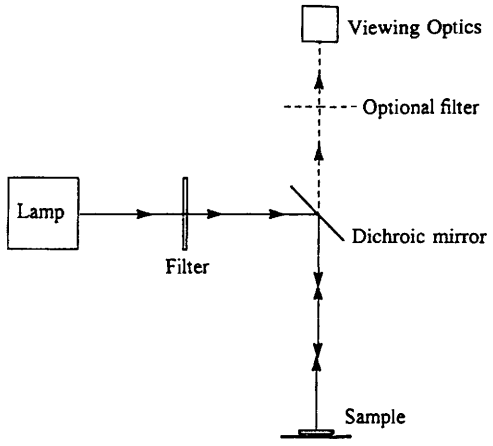


Figure 14 Diagram showing the parts of a fluorescence microscope associated with revealing the fluorescence of the sample.

in intensity as reaction proceeds. At the end of the reaction some styrylpyridinium groups remain and these are presumably groups which do not have a neighbour that is sufficiently close to enable the chemical reaction to occur.

Many of the benefits which accrue from using a fluorimeter of the type shown in Fig. 11 are due to the presence of the two monochromators. If these are replaced by filters the recording of spectra becomes impossible and for emission to be observed it requires that the excitation and observation wavelengths are sufficiently well separated. The *conventional* fluorescence microscope[23] relies upon the use of filters, and consequently fluorescence probes *etc.* being examined *via* the microscope should exhibit large Stokes shifts. The examples cited (*i.e.* compounds 1–12) possess this property and many well utilised probes exhibit fluorescence properties which are due to the photophysical processes displayed by compounds **1**–**12**. Microscopes such as the one shown in Fig. 14 are the standard work-horse of immunology laboratories where fluorescent labels are used to detect particular interactions.

In order to obtain sufficiently high light levels for visual observation, a super-high-pressure mercury lamp is the usual source of excitation. A filter is used on the excitation side which either transmits a wavelength associated with one of the main emission lines of the lamp, *e.g.* 365 nm, or transmits a spectrally wide band of light. The dichroic mirror (Fig. 15) reflects the excitation light onto the sample but transmits the fluorescence produced by the sample.

With the advent of charge coupled device (CCD) cameras it is possible to observe very low light intensities and such cameras can be mounted onto the microscope so as to increase the sensitivity of fluorescence detection. When the microscope is being used for studying chemical reactions it is often advantageous to mount either a photomultiplier tube or a monochromator equipped with a photomultiplier tube on the microscope. In this way fluorescence intensity changes during irradiation can be recorded and if the monochromator is properly equipped, fluorescence spectra can be recorded. Such a system can be enhanced further if a continuous wave (cw) *e.g.* an argon ion or helium cadmium, laser replaces the lamp. By placing a timed shutter between the laser and the microscope the system can in principle be used for determining fluorescence lifetimes (provided the photomultiplier tube is operating in the single photon counting mode) and to carry out bleach recovery experiments (see later). The layout of such a system is shown in Fig. 16.

Such an experimental set-up is ideal for examining samples and for carrying out photochemical reactions. The high intensity excitation beam can be used simultaneously to bring about a photochemical reaction and also monitor fluorescence changes which occur during the reaction (real time fluorescence spectroscopy). However, if the sample requires heating or if the sample is to be reacted in solution there is insufficient room between the microscope objective and the sample to allow positioning of the necessary equipment. This problem can be overcome by the use of an *inverted* fluorescence microscope. In such a microscope all the optical parts are sited below the microscope stage thereby leaving space between the stage and the ceiling of the room in which the microscope is being used to house any equipment. For purposes outlined later, we have constructed two accessories which fit on the top of the stage and which allow polymer films and similar samples to be heated[24] and another in which materials can be subjected to chemical treatment.[25] These accessories are shown in Fig. 17.

A very useful commercially available accessory is a microinjector which enables metered amounts of material (*e.g.* a dye) to be injected into a sample being examined on the stage of the microscope.

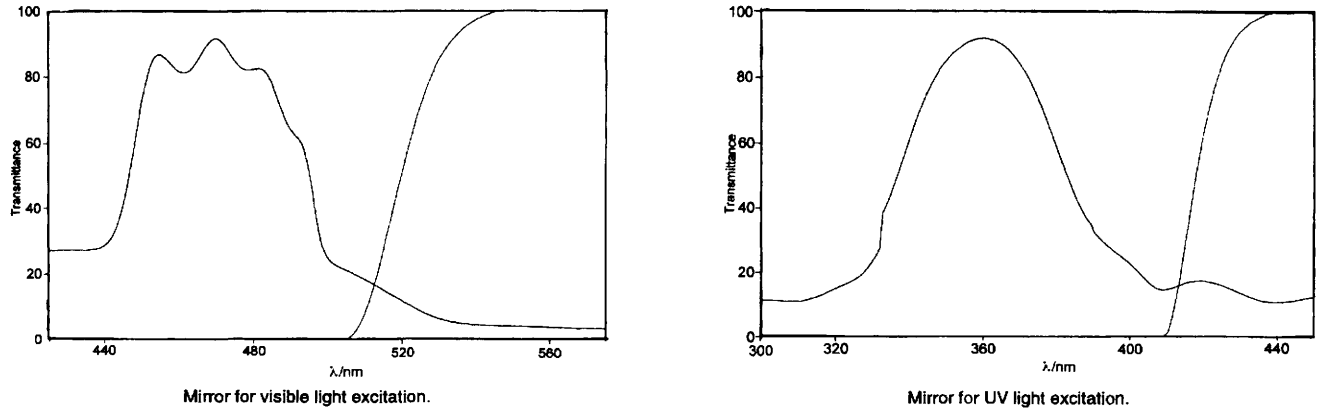


Figure 15 Spectral characteristics of a dichroic mirror used in a fluorescence microscope.

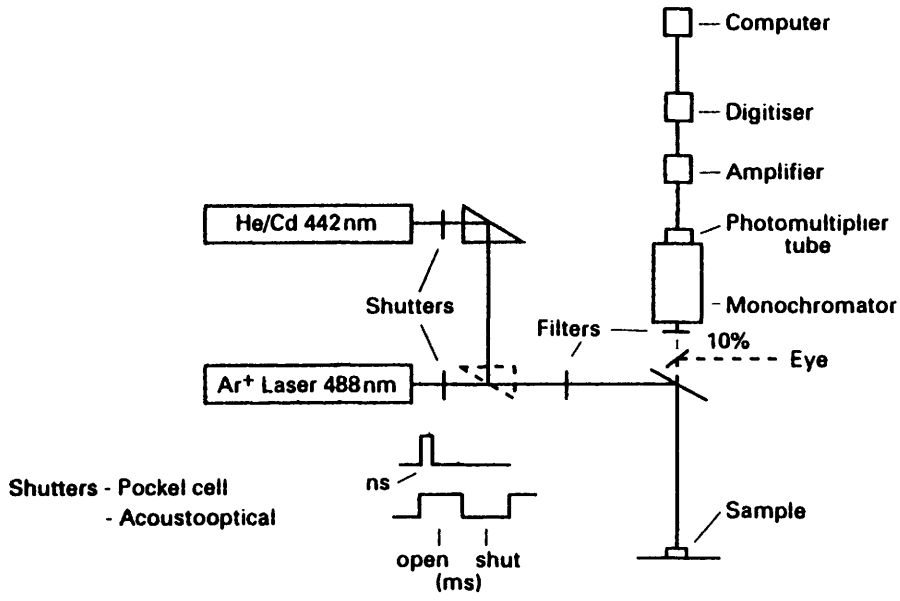


Figure 16 Diagram of a fluorescence microscope adapted for recording spectra, fluorescence lifetimes and for carrying out bleach recovery experiments

