Near Infrared Fluorescence Spectroscopy

by

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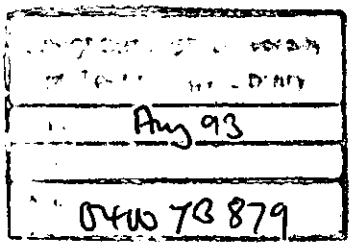

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In loving memory of my Father
and my Grandmother.

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Abstract

Fluorimetry in the very near infrared region ca. 600-1000nm is a new approach to photochemical analysis. The advantages include greatly reduced background fluorescence signals from important sample matrices (such as blood serum), reduced scattering, and reduced probability of sample decomposition. Also, the availability of low cost, efficient, stable and robust optical components (e.g. laser diodes and light emitting diodes), solid state detectors (e.g. single silicon photodiodes and diode arrays) and fibre optics, allows the construction of an inexpensive fluorimeter. In the near infrared region, there are some very bright fluorophores that can be adapted for use as fluorescent probes, labels for immunoassay, and as ion-pair agents.

The advantageous performance of most types of fluorimetric analysis now undertaken in the ultraviolet and visible region of the spectrum may therefore be extended into the longer wavelength region. Excellent limits of detection are attainable, and some near infrared fluorophores show invaluable fluorescence probe properties, such as Nile Red.

The most useful of the dye groups investigated were the phenoxazines and thiazines. Reactive derivatives of these dyes show great potential as fluorescent labels for immunoassay. These dyes have also been used as probes due to their solvatochromism and sensitivity to pH.

Glossary

A	absorptivity or Arrhenius constant
Abs.	absorbance
AC	alternating current
aq.	aqueous
AR	analytical reagent
b	path length in centimetres
BSA	bovine serum albumin
bp	boiling point
c	concentration (mol/l) or speed of light
ca.	circa (approximately)
CAS #	Chemical Abstracts Number (xxxxxx-xx-x)
CI	Colour Index Generic Name
CI No.	Colour Index Number (CI-xxxxx)
cm	centimetre(s)
cm^{-1}	wavenumbers
COT	cyclooctatetraene
DC	direct current
(dec)	decomposes (°C)
dil.	dilute
DMF	dimethylformamide
DMSO	dimethylsulphoxide
DODC	3,3'-diethyloxadicarbocyanine
DOTC	3,3'-diethyloxatricarbocyanine
DTTC	3,3'-diethyloxathiatricarbocyanine
°C	degrees Celsius
CW	continuous wave
E_A	activation energy
EGME	2-methoxyethanol: methyl cellusolve
Em	fluorescence (emission) maximum
ET(30)	Dimroth-Reichardt polarity scale
E_T^N	Normalised polarity scale
Ex	Excitation maximum
F	fluorescence intensity
F_o	initial fluorescence intensity
FT-IR	fourier transform infra-red
g	gram
GPR	general purpose reagent
h	Planck's constant (6.62×10^{-34} Js)
hv	light
HITC	1,1',3,3,3'3'-hexamethylindotricarbocyanine
H_2O	water
HPLC	high performance chromatography
hr.	hour
H_2SO_4	concentrated sulphuric acid
Hz	Hertz
I	insoluble
I	intensity of transmitted light
I_o	intensity of incident light
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
I.R.	infra-red
IR125	Indocyanine Green
J	Joules
K	Kelvin
kcal	kilocalories
kg	kilograms
kJ	kilojoules
l	litre
LED	light emitting diode
LOD	limit of detection
m	metre(s)
M	monomer or molarity (mol dm^{-3})
$^1M^*$	monomer in the singlet excited state
$^3M^*$	monomer in the triplet excited state
max.	maximum
mg	milligram(s)
MHz	megaherz
min.	minimum or minute
ml	millilitre(s)
mm	millimetre(s)
mol	mole
mol wt	molecular weight
N_o	Avogadro constant 6.022×10^{23} mol^{-1}
ND	not determined
ng	nanogram(s)
nmolar	nanomolar (10^{-9} molar)
nm	nanometre(s)
NMR	nuclear magnetic resonance

p.	page,
P	polarisation
pp.	pages
PC	personal computer
PDA	photo-diode array
Ph	phenyl or phosphorescence
pH	pH in aqueous media
pH(R)	pH meter reading
pH*	pH in non-aqueous medium (i.e. 50% (v/v) methanol/water)
pmolar	picomolar (10^{-12} molar)
PMT	photomultiplier
ppt.	precipitate
pptd.	precipitated
r	correlation coefficient
R	gas constant (8.314 $JK^{-1}mol^{-1}$)
RF	the ratio of the distance by a particular compound relative to the distance moved by the solvent front.
rpm	revolutions per minute
Q	quencher
$^1Q^*$	quencher in singlet excited state
$^3Q^*$	quencher in triplet excited state
s	soluble (around 1%) or second(s)
S_1	first excited singlet state
satd.	saturated
SLR	special laboratory reagent
soln.	solution
ss	slightly soluble (under 1%)
T	transmission or temperature (K)
t½	dye stability half life
T_1	lowest triplet state
THF	tetrahydrofuran
TLC	thin layer chromatography
TMS	tetramethylsilane
u.v.	ultra violet
v^1	first vibrational level
vs	very soluble (over 10%)
vss	very slightly soluble
v/v	volume for volume
w/v	weight for volume
w/w	weight for weight

α	photodecomposition decay constant (absorbance)
α^*	reference photodecomposition decay constant (absorbance)
α^*/α	photostability ratio (absorbance)
β	photodecomposition decay constant (fluorescence)
β^*	reference photodecomposition decay constant (fluorescence)
β^*/β	photostability ratio (fluorescence)
δ	correction factor to correct pH(R) to pH* (e.g. δ=0.11 for 50% (v/v) methanol/water mixture)
ε	molar absorptivity (l mol^{-1} cm^{-1})
ν	frequency (Hz)
λ	wavelength
µl	microlitre(s) (10^{-3} ml)
µm	micrometre(s) (10^{-6} m
µmolar	micromolar (10^{-6} molar)
τf	fluorescence lifetime (ns)
τp	phosphorescence lifetime (ms)
ϕf	fluorescence quantum yield
ϕp	phosphorescence quantum yield
?*	excited state

Aims of the Project

The aim of the project was to investigate the scope for fluorimetry in the near infrared region ca. 600-1000 nm. Work in fluorescence has traditionally been limited to 200-600 nm region due mainly to instrumental limitations. This should be overcome by the introduction of low cost, efficient, stable and robust optical components (i.e. laser diodes and light emitting diodes), solid state detectors (i.e. single silicon photodiodes and diode arrays) and fibre optics, that could allow the construction of a simple fluorimeter. The following questions laid the basis of the thesis:-

1. Could the excellent sensitivity and selectivity of fluorimetric analysis in the ultraviolet and visible region be extended and improved by working in the near infrared region?

2. Could near infrared fluorimeters be constructed? If so, could laser diodes and silicon photodiodes compete for sensitivity with conventional fluorescence instruments with xenon arc lamp light sources and photomultiplier detectors?

3. How useful are the theoretical advantages of greatly reduced background fluorescence, reduced scattering, and reduced probability of sample decomposition?

4. What dyes and dye groups are fluorescent in the near infrared region and what are their chemical, physical and photostability.

5. Do dyes in this region have the properties required to be probes and be used as ion pair reagents?

6. Are there any commercially available near infrared fluorophores with reactive groups that can be used for biochemical analysis?

7. If there are not, could a near infrared fluorophore be derivatised with a reactive group? and hence be used as a fluorescent label for immunoassay.

The above questions are addressed in the chapters of the thesis as follows:-

Chapter 1

The theory of fluorescence and the potential advantages of near infrared fluorescence are described.

Chapter 2

Materials and methods used in the project.

Chapter 3

Investigation of the chemical, physical, and photochemical properties of near infrared fluorescent dyes and dye groups. Evaluation of the dyes and dye groups are worth further investigation.

Chapter 4

Initial investigations prior to the project suggested that there were not any near infrared reactive dyes (labels) that would be able to link to proteins. This chapter investigates the modification of dyes evaluated in chapter 3 for biochemical analysis

Chapter 5

The probe properties of near infrared dyes were investigated to prove that dyes in this region could be used. Reactive dyes described in Chapter 4 were used in a typical immunoassay protocol to assess the potential of near infrared dyes with reactive groups as labels for immunoassay.

Chapter 6

This chapter gives an overview of the components that could be used to construct a fluorimeter for the near infrared region. Initial results using some components, such as a laser diode, a tungsten halogen lamp, a silicon photodiode array instrument and a silicon avalanche photodiode are shown. From this work, various suggested designs for fluorescence instruments are elucidated.

Chapter 7

This chapter reviews the initial exploration, scope and problems associated with near infrared fluorescence.