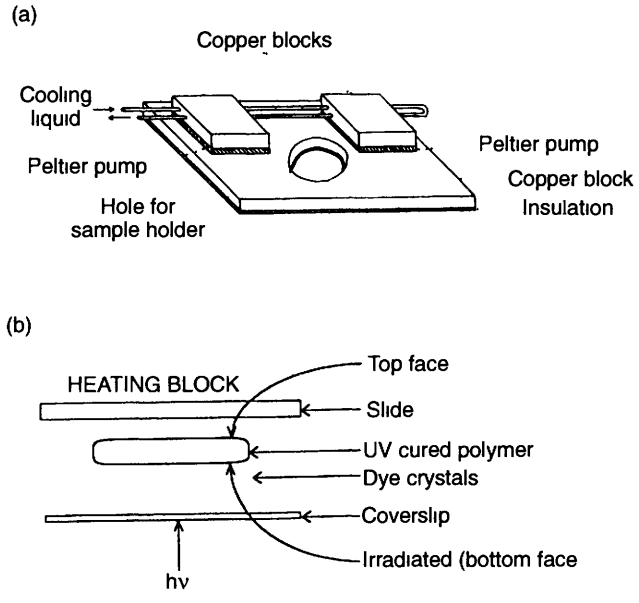


Figure 17 Accessories for use with an inverted fluorescence microscope (a) for heating a polymer film, (b) for carrying out chemical reactions

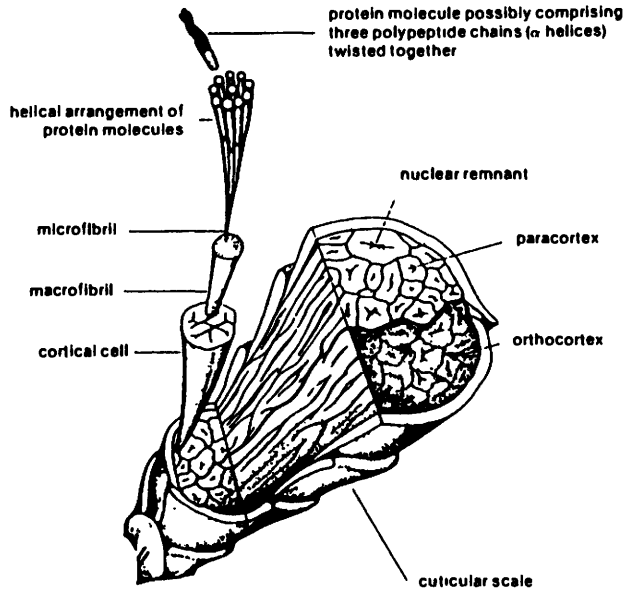


Figure 18 Sketch of a broken section of fine wool fibre showing the major cellular components and the detailed structures within them

3 Some Examples of the Use of Fluorescence Microscopy to Study Chemical Problems

3.1 The Chemistry of Wool

The structure of a wool fibre is very complex and Fig 18 shows in diagrammatic form some of its components The cuticle is normally only one cell thick except where the cells overlap and is rich in cystine Whilst most of the fibril structure is made up from keratin (a protein) molecules, the cortical cells are separated from each other by a cell membrane complex which is largely composed of lipids The cell membrane complex also separates the cuticle cells from the underlying cortical cells Whilst much of the chemistry of wool is based on the fact that it is a proteinaceous fibre, it is clear that this is very much an oversimplification of the real case Like other natural polymers, wool is affected by light The most obvious effect is that of photoyellowing, caused by wavelengths <380 nm, photobleaching is caused by wavelengths >380 nm The photodegradation processes also lead to an increase in the fluorescence of wool fibres[26] (Fig 19)

Clearly the colour changes indicate that photochemical reactions are occurring but in addition to these, others occur which lead to the wool fibres losing their strength These changes are exacerbated when the wool has been subjected to treatments such as oxidative bleaching or the application of fluorescent whitening agents (FWAs) It has been known for some time that wool possesses an intrinsic fluorescence the origin of which is far from understood When sections of wool fibres are examined by fluorescence microscopy it was very evident that the tips were far more fluorescent than the roots [26] By measuring the fluorescence intensity along the length of a 60 mm fibre (obtained from Merino sheep) it was found that most of the fluorescence was exhibited in the first 5 mm from the tip of the fibre [27] Given that the fleece is densely packed, this result is not surprising When a fibre from a more open-structured fleece was used the difference in fluorescence intensity at the root and tip was not so marked [28] Fluorescence spectra of the tips and roots of Merino wool (obtained by microspectrofluorimetry) were found to be very similar which suggested that the same species are present in tips and roots which give rise to the fluorescence A further finding which substantiated this deduction is that the bleach-recovery profiles for the tips and roots are similar In this type of experiment the sample is irradiated for a short time

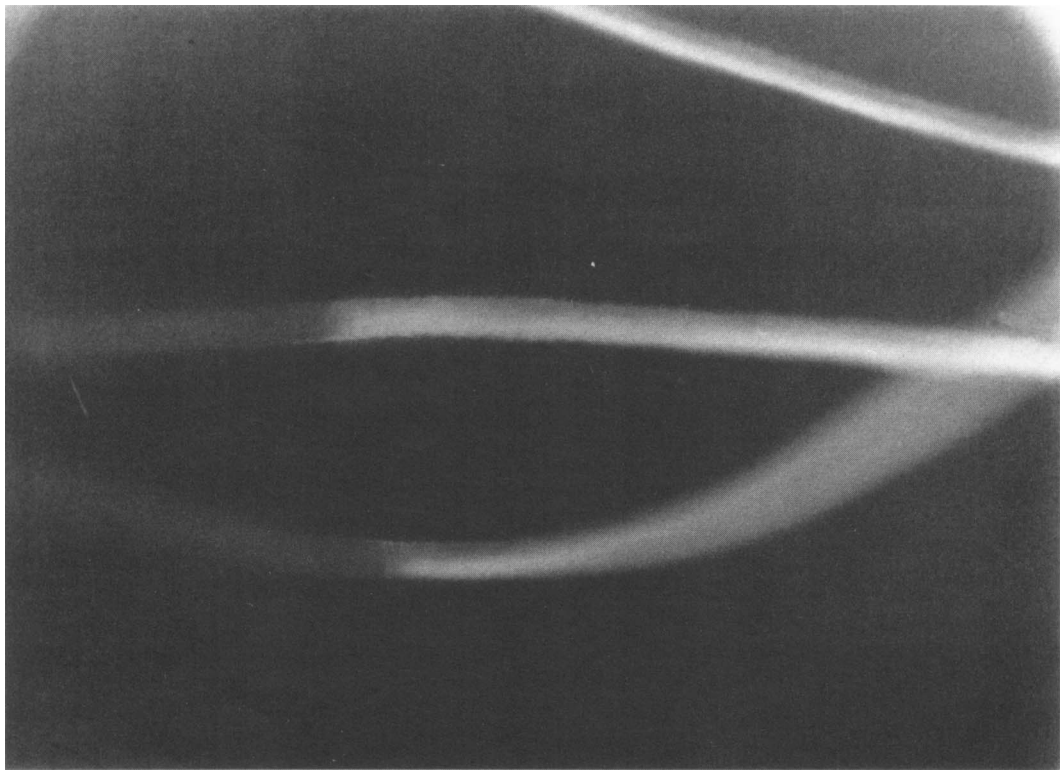


Figure 19 Wool fibres before irradiation (left hand side) and after irradiation (right hand side).