Chapter 1

1.0 Luminescence

Luminescence may be defined as emitted light from a substance other than black body radiation. Absorption of energy must take place before emission of a photon from the excited state of the molecule or atom. The form of excitation energy defines the type of luminescence.

Photoluminescence occurs as a result of a molecule being excited by a photon of electromagnetic radiation. *Fluorescence* is the almost immediate release of a photon from the singlet excited state. *Phosphorescence* is a delayed release of energy from the triplet state. Whereas, *delayed fluorescence* is the emission of a photon from the singlet excited state resulting from two intersystem crossings, first from the singlet to the triplet state, then from the triplet to the singlet.

The excitation energy of *chemiluminescence* is from the chemical energy of a reaction. *Electrochemiluminescence* is a type of chemiluminescence in solution when the excited state is produced by high energy electron transfer reactions caused by a high voltage. In *bioluminescence,* the excitation energy is supplied by enzyme catalysed reaction.

Triboluminescence (tribo is Greek to rub) is produced as a release of energy when certain crystals, such as sugar, are broken.

Cathodoluminescence results from the release of energy produced by exposure of a substance to cathode rays. *Anodoluminescence* arises from the irradiation by alpha particles.

Electroluminescence the luminescence from electrical discharges.

Thermoluminescence occurs when a material existing in high vibrational energy levels emits at a temperature below red heat, after being exposed to small amounts of thermal energy.

1.1 Fluorescence Principles

Fluorescence is the most common choice for quantitative trace analysis. The range of samples that can be studied by fluorescence is very large; organic, inorganic, synthetic and naturally occurring, small and large molecules. Samples may be dilute and concentrated solutions, gases, suspensions, or solid surfaces. The application may be either analytical, involve the study of molecular structure and interactions, or the location of a species. Fluorescence has also often been combined with separation techniques, for example high pressure chromatography [hplc] (Rhys Williams, 1984), thin layer chromatography [TLC] (Rhys Williams, 1984), and electrophoresis (Cheng et al., 1990).

1.1.1 Excitation

When light of wavelength between 200 and 1000 nm is used to irradiate a sample, a number of phenomena may occur: Most of the the photons pass straight through the sample and some are absorbed. Some of the photons are scattered by their collisions with other bodies.

The two scattering phenomena are:

Raman scattering involves a constant vibrational energy being added or subtracted from the incident photon and hence a subsequent shift in wavelength (Table 1.1). Raman scattering is sometimes confused with fluorescence. The intensity of Raman scattering is usually feeble compared with Rayleigh scattering. All solvents containing hydrogen atoms linked to either carbon or oxygen show a Raman band shifted approximately 3000cm^{-1} from the excitation radiation.

Table 1.1: Raman scattering in various solvents at 313, 366, 405 and 436 nm.

Solvents	Excitation Wavelength (nm)			
	313	366	405	436
water	350	418	469	511
acetonitrile	340	406	457	504
cyclohexane	344	409	458	499
chloroform	346	411	461	502

Rayleigh-Tyndall scattering occurs at the same wavelength as excitation and is due to elastic collisions. Rayleigh scattering is caused by solvent molecules and Tyndall scattering is caused by small suspended particles in solution.

Absorption occurs when a photon impinges on the molecule and is absorbed, an electronic transition takes place to a higher electronic state in 10^{-15} s (Figure 1.1). In a singlet state (S), each orbital electron is paired with another electron with spin opposite. When the molecule absorbs radiation the electron is raised to an upper excited state.

1.1.2 Energy loss pathways

Excited states loose energy by one or more of a number of pathways (Figure 1.1).

Vibrational relaxation and *Internal conversion* occurs within about a picosecond and brings the molecule to the lowest vibrational level ($v^1 = 0$) in the first excited singlet electronic level, S_1.

Fluorescence is the radiative transition from the lowest vibrational level of the singlet excited state (S_1) to to the ground state (S_0) in about a nanosecond. The emitted photon has lower energy than ~~than~~ incident radiation and so has a longer wavelength.

A molecule in S_1 may undergo *intersystem crossing* (ISC) to the lowest triplet state (T_1). The subsequent radiative transition from T_1 to S_0 (ground state) is *phosphorescence*. This is quantum mechanically forbidden, consequently the transition time is 100 microseconds to 100 seconds. This phenomenon is generally observed at 77 Kelvin. At room temperature, it will only normally be observed on a solid surface, or when the molecule is protected from collisional quenching by being enclosed in a micelle or by cyclodextrins.

Other mechanisms by which a molecule may lose its excitation energy include *photochemical decomposition* (the destruction of the sample by incident radiation) and by quenching (Section 1.4).

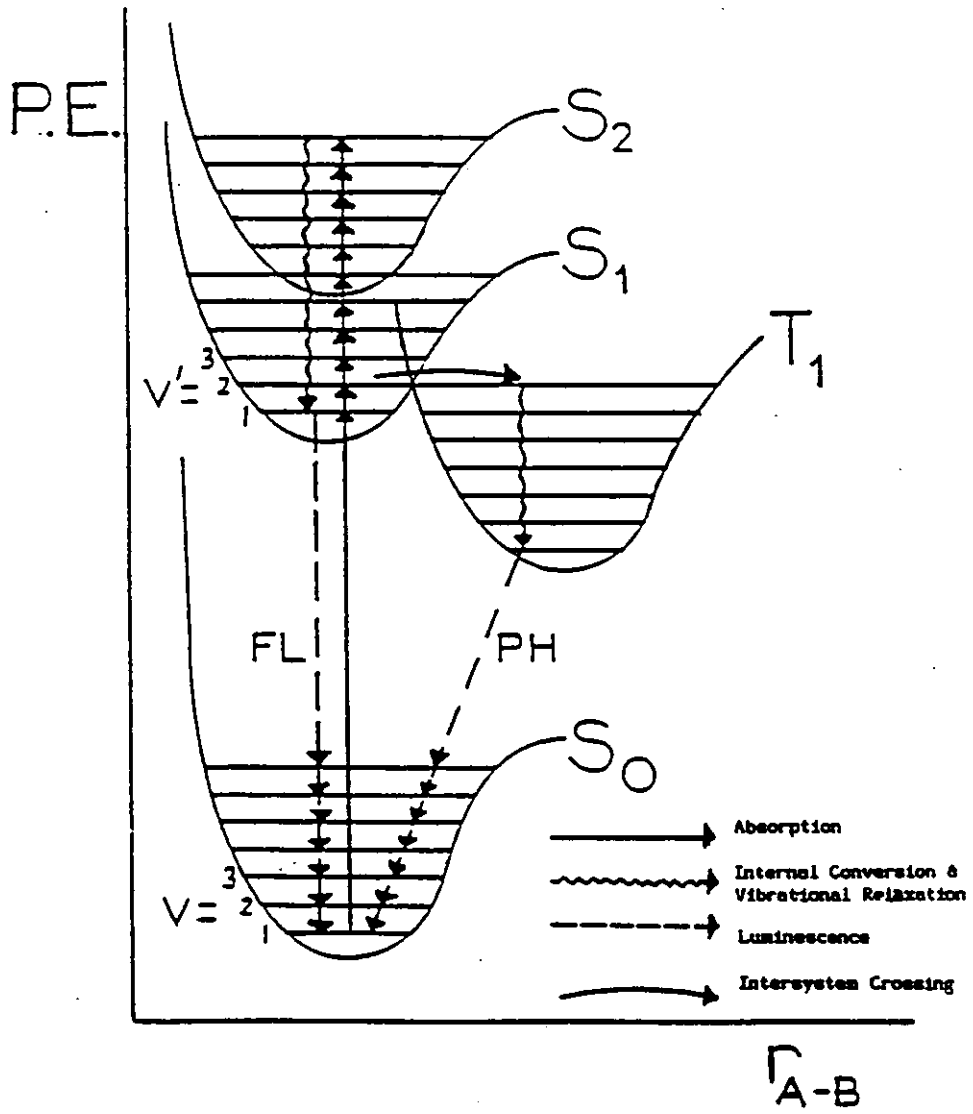


Figure 1.1: Potential energy diagram for a diatomic molecule.

1.2 Characteristics of Fluorescence

Fluorescence is more sensitive than absorption methods because the signal is measured directly against a very small background and is proportional to the intensity of the incident radiation for dilute solutions. In solution, picogram per millilitre levels can often be determined. This is two to three order of magnitude better than absorption methods, where the sensitivity is limited by the necessity of detecting a very small fractional decrease in the light transmitted by the solution.