(*e.g.* 1 μs), the intensity of fluorescence measured and then the fluorescence intensity measured after the sample has remained in the dark some time, *e.g.* 2 ms. If the fluorescent species undergoes reduction to give a leuco species, oxidation of this species in the dark period will regenerate the fluorescence. In other cases recovery of the fluorescence can be due to fluorescent species migrating into the viewing area of the microscope during the dark period. Such an experiment, carried out with the tips and roots of a fibre, showed that the fluorescent species was destroyed upon irradiation and that the destruction occurred over a similar time period for tips and roots.

The origin of the fluorescence of wool has been the subject of much debate. Conventional fluorimetry has been used to show that several species are responsible for fluorescence. When the wool is excited at 300 nm most of the emission (λ_{max} 350 nm) emanates from tryptophan. Excitation with light of wavelength >300 nm generates fluorescence having maximal intensity at >350 nm; *e.g.* excitation at 375 nm generates fluorescence having a λ_{max} at 430 nm. Species which may be responsible for this long wavelength emission include carbolines and other compounds which are derived by degradation of tryptophan. It is clear from the bleach-recovery experiments that these compounds are destroyed to non-fluorescent products upon irradiation and this is clearly the origin of the photobleaching effect. The destruction process can be accomplished chemically under both oxidising and reducing conditions which suggests that there is not a unique photodestruction process. It has also been established that the fluorescence of these compounds is quenched by the disulfide bonds present in cystine and this phenomenon may contribute to the lack of fluorescence exhibited by the root of the fibre (where little photooxidation of cystine has occurred) and the much greater fluorescence intensity of the tips (where much of the cystine and tryptophan has been oxidised[26]).

The dyeing of wool usually utilises the fact that it contains free amino groups (the ε-amino groups of lysine) and to a lesser extent sulfhydryl groups (present in cysteine and can be chemically produced by reduction of cystine). The presence of the amino groups means that anionic dyes (usually containing sulfonic acid groups) and reactive dyes (which rely upon the amino group acting as a nucleophile and thereby forming a covalent bond with the dye *via* a Michael addition reaction and in other cases *via* a nucleophilic substitution reaction). Since the wool fibres are more open at the tips due to photodegradation *etc*., dyeing occurs preferentially at the tips thereby leading to uneven dyeing. This problem can be overcome by the use of levelling agents which aid migration of the dye within the fibre. In an alternative approach to obtaining level dyeing, wool has been treated with chitosan (an amino-polysaccharide).[29] It was expected that the chitosan would adhere to the surface of the wool fibres and that the presence of the amino group would lead to rapid adsorption of anionic dyes. Having obtained a high concentration of dye on the surface, the normal processes whereby the dye is taken into the fibre were expected to take over. Fluorescence microscopy was used to demonstrate that chitosan-treated wool fibres, when treated with a fluorescent whitening agent, underwent rapid dyeing at a lower temperature, and that most of the dye was located on the exterior of the fibre. By raising the temperature of the dye-bath, the dye on the surface of the fibre migrated into the interior of the fibre leading to a greater degree of level dyeing than was observed with wool that had not been treated with chitosan.

By construction of the appropriate accessory (Fig. 17) and using it with an inverted microscope, it proved possible to follow the dyeing of wool *in situ*.[19] To follow the dyeing of a fibre *in situ* it is essential that the dye-bath solution does not fluoresce (or at least is only weakly fluorescent), so that the fluorescence of the fibre can be readily detected by the dye, or if the intensity of fluorescence is being monitored, that the detection device is only seeing fluorescence from the fibre. This requirement was accommodated by the dyes **12** (Fig. 10) and *N*-(9-acridinyl)maleimide. Both dyes react with primary amines and sulfhydryl groups but selectivity can be obtained by control of pH. Nucleophilic attack upon the bromoacrylamido group present in **12** leads to loss of bromine thereby removing the internal quenching group and rendering the stilbene dye fluorescent. The maleimido group present in *N*-(9-acridinyl)maleimide quenches the fluorescence of the acridinyl group and consequently where the maleimido group is converted to a succinimido group *via* nucleophilic attack of an amino or sulfhydryl group, the fluorescence of the acridinyl group is restored. The dyeing of wool fibres by these dyes was carried out on the microscope stage and the process of the dyeing followed by recording the change in fluorescence intensity of the fibres with time. Figs. 20 and 21 show the results obtained in this way and Figs. 22 and 23 show photographs of the wool fibres before and after dyeing.

Figs. 20 and 21 demonstrate that the dye-bath solution exhibits little fluorescence, that the wool fibres exhibit an increasing amount

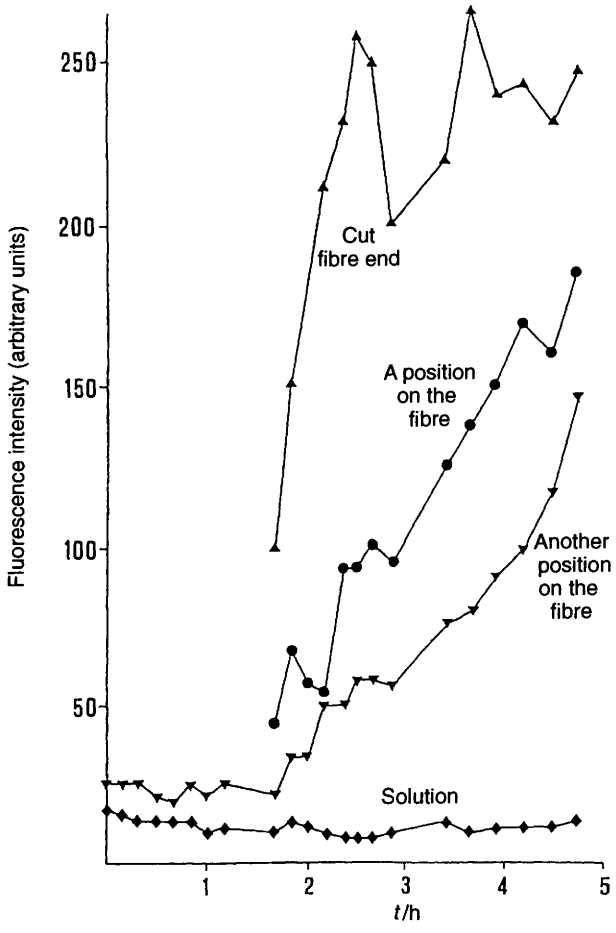


Figure 20 Change in fluorescence intensity of a fibre present in a dye-bath containing **12** as a function of time.