Fluorescence methods possess greater selectivity than spectrophotometric methods, because there is a choice of wavelength not only for the radiation emitted but also for the light which it excites it. This allows the possibility of determining simultaneously two constituents that emit at the same wavelength but have respective excitation wavelengths separated sufficiently. Also fluorescence lifetime and fluorescence depolarisation can be used to differentiate between analytes in a sample. Furthermore, not all compounds that absorb light fluoresce so there is less interference and this makes sample preparation less stringent than that required for absorption measurements. The careful selection of parameters means that physical separation of complicated mixtures can be eliminated. The most commonly used is a change in pH. Closely related substances in the same solution without separation can be determined by shifting the pH as well as the excitation and emission wavelengths. This is used in cytochemistry (Haugmann, 1992), and homogeneous immunoassay (Hemmilla, 1985).

The factors affecting fluorescence can be determined assuming a number of assumptions. Equation 1.1 defines the fluorescence and concentration relationship.

$$F = \phi_f I_0 (1 - e^{-\varepsilon bc}) \qquad (1.1)$$

Where F is the fluorescence intensity, ϕ_f is the fluorescence quantum efficiency (Section 1.2.4), I_0 is incident power radiation, b is the path length of cuvette in centimetres, c is the analyte concentration in mol dm^{-3} and ε is molar absorptivity in mol^{-1} cm^{-1} dm^3. This indicates that an increase in either quantum efficiency, incident radiation, path length or molar absorptivity increases fluorescence.

Equation 1.2 may be reduced to an equation comparable to Beer's law if the absorptivity is below 0.05.

$$F = (\phi_f \times I_0 \times \varepsilon bc) \times \text{modular factors} \qquad (1.2)$$

The modular factors are constant for a particular instrument and these include the responsivity of the detector, the efficiency of the monochromator and so on.

Equation 1.3 can be reduced to:

$$F = K \times c \qquad (1.3)$$

K is a constant that includes all the other terms. This is only valid at low analyte concentrations. At higher concentrations the inner filter effect causes the deviation from linearity.

1.2.1 Luminescent lifetime

Luminescent lifetime (τ) is the mean lifetime of the excited state and varies from 1-20 nanoseconds for fluorescence (τ_f) and 0.001-10 seconds for phosphorescence (τ_p). The fluorescent lifetime is defined by the following.

$$F = F_o e^{-t/\tau_f} \qquad (1.4)$$

Where t is the time after removing the excitation source and F_o is the maximum fluorescent intensity during excitation.

1.2.2 Spectra

The *excitation spectrum* is independent of fluorescence wavelength and should be identical to that of the absorption spectrum. This is seldom the case due to instrument artifacts.

The *emission* or *fluorescence spectrum* results from the re-emission of radiation absorbed by the molecule. The quantum efficiency and the shape of the emission spectrum are independent of excitation. If the exciting radiation is at a wavelength different from the absorption maxima, less radiant energy will be absorbed and hence less will be emitted. Each absorption band to the first electronic state will have corresponding emission spectra that are approximately mirror images of each other.

1.2.3 Stokes shift

This is the wavelength difference between the excitation and emission maxima. It indicates the energy dissipated by non-radiative processes during the excited state lifetime before its return to the ground state.

1.2.4 Fluorescence quantum efficiency

The fluorescence quantum efficiency (ϕ_f) is the ratio of the total energy emitted per quantum of energy absorbed.

$$\phi_f = \frac{\text{number of quanta emitted}}{\text{number of quanta absorbed}} \tag{1.5}$$

1.2.5 Fluorescence polarisation

Fluorophores preferentially absorb photons whose electric vectors are aligned parallel to the transition moment of the fluorophore. The transition moment has a defined orientation in the fluorophore. In solution, fluorophore molecules are randomly orientated. Upon excitation with polarised light, the molecules whose absorption transition dipole is parallel to the electric vector of the excitation are selectively excited resulting in polarised fluorescence emission. The transition moments for absorption and emission within each fluorophore and the relative angle between these moments determines the maximum measured anisotropy. The fluorescence anisotropy (r) and polarisation (P) are defined by:

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \tag{1.6}$$

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \tag{1.7}$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities of the vertically ($_{\parallel}$) and horizontally ($_{\perp}$) polarised emission, when the sample is excited with vertically polarised light. Anisotropy and polarisation are both expressions for the same phenomenon.

1.3 Conventional Fluorescence Instrumentation

A fluorimeter (Figure 1.2) consists of a light source (usually a xenon arc lamp) excitation dispersing element, sample area containing either a cuvette or flow cell, an emission dispersing element, a photodetector (usually a photomultiplier [PMT]) and a data read-out device. The fluorescence from the sample is collected at right angles to the excitation light.

1.3.1 Filter fluorimeter

Filter instruments are inexpensive, very sensitive and simple in design. The dispersing elements are both filters. The primary (excitation) filter has a narrow bandpass with peak transmittance at the absorption maxima of the sample. The secondary (emission) filter is normally a "cut-off" filters whose short wavelength transmission does not significantly overlap the long wavelength transmission of the primary filter in order to prevent scattered radiation reaching the photodetector. Filters are more efficient than grating monochromator.

1.3.2 Spectrofluorimeter

Both dispersing elements of spectrofluorimeters are grating monochromators. The excitation grating is normally blazed at 250 to 300 nanometres and the emission grating at 350 to 500 nanometres. The bandpass and light intensity striking the sample or detector is determined by the slit width. Grating spectrofluorimeters are far more versatile than filter instruments due to their ability to scan spectra and select discrete wavelengths.

The effectiveness with which the scattered exciting radiation is excluded from the luminescent radiation by an instrument largely determines the signal to noise ratio and hence limit of detection. Thus, holographic ruled gratings with filters between the emission monochromator and photodetector reduce drastically scatter and second order spectra. Modulation of light source and a lock-in amplifier provided in many instruments, such as the Perkin Elmer (Beaconsfield, Buckinghamshire, UK) LS-50 Spectrofluorimeter, will extend the limit of detection.

1.3.3 Combination spectrofluorimeter

These have an excitation filter and an emission monochromator, for example the Perkin Elmer (Beaconsfield, Buckinghamshire, UK) LS-2B Spectrofluorimeter.

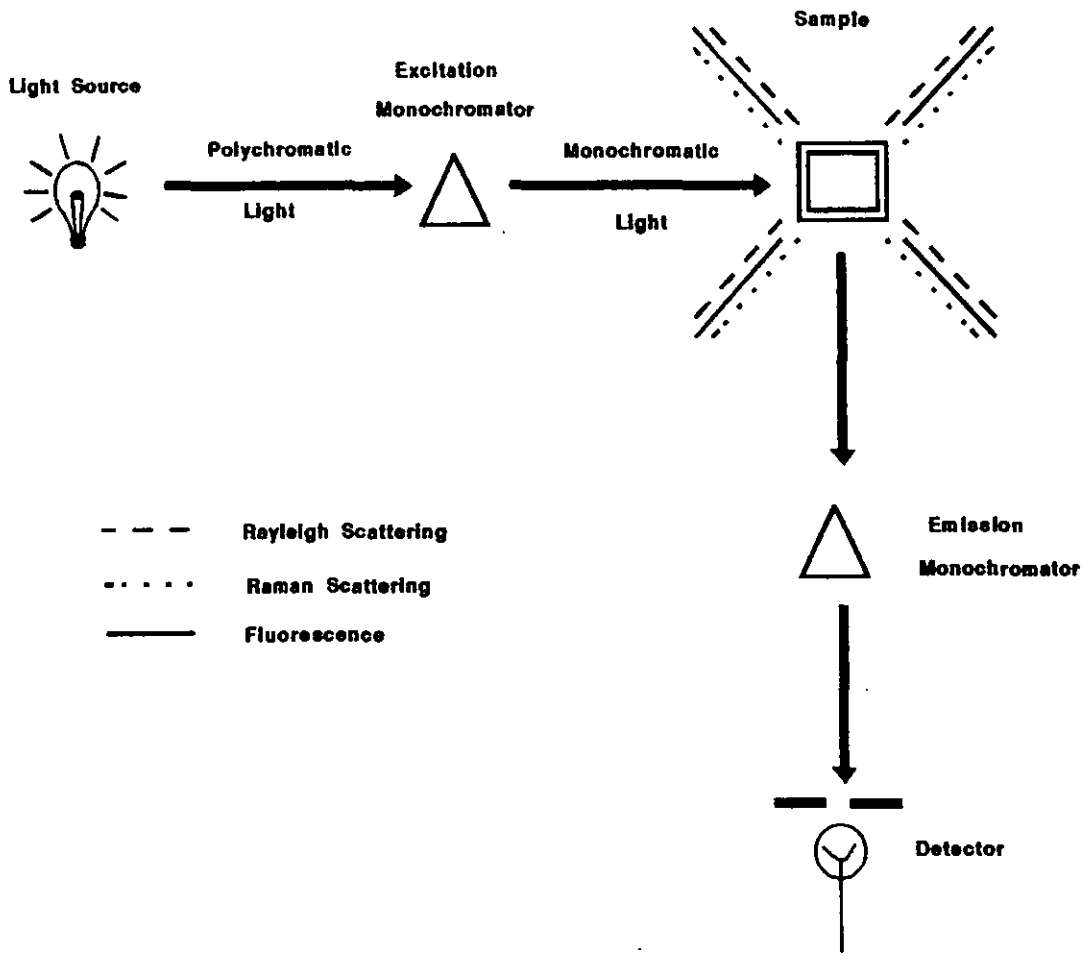


Figure 1.2: A schematic diagram of a spectrofluorimeter.