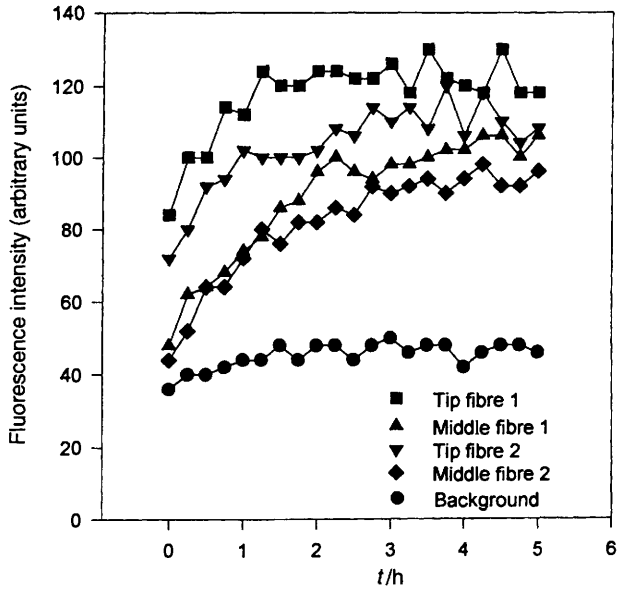


Figure 21 Change in fluorescence intensity of wool fibre present in a dye-bath solution containing *N*-(9-acridinyl) malemide, as a function of time.

of fluorescence as their immersion time in the dye-bath increases and that the cut end of the fibres takes up the dye more rapidly than the centre portion of the fibre. This latter observation can be attributed to some of the dye entering the fibre *via* the exposed centre of the fibre rather than through the cuticular layer. Such experiments pave the way for a more detailed investigation of how different parameters, *e.g.* varying the amount and consistency of dye-bath agents such as surfactants and levellers, affect the dyeing process.

In order to achieve the whiteness required by customers, fluorescent whitening agents (FWAs) are applied to wool. These colourless dyes absorb ultraviolet radiation and emit blue light thereby making up for the blue light which is absorbed by the yellow coloured species which are responsible for the wool having an off-white colour. Unfortunately, FWAs photodegrade to give yellow products *via* singlet oxygen and radical mediated reactions, and in

Figure 22 Wool fibres before dyeing.

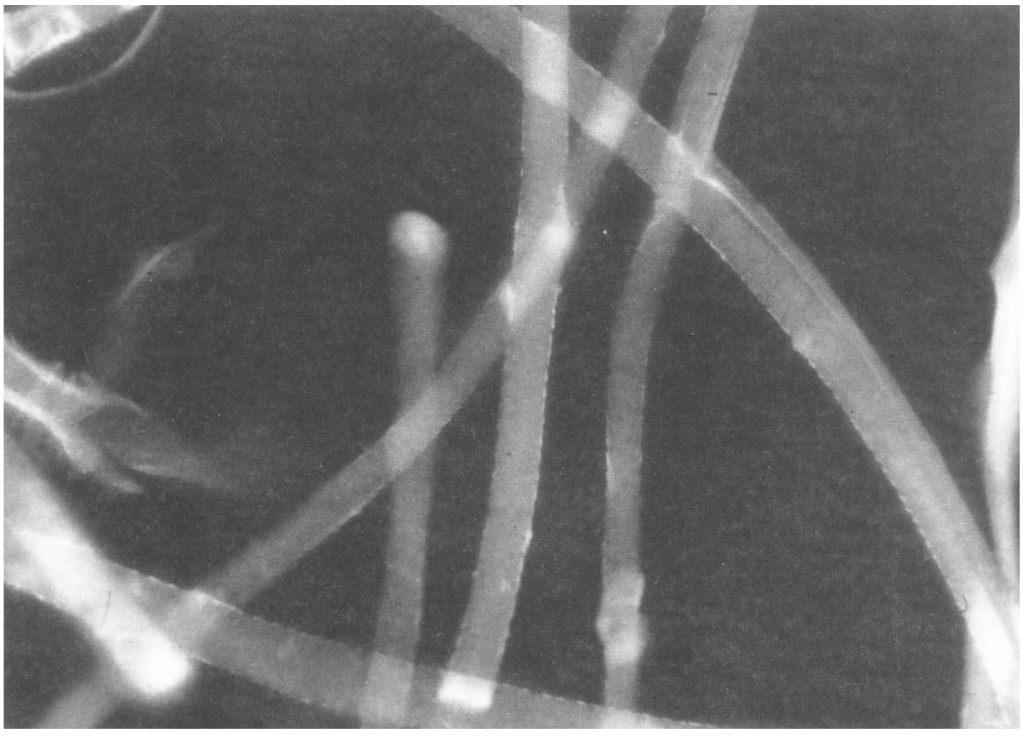


Figure 23 Wool fibres after dyeing with **12** for 6 h at 80 °C.

the process increase the rate of yellowing of the wool. This process can be readily monitored using a fluorescence microscope. Fig. 24 shows that wool fibres treated with an FWA exhibit little fluorescence after they have been exposed to light.

The degradation of FWAs on the surface of wool has been monitored in real time using fluorescence microscopy. Wool was dyed with FWAs based on the stilbene and pyrazoline chromophores and then irradiated on the stage of a fluorescence microscope with simultaneous recording of the fluorescence intensity of the sample.[30] Using this technique, the ability of additives such as Blankit D (80% formaldehyde sulfoxylate) and thiourea dioxide to arrest the degradation of the FWAs was investigated. A diagrammatic representation of one of the many results is shown in Fig. 25. It was found that the degradation of the FWAs was preceded by an induction period which was attributed to the wool protecting the FWA by reaction of its cystyl residues with any generated singlet oxygen. However, the protective action of the cystyl residues is sacrificial by nature since in the process it becomes oxidised with the final product being cysteic acid. Once the cystyl residues in the wool (particularly the cystyl-rich cuticle) have been consumed, degradation of the FWAs commences. The role of singlet oxygen is not as great for the stilbenes as it is for the pyra-