1.4 Fluorescence Quenching

Fluorescence quenching refers to any process which decreases the fluorescence intensity of a given substance. Quenching may be either by a *photophysical* or *photochemical* pathway. Photochemical reaction causes photodegradation or photobleaching producing degradation products (see Chapter 3.7). Photophysical quenching produces new ground state products and can be divided into *concentration quenching* (the quenching species is itself "M"), and *impurity quenching* (another chemical species "Q"). Figure 1.3 summarises the various quenching processes.

1.4.1 Concentration quenching

Inner filter effect is the re-adsorption of light emitted from the fluorophore and is the major cause for the departure from the linear relationship of fluorescence intensity and concentration.

Excimer (EXCIted state diMER): Many aromatic organic dyes form dimers and higher aggregates in water, and these have normally have a strong absorption band at shorter wavelength and a emission band at longer wavelength caused by the association of the ground state and excited state of the same species, the *excimer* $^1(MM)^*$.

$$M \overset{h\nu}{\rightleftharpoons} {}^1M^* \overset{M}{\rightleftharpoons} {}^1(MM)^* \longrightarrow M + M + h\nu' \qquad (1.8)$$

The dimerisation of Rhodamine B and Rhodamine 6G is severe enough to prevent fluorescence. Dimerisation increases with increasing dye concentration and decreasing temperature. Dimers form due to the hydrophobicity of the dye molecules. Dimerisation of rhodamines increase with the number and size of alkyl substituents (Drexhage, 1973b). Aggregation does not usually occur in organic solvents even at very high concentration.

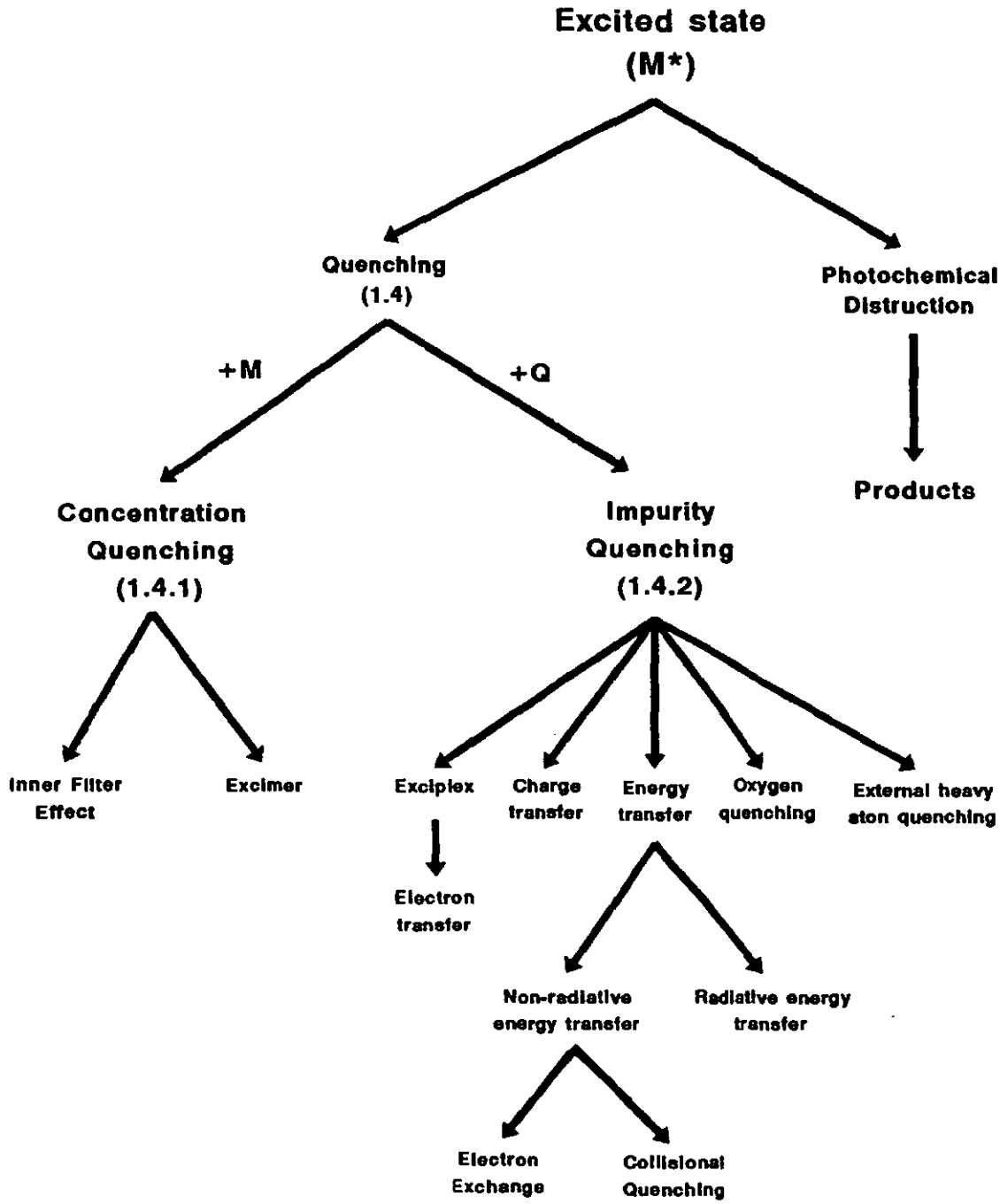


Figure 1.3: Quenching processes of the excited state (M*). M is another fluorophore molecule either in excited or ground state. Q is a quencher.

1.4.2 Impurity quenching

This involves another chemical species (Q) as the quencher.

An *exciplex* (EXCIted state comPLEX), $^1(MQ)^*$, is formed by the association of one excited ($^1M^*$) and one ground state species (Q). This occurs in solutions of mixed solutes, for example, the addition of diethylaniline to a solution of anthracene in toluene quenches the anthracene fluorescence and replaces it by emission at longer wavelength (Weller, 1967).

$$M \xrightarrow{h\nu} {}^1M^* \xrightarrow{Q} {}^1(MQ)^* \qquad (1.9)$$

Electron transfer occurs between the quenched species (M) and the polar solvent (the quencher: Q) that form the exciplex, $^1(MQ)^*$. For example, the fluorescence of amine substituted aromatics in highly polar solvents (such as acetonitrile) is bathochromically shifted and quenched. The complete quenching of the exciplex is caused by the complete dissociation of the exciplex into solvated radical ions, $M\cdot_s^-$ and $Q\cdot_s^+$, by complete electron transfer.

$$^1(MQ)^* \longrightarrow M\cdot_s^- + Q\cdot_s^+ \qquad (1.10)$$

In non-polar solvents electron transfer is incomplete so the exciplex relaxes by fluorescence or radiationless return to regenerate monomer and quencher in their ground state.

Charge transfer takes place between the excited state species and the counter-ion. The tendency of charge transfer quenching of anions decreases as follows.

$$I^- > SCN^- > Br^- > Cl^- > ClO_4^-$$

The effect of the anion on fluorescence depends upon the concentration and polarity of the solvent. For example Rhodamine 6G iodide and perchlorate in ethanol have identical fluorescence (Drexhage, 1973a,b), but the fluorescence of Rhodamine 6G iodide is completely quenched in non-polar solvents (such as diethyl ether). In polar solvents (e.g. ethanol), the quenching anions are completely dissociated so the anion does not have sufficient time to reach the excited species during the fluorescent lifetime. Whereas in non-polar solvents (e.g. chloroform and hexane), they are not dissociated, so the anions are readily available for charge

transfer.

Energy transfer: The excited donor molecule M* collapses to the ground state (M) with the simultaneous energy transfer to the acceptor molecule (Q), which is promoted to an excited state (Q*).

$$M^* + Q \longrightarrow M + Q^* \qquad (1.11)$$

The acceptor may also be in an excited state:

a. *Radiative energy transfer* is the capture by the acceptor (^0Q) of photons emitted by the donor (M*). The acceptor is promoted to the excited state (Q*) and the donor falls to the ground state (^0M).

$$M^* \longrightarrow {}^0M + h\nu \qquad (1.12)$$
$$h\nu + Q \longrightarrow Q^* \qquad (1.13)$$

The rate of radiative energy transfer depends on the number of acceptor molecules in the path of the emitted photon, the quantum efficiency of the donor, the ability of the acceptor to absorb light, the amount of spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor and the shape and size of the vessel.

b. *Non-radiative energy transfer*

Collisional energy transfer increases with increasing temperature caused by the increased collisions of the molecule in the excited state with vessel walls and other molecules.

Electron exchange interaction (as shown in Equation 1.14): The excited state molecule (M*) and the quencher (^1Q) forms the exciplex, (M——Q)*, which then dissociates. Electron exchange from the molecule to the quencher ($^3Q^*$) and the fluorophore falls to the ground state (^0M).

$$M^* + {}^1Q \longrightarrow (M{-}{-}Q)^* \longrightarrow {}^0M + {}^3Q^* \qquad (1.14)$$

Quenching by oxygen and paramagnetic species: The quenching of the excited states of many molecules by oxygen is diffusion-controlled.

$$^1M^* + {}^3O_2 \longrightarrow {}^3M^* + {}^3O_2 \qquad (1.15)$$

Molecular oxygen dissolved in the dye solutions exhibits two competing processes.

The first, increases the number of singlet / triplet transitions and so reduces fluorescence quantum yield. The rate constant for the intersystem crossing (k_{ST}) can be written as:

$$k_{ST} = k^{\circ}_{ST} + k_Q[O_2] \qquad (1.16)$$

The quenching of the excited singlet state rate constant (k_Q) is about 2×10^{10} sec^{-1}. $[O_2]$ is the oxygen concentration in mol dm^{-3}, and k°_{ST} is the rate of intersystem crossing in the absence of oxygen. For example, 1,1',3,3,3'3'-hexamethylindotricarbo-cyanine (HITC: Figure 3.8 [XXIV]) has a 10% greater intersystem crossing constant in methanol than in dimethylsulphoxide (DMSO), both being air equilibrated (Hirth et al., 1973).

Dissolved oxygen may also enhance non-radiative triplet to ground state transitions. The rate constant for triplet state quenching is smaller than that for the singlet state. The process that dominates establishes whether oxygen increases or decreases fluorescence. Fluorescence quantum efficiency of polymethines are reduced as the concentration of dissolved oxygen increases (Hirth et. al., 1973). Fluorescence intensities can be increased by addition of specific triplet quenchers to de-gassed solutions, in order to reduce the triplet lifetime. Hirth et al. (1973) stated that cyclooctatetraene (COT), a triplet quencher, added to a solution of 3,3'-dimethyloxatricarbocyanine iodide (DMOTC) in methanol increased the fluorescence intensity by a factor of twelve.

External heavy atom quenching: Fluorescence is quenched by solvent molecules containing heavy atom substituents, e.g. iodomethane or iodobenzene. These enhance the rate of intersystem crossing.

$$^1M^{\bullet} + Q \longrightarrow {}^3M^{\bullet} + Q \qquad (1.17)$$

1.5 Environmental influence on fluorescence

Environmental factors can severely influence the fluorescence character of molecules. A change in pH can ionise the fluorophore, commonly only one of the ionic forms is fluorescent, and so the control of pH is very important. Protonation has a higher rate constant than fluorescence so it is possible to observe the absorption spectrum of the neutral molecule and the fluorescence spectrum of the ionised molecule (e.g. β-naphthol at pH 3).

An increase in temperature usually reduces the fluorescence intensity because of the increased collisional quenching. In some cases the temperature dependence of fluorescence is severe, as much as 5% per °C. The thermostatic control of the sample cell should be a routine precaution. An increase in viscosity of the solvent increases the fluorescence, since collisional interactions are reduced.

1.5.1 Solvatochromism

The modification in shape, position or intensity of both the absorption and fluorescence bands by varying the solvent is called solvatochromism. The Franck-Condon principle states that an electronic transition takes place so rapidly that a vibrating molecule does not change its internuclear distance appreciably during the transition (Banwell, 1985). In other words, adsorption happens in about 10^{-18} seconds before the molecule and the solvent molecules arranged around it (the solvation sphere) rearrange in about a picosecond from the Franck-Condon excited state to their new lower energy equilibrium excited state positions. Emission from this new equilibrium excited state then occurs in 10 nanoseconds. A bathochromic shift or red shift to longer wavelength is observed, if the Franck-Condon excited state has a higher dipole than the ground state. A hypsochromic shift to shorter wavelength (also known as a blue shift) is observed, if the Franck-Condon excited state has a decreased dipole relative to the ground state. Figure 1.4 shows pictorially the influence of these shifts on the absorbance spectra.

Reichardt (1965) described solvent polarity as the sum total of the coulombic, directional, inductive, dispersion, charge transfer and hydrogen bonding intermolecular interactions between the solvent and the solute. This does not include chemical changes of the solute stemming from protonation, oxidation, complex formation and so on. The most comprehensive empirical polarity scale is the Dimroth-Reichardt $E_T(30)$ scale and is derived from the $\pi \rightarrow \pi^*$ transition energy of Pyridium-N-phenoxide (Dye number 30 in the original paper hence the $E_T(30)$ scale).

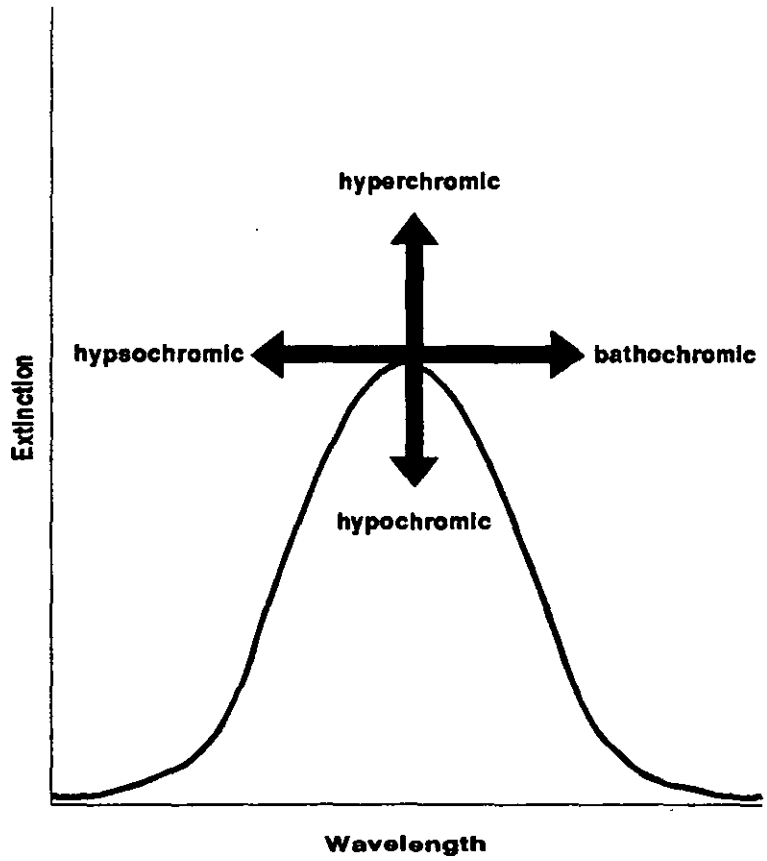


Figure 1.4: Shifts in absorbance maxima to longer and shorter wavelength, respectively are called BATHOCHROMIC and HYSOCHROMIC shifts. An increase and decrease in the magnitude of the extinction coefficient (absorbance) are termed HYPERCHROMIC and HYSOCHROMIC changes, respectively.

Presently, there are over 204 solvents and mixed solvent systems determined by this scale (Reichardt 1965, 1990) and 37 solvents are given in Table 1.2. $E_T(30)$ has kilocalories per mole as units (non-SI units) and consequently should be abandoned in the framework of SI units. Reichardt and Harbusch-Görnett (1983) recommended the E_T^N scale as specified by equation 3.2 to be used instead of the $E_T(30)$ polarity scale, The E_T^N values are calculated as follows using water and tetramethylsilane (TMS) as the extreme reference solvents.

$$E_T^N = \frac{E_T(\text{solvent}) - E_T(\text{TMS})}{E_T(\text{water}) - E_T(\text{TMS})} \quad (1.18)$$

$$= \frac{E_T(\text{solvent}) - 30.7}{32.4} \quad (1.19)$$

The E_T^N scale (Table 1.2) ranges from 0.000 for tetramethylsilane (the least polar solvent), to 1.000 for water (the most polar solvent). An E_T^N value of 0.500 for cyclohexanol indicates that the solvent displays half of the polarity of water. Solvents can be crudely divided into three groups according to their E_T^N values being based on their specific solvent/solute interactions: (i) protic hydrogen-bonding solvents (E_T^N 0.5...1.0); (ii) dipolar non hydrogen-bonding solvents (E_T^N 0.3...0.5) and (iii) a-polar non hydrogen-bonding solvents (E_T^N 0.0...0.3).