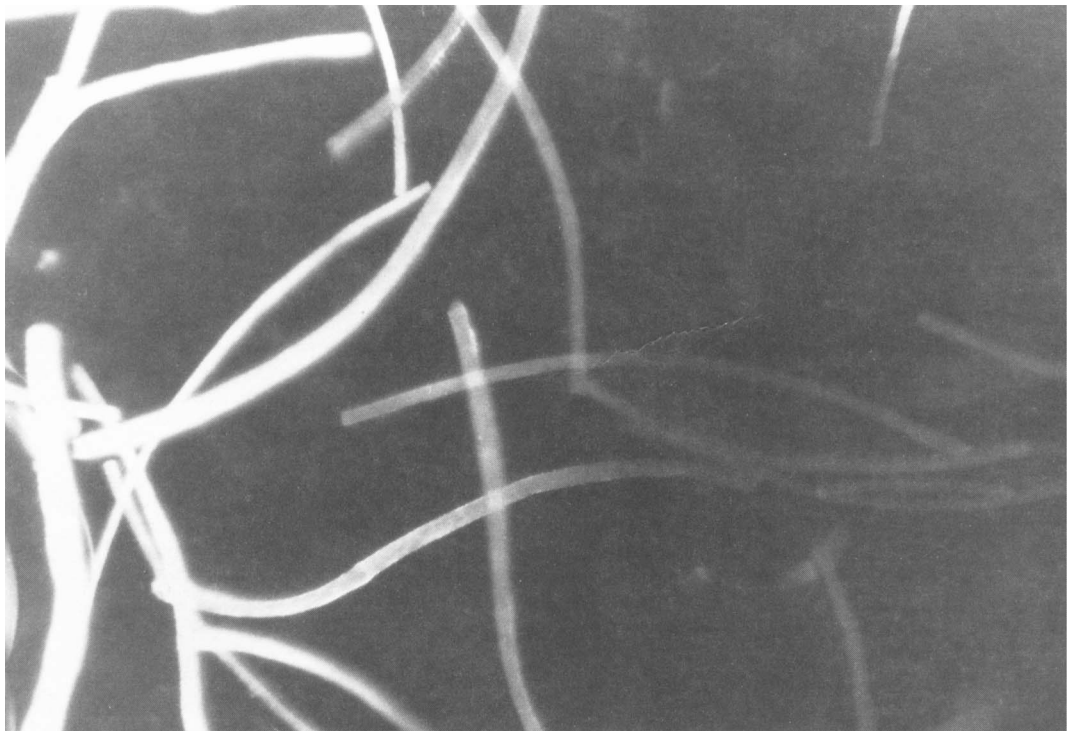


Figure 24 Wool fibres, treated with an FWA, before irradiation (left hand side) and after irradiation (right hand side)

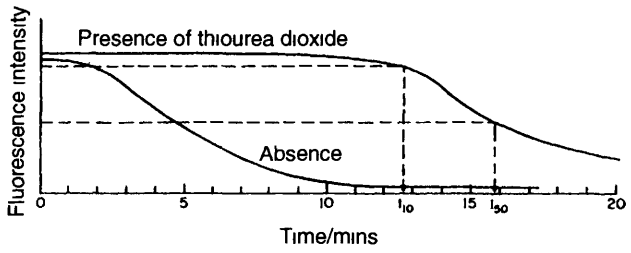


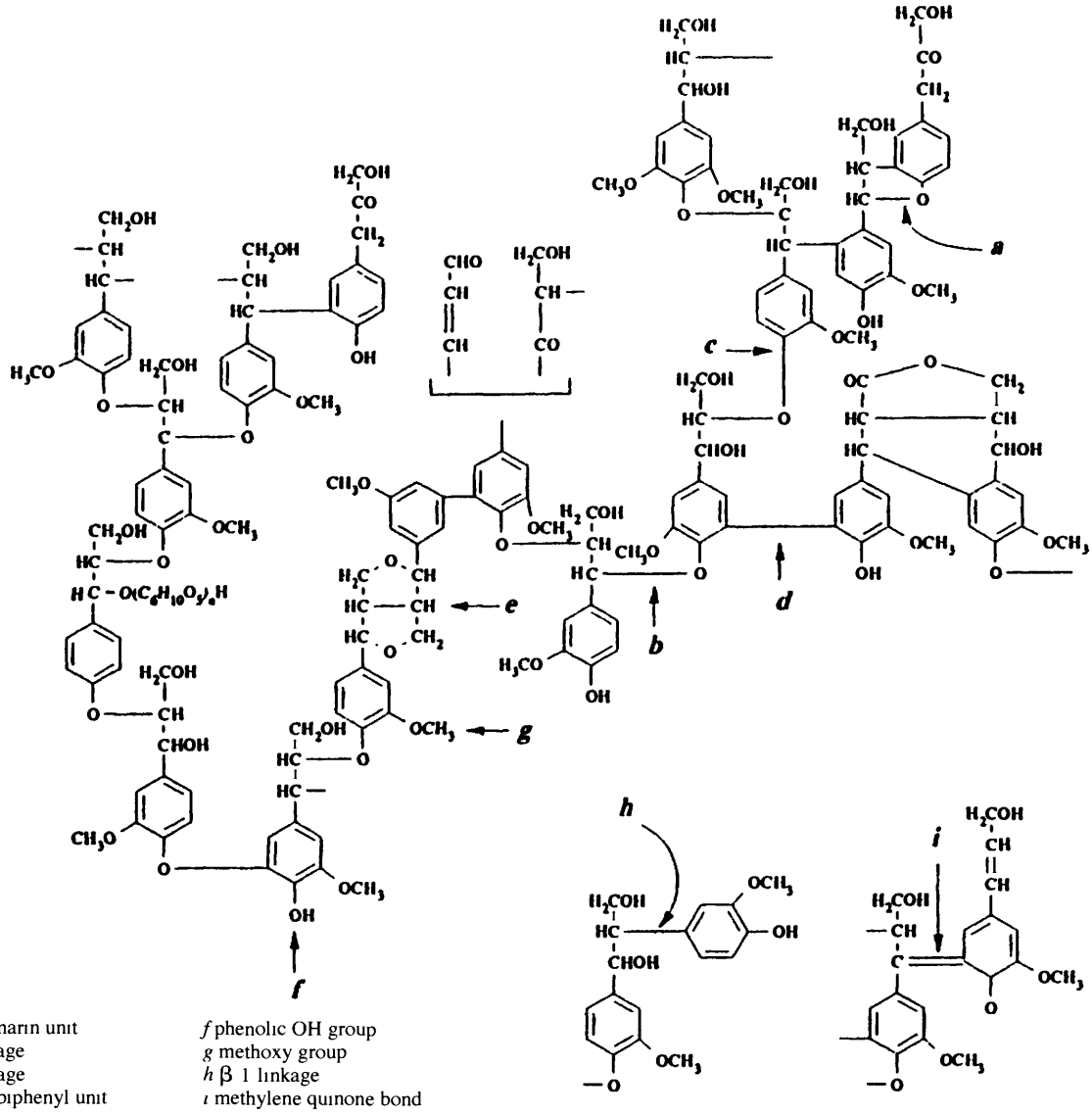
Figure 25 Degradation of an FWA as monitored by real time fluorescence spectroscopy Real time degradation of pyrazoline H treated wool in the presence and absence of a 2% solution of thiourea dioxide, where t_{10} is the time for the fluorescence intensity to decrease by 10%, t_{50} is the time for the fluorescence intensity to decrease by 50% and $t_{40}' = t_{50} - t_{10}$

zolines and there is evidence that a significant part of the degradation of the stilbenes involves a reductive interaction between the wool and the FWA

3.2 The Photobleaching and Photoyellowing of Paper Containing Lignin

The mechanical strength of plant materials is due to the laying down of a polymer, lignin, in the cell walls Lignin is derived from phenyl alanine *via* a series of enzymatically induced hydroxylation and oxidation reactions It does not have a unique structure and the chemical structure of an extracted lignin will reflect the nature of the species from which the lignin was extracted, and the season in which the lignin was laid down Despite the complexity of the material, several important structural motifs have been identified (Fig 26) and many of these contain photoactive chromophores such as the α-O-4 and β-O-4 units, quinones and phenolic residues If pulp produced from wood without extracting the lignin is converted into paper, the product has a light brown appearance Bleaching of the pulp can be used to produce papers having an acceptable whiteness but such papers readily undergo photoyellowing As with wool, the yellowing is largely produced by light wavelengths <396 nm with longer wavelength light leading to photobleaching There is abundant evidence to support the view that the lignin is to a large extent responsible for the photoyellowing Extensive detailed work has unravelled many of the processes which lead to the formation of coloured products but as yet little precise information is available as to the structure of the degradation products [31]