Characterising solvent polarity by a single parameter is an over simplification. A series of multicomponent equations in which solute-solvent interactions are separated have been proposed and were reviewed by Reichardt (1965, 1990) and Suppan (1990) However, the interdependency of the parameters means that there is little more information imparted by these quantitative measures than by empirical scales.

Table 1.2: Solvent polarity scales at 25 °C arranged in order of decreasing polarity: Dimroth-Reichardt $E_T(30)$, and E_T^N (Reichardt, 1990).

Solvent	$E_T(30)$ (kcal mol^{-1})	E_T^N
water	63.1	1.000
phenol	61.4	0.948
glycerol	57.0	0.812
ethylene glycol	56.3	0.790
methanol	55.4	0.762
formic acid	54.3	0.728
N,N-dimethylformamide	54.1	0.722
ethanol/water (80:20)	53.7	0.710
ethanol	51.9	0.654
acetic acid	51.7	0.648
propan-1-ol	50.7	0.617
benzyl alcohol	50.4	0.608
butan-1-ol	50.2	0.602
isobutyl alcohol	48.6	0.552
propan-2-ol	48.4	0.546
octan-1-ol	48.3	0.543
butan-2-ol	47.1	0.506
acetonitrile	45.6	0.460
dimethylsulphoxide (DMSO)	45.1	0.444
t-butyl alcohol	43.3	0.389
acetone	42.2	0.355
1,2-dichloroethane	41.3	0.327
nitrobenzene	41.2	0.324
dichloromethane	40.7	0.309
pyridine	40.5	0.302
1,1-dichloroethane	39.4	0.269
chloroform	39.1	0.259
ethyl acetate	38.1	0.228
tetrahydrofuran (THF)	37.4	0.207
1,4-dioxane	36.0	0.164
diethyl ether	34.5	0.117
benzene	34.3	0.111
toluene	33.9	0.099
carbon tetrachloride	32.4	0.052
n-hexane	31.0	0.009
cyclohexane	30.9	0.006
tetramethylsilane (TMS)	30.7	0.000

1.6 Near infrared fluorescence

Fluorescence and in particular fluorogenic labelling is used to increase the sensitivity of other methods, for example chromatography, immunoassay and so on. The fluorophores currently used emit are in the visible region (300 to 600 nanometre). The most commonly used fluorophore is Fluorescein (Figure 3.2: [IV]). The absorption and emission bands lie in the spectral region where interference is likely from intrinsic fluorophores. The interferences are less than those at shorter wavelength but are still a severe limiting factor. The practical applicability of of any fluorogenic labelling method depends on its intrinsic sensitivity (primarily determined by the molar absorptivity and fluorescence quantum yield) and on the presence of interfering fluorophores.

There are very few fluorophores absorb and emit in near infrared region (600 to 1000 nanometres) so there are less likelihood of background fluorescence. Most biological compounds are fluorescent between 300 and 400 nanometre.

The intensity of Rayleigh-Tyndall scattering at right angles to the direction incident light beam is inversely proportional to the forth power of the wavelength. Thus scattering of blue light (450 nm), as compared with red light (700 nm), is approximately six times greater. Also Raman Scattering can be easily resolved at longer wavelength because the separation between the excitation wavelength and Raman becomes greater at longer wavelength so there is less chance of mistaking a Raman peak as fluorescence as shown in Table 1.3.

Table 1.3: Raman scattering for the water band at 3450 cm^{-1} at various excitation wavelengths.

Excitation Wavelength (nm)	Scattering Wavelength (nm)
200	215
280	310
300	335
350	398
480	575
650	838
700	923
750	1012
800	1105
850	1203

The fluorescence spectra at excitation wavelengths 350, 450, 650 and 750 nm (Figures 1.5 and 1.6) of a 1% human blood sera shows that background scattering and fluorescence is reduced by at least 100 in the near infrared region as compared to the visible region. There is also lower photodecomposition of the sample at longer wavelengths because lower energy radiation is insufficient to excite the analyte, usually a protein. These features make the near infrared region make it ideal for fluorogenic labelling.

Long wavelength absorption and fluorescence requires extensive conjugation in the molecule. There are three major families of dyes that are known to have fluorescence maxima in the near infrared: the polymethines, azines and phthalocyanines. Phycobiliproteins, large polyaromatic aromatic hydrocarbons (Rauhut et al., 1975), anthraquinones, indigonoid and higher conjugated formes of the xanthenes can exhibit fluorescence in the near infrared.

The limit to fluorescence in the near infrared region is about 1000 nm. A molecule that absorbs in the near infrared has a low lying excited singlet state (S_1) and even lower excited triplet (T_1) state. Thermal excitation to the triplet state with its biradical unpaired electrons cause it to become highly reactive towards any solvent molecules, dissolved oxygen, impurities or other dye molecules to yield decomposition products. The decomposition is pseudo first order.

$$k_1 = A \times \exp(-E_A/RT) \qquad (1.20)$$

$$t_{½} = \frac{\ln 2}{k_1} \qquad (1.21)$$

Where A is the Arrhenius constant (10^{-10} to 10^{-14} seconds), E_A is the activation energy, R is the gas constant (8.314 J K^{-1} mol^{-1}) and T is the absolute temperature (K). The minimum practical stability of a dye in a solution is one day the above relationship yields an activation energy of 100 kJ mol^{-1} corresponding to 1000 nm.

Unfortunately, until recently the near infrared region has been almost inaccessible to inexpensive instrumentation. The sensitivity of the most widely used detector, the photomultiplier tube (PMT), is significantly reduced in this region. This has changed rapidly with the introduction of solid state detectors based primarily on silicon semiconductors, such as photodiodes, diode arrays and avalanche photodiodes. Diode arrays have been in the near infrared region to measure fluorescence (Summerfield and Miller, 1993).

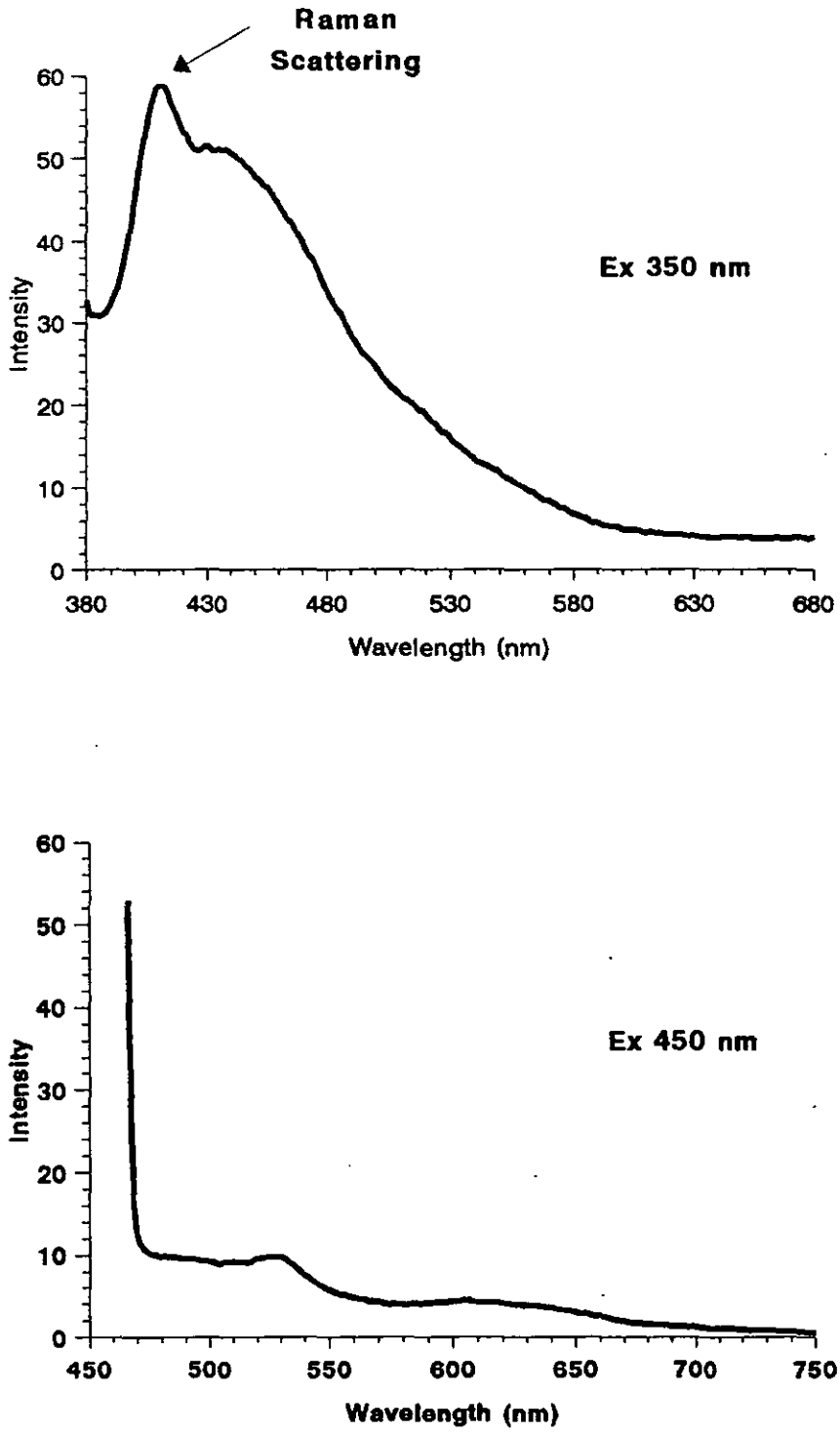
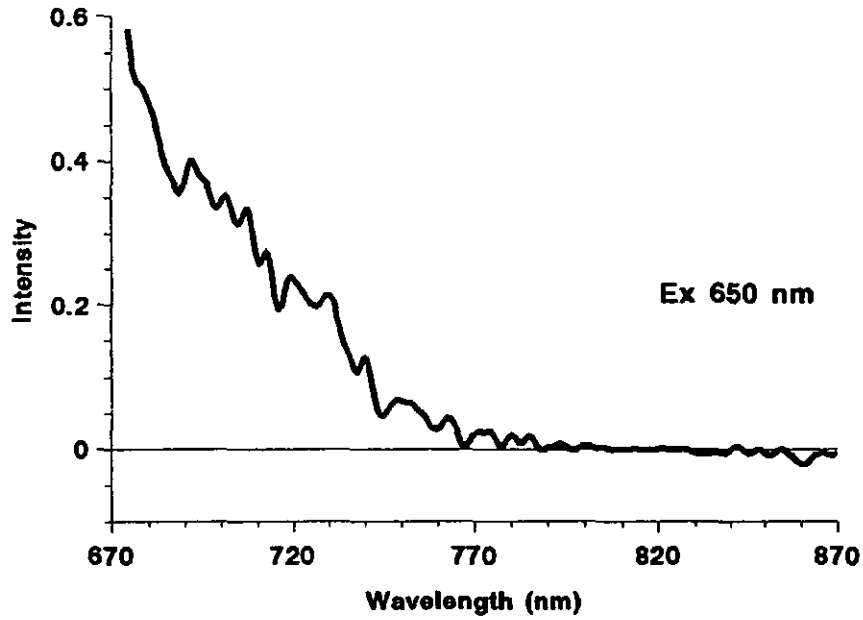


Figure 1.5: Background scattering and fluorescence of a 1% solution of human blood sera excited at 350 and 450 nm.



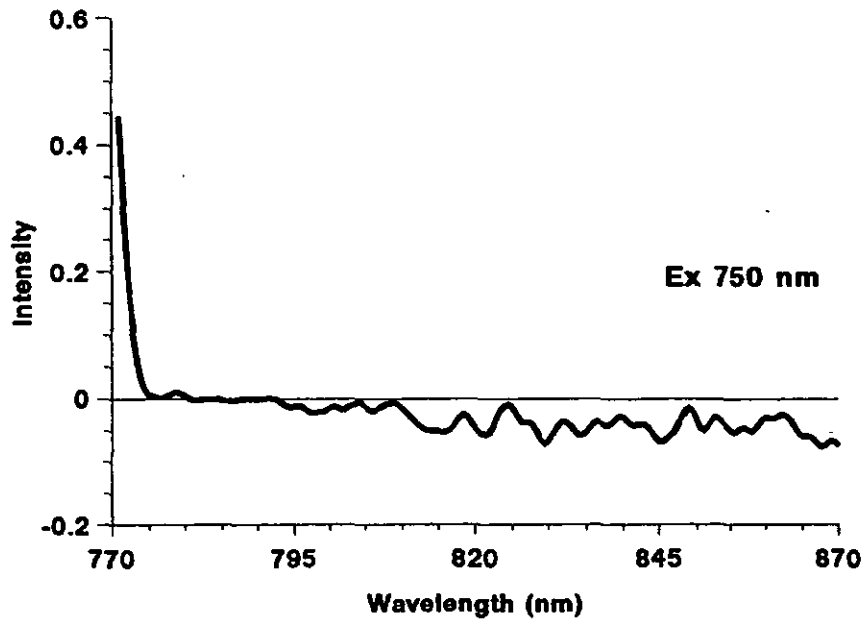


Figure 1.6: Background scattering and fluorescence of a 1% solution of human blood sera excited at 650 and 750 nm.

Robust semiconductor light sources, such as light emitting diodes (LEDs) and laser diodes are inexpensive and very stable. The limitation of using laser diodes as sources for fluorescence is their wavelength. The laser diodes presently available show great promise especially with the introduction of a 635 nm diode laser by Philips and shorter wavelength laser diodes are expected in the near future. Laser diodes between 660 and 780 nanometres have been used by various workers in fluorimetry, such as Imasaka et al. (1985, 1989) and Johnson et al. (1989). Pulsing of laser diodes allow their use in in fluorescence lifetime determination or phosphorimetry. The Imasaka group from Japan [Okazaki et al. (1988), Imasaka et al. (1984, 1985, 1988b, 1989)] have used laser diodes in various areas of molecular fluorimetry. Imasaka (1982, 1988a) has reviewed this area in some detail.

Light emitting diodes (LEDs) with peak wavelength of 630-635 nanometres have been used to excite fluorescence (Smith, Jones and Winefordner, 1988).

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Chapter 2

Materials and Methods

2.1 Instrumentation

2.1.1 Absorbance Measurements

Absorbances at fixed wavelengths were routinely measured on a Pye Unicam (Unicam, Cambridge, UK) PU8600 ultraviolet-visible spectrophotometer fitted with tungsten and deuterium lamps. Spectra were recorded on a Kontron UVIKON 810 Spectrophotometer. Measurements were carried out using either one or four centimetre path length silica cuvettes (Hellma, Essex, UK). The reference cell contained pure solvent of the solution under investigation.

2.1.2 Fluorescence Measurements

Fluorescence measurements were carried out on either a Perkin Elmer MPF-44B (Beaconsfield, Buckinghamshire, UK) or a Perkin Elmer LS50 Spectrofluorimeter, both were fitted with a Hamamatsu R928 PMT. The former had a temperature controlled cuvette holder. Periodically the emission monochromator was checked using a series one centimetre acrylic blocks containing a range of fluorophores (Starna, Essex, UK). The excitation monochromator was checked by scanning through the Rayleigh scatter peak with narrow slits.

Most of the measurements were carried out using one centimetre or four millimetre path length silica cuvettes (Hellma, Essex, UK). Other cuvettes made of acrylic (Sarstedt, Leicester, UK), polystyrene (Sarstedt, Leicester, UK) and glass (Hellma, Essex, UK) of one centimetre were also used. The region of 80% transmission for various materials used for making cuvettes.

Table 2.1: Usable wavelength ranges for materials used to make cuvettes.

	Wavelength (nm)
Special Optical Glass	320 - 2400
Silica	220 - 2400
Far ultraviolet grade silica	185 - 2400
Polystyrene	400 - 1100
Acrylic	390 - 1100

The reflective coated silica cuvette comprised of two adjacent sides coated with a very thin aluminium layer (approximately ten micrometers thick) and this was lacquer coated for protection. Great care was taken taken not to allow this cuvette come into contact with acid which would react with the aluminium. Gold splutter coated acrylic cuvettes consisted of two adjacent sides splutter coated with gold and this was in turn coated with insulating tape.

Limits of detection (LOD) were determined on the Perkin Elmer (Beaconsfield, Buckinghamshire, UK) MPF-44B Spectrofluorimeter at 25 °C in hplc methanol (BDH, Poole). The fluorescence intensity of at least five solutions were measured. The LOD was defined as the intercept of the line with three standard deviations of the background (Miller and Miller, 1988).

2.1.3 Instrumental Dye Identification

Elemental microanalysis by CHN analysis was carried out by MEDAC Ltd., Brunel University, UK. Electron impact mass spectrometry was carried out by Mr Greenfield, Chemistry Department, Loughborough University of Technology.

Infrared spectra were recorded using Pye Unicam SP3-100 infrared spectrophotometer (Unicam, Cambridge, UK). Fourier Transform Infrared (FT-IR) spectra were recorded on either the Perkin Elmer (Beaconsfield, Buckinghamshire, UK) 1600 Series FT-IR Spectrometer or the Niconex 20DXC FT-IR Spectrometer (Nicolet, US).

Proton NMR were recorded on a 250 MHz Bruker AC250F NMR Spectrometer. The compounds were analysed in deuterated solvents with tetramethylsilane (TMS) as the internal standard.