Lignin is fluorescent and consequently the structure of a sample of wood can be examined by fluorescence microscopy By use of the chemical reactor shown in Fig 17, the *in situ* delignification of wood using a Kraft liquor at 95–100 °C was continuously monitored The loss of lignin was readily apparent and after a 4 h treatment, some lignin still remained attached to the cellulosic



a phenylcoumarin unit
b α O 4 linkage
c β O 4 linkage
d 5 5′ bond, biphenyl unit
e pinoresinol unit
f phenolic OH group
g methoxy group
h β 1 linkage
i methylene quinone bond

Figure 26 Structure of lignin after Freudenberg (C K Freudenberg and A C Neish, *Mol Biol Biochem Biophysics*, 1968, **2**, 103)

framework The fluorescence of this lignin was different in appearance to that observed prior to the Kraft liquor treatment which would indicate that it has a different structure Whether this structure has been produced by the chemical treatment or not is not clear The presence of lignin in the wood structure can also be detected by staining with dyes such as Basocryl gold and Astrazon red Since lignin is fluorescent, it is not surprising to find that papers made from high yield pulps, *i e* pulps produced without delignifying the wood, also exhibit fluorescence [32] The wavelength distribution of the fluorescence is highly dependent upon the excitation wavelength that is used and this shows that more than one chromophore is responsible for fluorescence When such papers were subjected to bleach-recovery experiments, (using the modified fluorescence microscope) with an argon ion laser operating at 488 nm as the illumination source, it was found that the recovery was relatively slow (>2 s) This is attributed to lignin diffusing into the area where the lignin had previously been photodegraded and is an indication of the lignin being a relatively mobile species Chemical and photochemical reduction of papers made from high yield pulps increased the overall fluorescence intensity of the papers and fluorescence spectra showed that this was due principally to species emitting at 400 nm It is possible that the reduction process has transformed species which act as inner filters or quenchers of fluorescence into innocuous products, *e g* quinones into dihydroxybenzenes, coniferaldehyde into coniferyl alcohol These changes are unfortunately not permanent and upon illumination, these reductively bleached papers undergo rapid photoyellowing Fluorescence microscopy and microspectrofluorimetry have been used to study these and related processes [33] Of particular value was the use of sequential irradiation In this procedure the sample is irradiated with UV light (broad band pass filter centred at 365 nm) with continuous monitoring of fluorescence intensity for a set period and then it is irradiated with visible light (450–490 nm) with continuous monitoring of fluorescence intensity for a set period This procedure is facilitated by the microscopes having the appropriate filters fitted and therefore change of excitation and monitoring wavelengths can be readily accomplished A typical result is shown in Fig 27

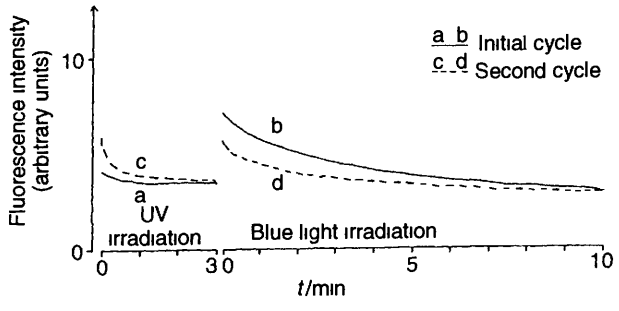


Figure 27 Changes in fluorescence intensity caused by sequential UV and visible irradiation of a paper made from a high yield pulp (SGWP)

Irradiation with UV light leads to a decrease in fluorescence intensity as does irradiation with visible light However, following irradiation with visible light, it is found that the initial intensity of the UV-stimulated fluorescence is substantially greater than that recorded at the end of the first UV excitation period Irradiation of the paper with UV light in the second cycle leads to an increase in the intensity of the fluorescence initially recorded in the second cycle of irradiation with visible light These observations are most readily accommodated by the view that lignin exhibits photochromism and that the photochromic process exhibits fatigue (Fig 28)

That the observed changes in fluorescence are due to lignin is attested by the fact that sections of wood, when subjected to the sequential irradiation procedure, exhibits the same characteristics as the paper

When either the paper or wood is subjected to oxidative or reductive bleaching, the intensity of the UV-stimulated fluorescence increases markedly Not only is this apparent when the sequential irradiation procedure is employed, but also the

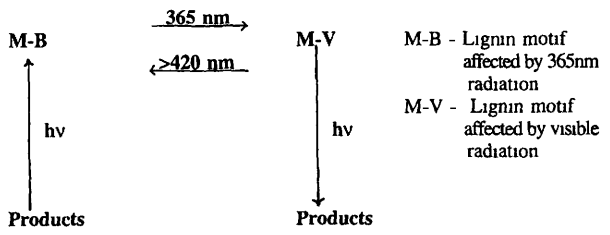


Figure 28 Photochromic behaviour exhibited by lignin

irreversible destruction of the chromophores giving rise to the UV-stimulated fluorescence Some differences were, however, observed between the behaviour of reduced wood and paper since the paper contains species which fluoresce in the visible which are not reduced by borohydride These species are thought to be stilbenes which are produced during pulping *via* a mechanochemical process The stilbenes are remarkably chemically active and represent an important seat of photochemical instability

3.3 Determination of T_g Values

The chemical characterisation of a crosslinked synthetic polymer is usually difficult since normally the material is insoluble in most solvents A property of some importance is its T_g value, *i e* the temperature at which the polymer softens, thereby allowing some molecular movement Techniques such as differential scanning calorimetry (DSC) and dynamic mechanical thermal analysis are frequently used but they have their limitations A method employing fluorescence microscopy has been developed which allows the T_g of films, including thin films, and fibrous materials to be determined The basis of the method lies in the technique of disperse dyeing which is used to dye synthetic fibres such as polyesters and polyamides In this mode of dyeing the substrate is heated in a dye-bath, the temperature of which is sufficiently high as to cause the substrate to soften The dye, which is present in the dye-bath in dispersed form, enters the softened fibre In the attachment for the inverted fluorescence microscope shown in Fig 17 a polymer film or other substrate is laid down on the top of a few dye crystals It is important that the dye used is reasonably soluble in the softened polymer and that it exhibits characteristic, easily visible fluorescence In our hands perylene and 3,7-bis(4-*n*-propoxyphenyl)benzo[1,2-*b* 4,5-*b*']difuran-2,6-dione have proved to be very suitable Using the attachment shown in Fig 17, the film is heated slowly When the T_g is reached the dye enters the polymer imparting a beautiful colour to the polymer (Figs 29 and 30) T_g values of several UV-cured films have been determined (Table 1, p 252)

To ensure that the technique was reliable the T_g of the poly(isobornyl acrylate) film was also determined by DSC and was found to be very similar In another check the T_g of a commercial sample of polystyrene was determined by both techniques with the same result Given the system of heating the sample there was some concern that the heat flow from the surface nearest the heater to the surface farthest away from the heater may be so slow that accurate T_g values would only be obtained by ramping the temperature at an incredibly slow rate To test this point the T_g of a polyester fabric was determined both as a single and triple layer and the fact that similar results were obtained suggests that heat transfer in the system is not a problem The technique has been used to show that UV-initiated polymerisation of isobornyl acrylate produces a film having a T_g of 69 °C and that if films of isobornyl acrylate containing increasing amounts of ethoxylated phenol acrylate are polymerised the T_g of the resulting films decreases as the amount of the latter is increased (Table 2, p 252)

In many UV-initiated free radical polymerisation processes the photoinitiator system consists of an aromatic ketone admixed with a tertiary amine The triplet state of the aromatic ketone reacts with the amine to give an α-aminoalkyl radical which then initiates polymerisation There has been some evidence presented which supports the view that if the amines are used at a sufficiently high

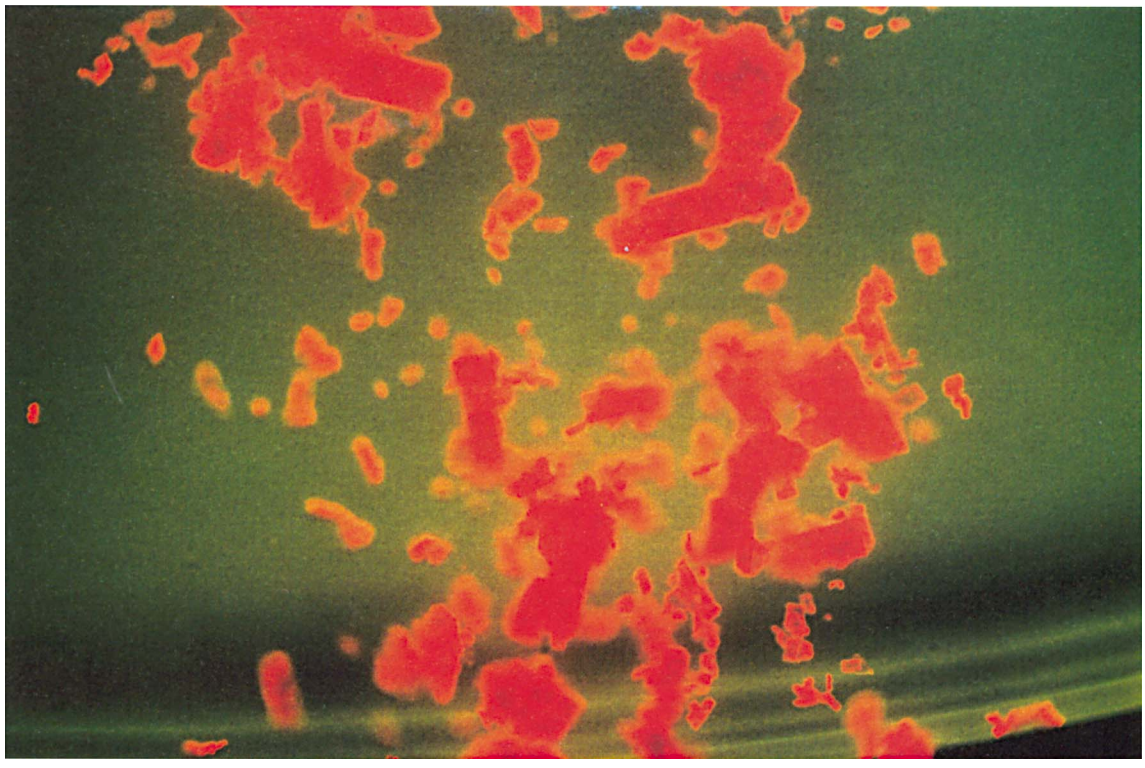


Figure 29 Polymer film together with crystals of a benzodifuranone dye before migration of the dye has occurred.

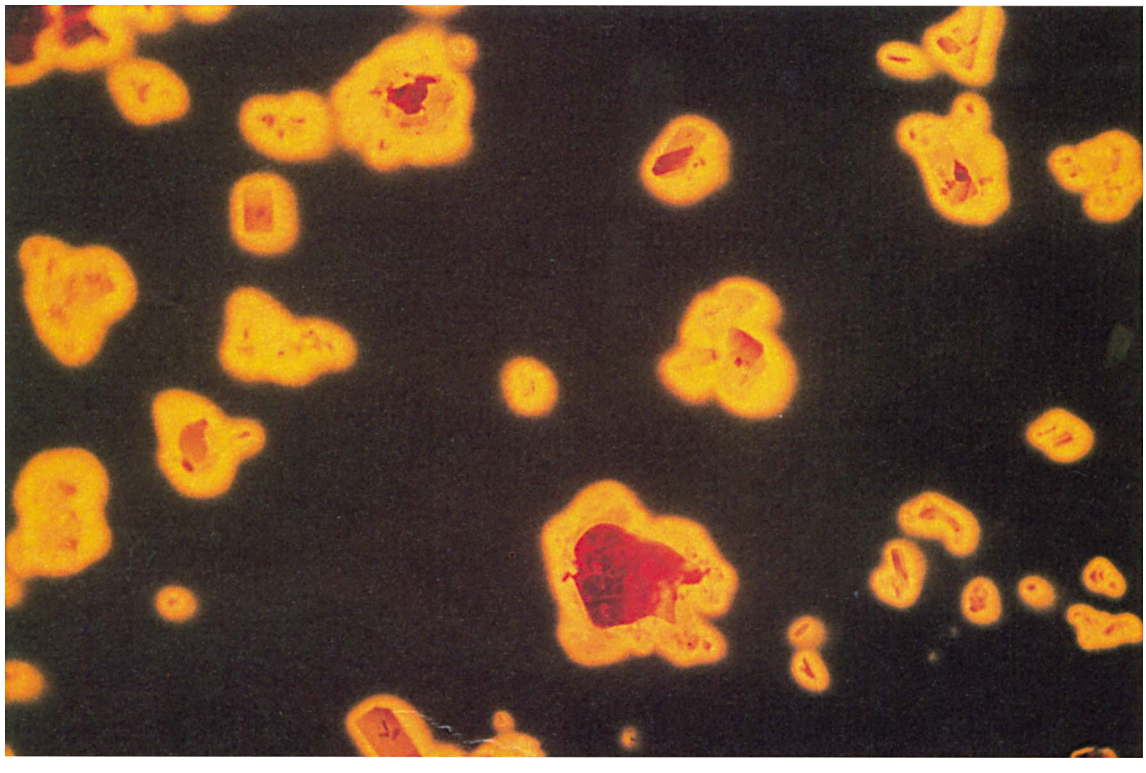


Figure 30 Polymer film together with crystals of a benzodifuranone dye showing the dye dissolving in the polymer film.

concentration they can act as plasticisers. This contention is admirably supported by the results shown in Table 3 where it can be seen that use of ethyl 4-dimethylaminobenzoate at a concentration of >2% m/m leads to a decrease in the T_g values of the cured films.

Undoubtedly the technique described will undergo further developments, *e.g.* automation, and also undergo modification so as to study the migration of fluorescent species in films and across the points of contact between one polymer and another.

Acknowledgements The work described would not have been possible without the sterling efforts and intellectual input of many of my research students, postdoctoral fellows and colleagues in industry. Similarly without the financial input from the SERC, the International Wool Foundation, the Australian Wool Corporation,

Table 1 T_g values of UV-cured films using fluorescence technique

Reactive diluents	Film formed	T_g/°C
Isobornyl acrylate	Very hard brittle film	69
Epoxidised soya bean oil	Very flexible rubbery film	44
Polyester acrylate	Flexible strong film	53
Poly(propylene glycol) monoacrylate	Very soft flexible film	Dye migrated at room temperature
Ethoxylated phenol monoacrylate	Soft flexible film	Dye migration at room temperature
Urethane oligomer/ poly(ethylene glycol 200) diacrylate 75/25	Very flexible strong film	63
Urethane oligomer/ ethoxylated phenol monoacrylate 75/25	Very flexible strong film	40
Urethane oligomer/ Tri(propylene glycol) diacrylate 75/25	Strong film slightly flexible	60
Poly(ethylene glycol 200) diacrylate	Weak flexible film	64
Poly(ethylene glycol 400) diacrylate	Soft flexible film	28
1,6-Hexanediol diacrylate	Brittle film slightly flexible	52
Tri(propylene glycol) diacrylate	Hard film slightly flexible	59

Table 2 Effect upon T_g of films produced from a mixture of isobornyl acrylate and ethoxylated phenol acrylate

Poly(isobornyl acrylate)/ethoxylated phenol acrylate	T_g/°C
100/0	69
95/5	63
90/10	58
85/15	45
80/20	34
75/25	Dye migrated at room temperature

Table 3 Effect of added amine upon the T_g of cured isobornyl acrylate films

Amine (%)	*N* Methyldiethanolamine T_g/°C	Ethyl 4-dimethylaminobenzoate T_g/°C
0	22	43
1	—	53
2	31	65
4	35	42
6	38	35
8	20	31
10	20	30

the EEC (contracts MA1B 0127 – C (EDB) and MA2B0018), the Groupemente de Recherche Papiers et Dérivés (CNRS, CTP Grenoble), Du Pont Demours, Deutschland, SmithKline Beecham Ltd and the Cancer Research Campaign it would have been impossible to carry out the work

4 References

1 C A Parker, *Photoluminescence of Solutions*, Elsevier, Amsterdam, 1968, E J Bowen, *Luminescence in Chemistry*, Van Nostrand, London, 1968
2 *The Chemistry and Application of Dyes*, ed D R Waring and G Hallas, Plenum Press, New York, 1990
3 For a description of isomerisation processes exhibited by excited states see N J Turro, *Modern Molecular Photochemistry*, Benjamin/ Cummins, Menlo Park, California, 1978
4 R S Davidson and M M Hilchenbach, *Photochem Photobiol*, 1990, **52**, 431
5 B S Hudson, D L Harris, R D Lucheser, A Ruggier, A Cooney-Freed and S A Cavalier, in *Applications of Fluorescence in the Biomedical Sciences*, ed D L Taylor, A S Waggoner, R F Murphy, F Lanni and R R Birge, A R Liss, New York, 1986, p 159
6 A Weller, *Progr React Kinet*, 1961, **1**, 187
7 G S Beddard, S Carlin and R S Davidson, *J Chem Soc, Perkin Trans 2*, 1977, 262
8 L Tanasugarn, P McNeil, G Reynolds and D L Taylor, *J Cell Biol*, 1984, **98**, 717
9 Z R Grabowski, K Rotkiewicz, A Siemarczuk, J Cowley and W Baumann, *Nouv J Chim*, 1979, **3**, 443, W Rettig, *Angew Chem, Int, Ed Engl*, 1986, **25**, 971
10 R S Davidson and J B Hobbs, in *Natural Products*, ed J Mann, R S Davidson, J B Hobbs, D V Banthorpe and J V Harborne, Longman, Harlow, 1994, ch 3, p 131
11 J C Song and D C Neckers, *American Chemical Society, Division of PMSE, Papers. 1994*, **71**, 71
12 R S Davidson, in *Molecular Association I*, ed R Foster, Academic Press, 1975, p 215
13 G S Beddard, R S Davidson and T D Whelan, *Chem Phys Lett*, 1977, **56**, 54
14 A P de Silva and H Q N Gunaratne, *J Chem Soc, Chem Commun*, 1990, 186
15 S A Jonker, F Ariese and J W Verhoeven, *Recl Trav Chim Pays-Bas*, 1989, **108**, 109, S A Jonker, K Van Dijk, K Goubitz, C A W Reiss, W Schuddeboom and J W Verhoeven, *Mol Cryst Liq Cryst*, 1990, **183**, 273
16 A P de Silva and S A de Silvo, *J Chem Soc, Chem Commun*, 1986, 1707, E U Akkaya, M E Huston and A W Czarnik, *J Am Chem Soc*, 1990, **112**, 3590
17 M Kasha, *J Chem Phys*, 1952, **20**, 71
18 R S Davidson, R Bonneau, J Joussot Dubien and K R Trethewey, *Chem Phys Lett*, 1980, **74**, 318
19 R S Davidson, G Ismail and D M Lewis, *J Soc Dyers Colourists*, 1988, **104**, 86
20 J F McKellar and N S Allen, *Photochemistry of Man-made Fibres*, Applied Science Publishers, Barking, Essex, 1979
21 J W Longworth, in *Creation and Detection of the Excited State IA*, ed A A Lamola, Marcel Dekker, New York, 1971, p 343
22 E S Cockburn, R S Davidson and J E Pratt, *J Photochem Photobiol A Chem*, 1996, **94**, 83
23 F W D Rost, in *Fluorescence Microscopy*, vol I, Cambridge University Press, Cambridge, 1992
24 R S Davidson, L Merritt and G Bradley, in *Aspects of Analysis*, Proceedings of a Conference organised by the Paint Research Association, Egham, Surrey, 1994
25 G Bradley, S Collins and R S Davidson, *Rev Sci Instr*, in the press
26 S Collins, R S Davidson, P H Greaves, M Healey and D M Lewis, *J Soc Dyers Colourists*, 1988, **104**, 348
27 S Collins, R S Davidson and M E C Hilchenbach, *Dyes and Pigments*, 1994, **24**, 151
28 K Schafer, *J Soc Dyers Colourists*, 1993, **109**, 202
29 R S Davidson and Y Xue, *J Soc Dyers Colourists*, 1994, **110**, 24
30 S Collins and R S Davidson, *J Photochem Photobiol A Chem*, 1994, **77**, 277
31 C Heitner, in *Photochemistry of Lignocellulosic Materials*, ACS Symp Ser 531, ed C Heitner and J L Scaiano, American Chemical Society, Washington DC, USA, 1993, p 2, A Castellan, *L'Actualite Chimique*, 1994, Supplement 7, 148
32 R S Davidson, L A Dunn, A Castellan and A Nourmamode, *J Photochem Photobiol A Chem*, 1991, **58**, 349

33 H Choudhury, S Collins and R S Davidson, *J Photochem Photobiol A Chem*, 1992, **69**, 109, A Castellan and R S Davidson, *J Photochem Photobiol A Chem*, 1994, **78**, 275, A Castellan, H Choudhury, R S Davidson and S Grelier, *J Photochem Photobiol A Chem*, 1994, **81**, 117, A Castellan, H Choudhury, R S Davidson and S Grelier, *J Photochem Photobiol A Chem*, 1994, **81**, 123