Melting point measurements were made on an Electrothermal Digital Melting Point Apparatus (Fisons, Loughborough, UK) and were uncorrected.

2.1.4 pH Measurements

The pH values of solutions used were measurements using a glass electrode on the Ciba-Corning Delta 120 pH meter (Fisons, Loughborough), calibrated before use using aqueous standard buffers. Unless otherwise stated all pH and pK_a values are in aqueous medium.

2.1.4.1 pH scale*

Solvent molecules are involved in acid-base equilibria as acceptors or donors, so that the acidic or basic strength of a substance varies with the nature of the

solvent. The lower alcohols resemble water, in that they can form the ROH_2^+ and RO^-, but their dissociation is less than water ($pK_{methanol}$ = 16.7, $pK_{ethanol}$ = 19.1 as compared to pK_w = 14.0). Consequently, substances dissolved in alcohols are weaker acids and bases than in water. Other factors influencing acidic and basic strengths in solution, include dielectric constant and solute-solvent interactions which in mixed solvents, can lead to the further complications selective ordering of solvent molecules around ionic species.

The pH* scale are different for each medium because of the difference in solvent acidity, basicity and dielectric constants, and differences in ion activities and mobilities. From their method of measurement and definition, pH* values are consistent with the thermodynamic acid-base equilibria in the solvent system to which they apply.

$$pH^* = pK_a^* + \log \frac{\text{(basic form)}}{\text{(acidic form)}} \qquad (2.1)$$

However, pH* values for different solvents cannot be compared with one another or with pH values for aqueous solutions. Consequently, solutions of different solvent composition may give the same pH meter reading yet behave in quite different ways in acid-base reactions.

A conventional pH meter was used to measure acidity in the partially aqueous solvent mixture of 50% (v/v) methanol/water. The glass electrode responds in a reproducible way to hydrogen ions in media which contains at leas a few per cent of water and also in some anhydrous solvents (Perrin and Dempsey, 1979). The approximate method of measuring pH* was used as proposed by Bates et al. (1963). The pH meter was standardised using aqueous buffers, the meter readings, pH(R), obtained in the partially aqueous medium differs by an amount δ from the reading of pH* attained when the pH meter was standardised using appropriate pH* buffers. That is:-

$$pH^* = pH(R) - \delta \qquad (2.2)$$

The quantity δ is constant for a given solvent concentration, temperature and solute concentration. The value for 50% (v/v) methanol/water is 0.11 (Douheret, 1967).

A comparison with pH readings (pH(R)) for the same concentrations these buffers in partially aqueous buffers was investigated in partially aqueous solutions

in 50% (v/v) methanol/water. The differences between pH and pH* was found to be small, between 0.19 and 0.63, are tabulated below.

Table 2.2: The difference between aqueous buffer pH and 50% methanol/buffer pH*.

pH	pH(R)	pH*	(pH*-pH)
2.00	2.30	2.19	0.19
4.00	4.68	4.57	0.57
6.14	6.88	6.77	0.63
7.17	7.93	7.82	0.65
7.51	8.20	8.09	0.59
8.50	9.10	8.99	0.49
10.28	10.58	10.47	0.19

Aqueous solutions containing appreciable quantities of surfactants can be considered as special cases of partially aqueous systems. According to Perrin and Dempsey (1979) the pH of phthalate, phosphate or borate buffers as measured by a glass electrode did not significantly change by adding 3% (w/v) in cetomacrogol (a non-ionic detergent), 3% (w/v) sodium dodecyl sulphate (an anionic surfactant) or 3% (w/v) cetyl pyridinium bromide (a cationic surfactant). The maximum effect, a decrease of 0.14 pH unit, was found for cetyl pyridinium bromide with the phthalate buffer. All the surfactants were above their critical micelle concentration.

2.2 Solvents and Reagents

All solvents were high pressure liquid chromatographic (hplc) or analytical reagent (AR) grade (British Drug Houses Ltd. (BDH), Poole, Dorset, UK) except where otherwise stated. Polished water was obtained from the LiquiPure (Northampton, UK) Modulab Water Purification System in which pre-filtered, passed through a carbon filter, two mixed bed deionisers and organic scavenger.

2.2.1 Derivatising agents

cyanuric chloride	AR	Aldrich
3-bromopropyl-1-ene (allyl bromide)	AR	Sigma
phosphorus oxychloride	AR	Aldrich
sulphonyl chloride	AR	Sigma

2.2.2 Organic Compounds

acetic acid	SLR	BDH
2-amino-2-(hydroxymethyl)propane-1,3-diol hydrochloric acid [Tris]	AR	Sigma
2-amino-2-(hydroxymethyl)propane-1,3-diol hydrochloric acid [Tris]	SLR	Fisons
citric acid monohydrate	AR	Fisons
formic acid	Analar	BDH
urea	SLR	Fisons

2.2.3 Inorganic Compounds

ammonium hydroxide	SLR	Fisons
boric acid	AR	Fisons
hydrochloric acid	SLR	Fisons
20 volume hydrogen peroxide	AR	Fisons
magnesium sulphate	GPR	BDH
nitric acid	SLR	Fisons
potassium chloride	Analar	BDH
potassium dihydrogen orthophosphate	Analar	BDH

sodium azide	SLR	Fisons
sodium chloride	SLR	Fisons
sodium dithionite	GPR	BDH
sodium dithionite	SLR	Fisons
sodium carbonate	SLR	Fisons
sodium dihydrogen orthophosphate	AR	Fisons
sodium hydrogen carbonate (anhydrous)	SLR	Fisons
disodium hydrogen orthophosphate (anhydrous)	SLR	Fisons
disodium hydrogen orthophosphate decahydrate	AR	Fisons
sodium hydroxide pellets	SLR	Fisons
sulphuric acid	SLR	Fisons

2.2.4 Dyes

Oxazines

Cresyl Violet (Oxazine 9)	99.9%	KODAK
Nile Blue A chloride (CI Basic Blue 12)	80%	Sigma
Nile Blue A chloride (CI Basic Blue 12)	90%	Aldrich
Nile Blue A perchlorate	99%	KODAK
Nile Red (Nile Blue Oxazone)	99.9%	KODAK
Nile Red (Nile Blue Oxazone)	95%	Sigma
Oxazine 4 (LD690)	99.9%	KODAK
Oxazine 720 (Oxazine 170)	99.9%	KODAK
Oxazine 725 (Oxazine 1, CI Basic Blue 3)	99%	Lambda Physik
Oxazine 750	99%	Exciton

Polymethines

3,3'-diethyloxadicarbocyanine iodide [DODC]	99.9%	KODAK
3,3'-diethyloxatricarbocyanine iodide [DOTC]	99.9%	KODAK
3,3'-diethylthiatrcarbocyanine perchlorate [DTTC]	99.9%	KODAK
1,1',3,3,3'3'-hexamethylindotricarbocyanine perchlorate [HITC]	99.9%	KODAK

IR125 [indocyanine green]	99.9%	KODAK
Merocyanine 540	95%	Sigma
Styryl 7	99.9%	KODAK
Styryl 11	99%	Exciton
Thiazines		
Azur A		Aldrich
Azur B (Methylene Azur)	84%	Sigma
Methylene Blue BP (CI Basic Blue 9)		Boots
Methylene Green (CI Basic Blue 5)		BDH
Bernsthen Methylene Violet		BDH
Toluidine Blue (Tolonium Blue O)	95%	Fluka
Xanthenes		
Fluorescein isothiocyanate [FITC]		Sigma
Fluorescein sodium (Uranin)		Sigma
Pyronine B		Boots
Rhodamine B (CI Basic Violet 10)		BDH
Rhodamine 6G (CI Basic Red 1)		BDH
Rhodamine 800 perchlorate (LD800)		Lambda Physik
Rose Bengal (CI Solvent Red 141)	70%	KODAK
Sulphorhodamine 101 (Sulphorhodamine 640)		KODAK
Texas Red	90%	Molecular Probes

2.2.5 Proteins

α_1-acid glycoprotein		Sigma
bovine serum albumin ,essentially fatty acid and globulin free	98%	Sigma
human IgA (lyophilised)	Grade A	Calbiochem-Bahring
human IgG (lyophilised, essentially salt free)		Sigma
bovine insulin		Sigma

β-lactoglobulin from bovine milk		Sigma
transferin, apo-, human (siderophilin):low endotoxins, <10 ng of iron per mg of protein,	98%	Sigma
transferin human (siderophilin): substantially iron free		Sigma
anti-human transferin antibody, developed in Goat, fractionated antiserum		Sigma

2.2.6 **Detergents (surfactants)**

Non-ionic surfactants

Brij 35 (polyoxyethylene lauryl ether)	ICI
Tween 20 (Polysorbate 20: polyoxyethylenesorbitan mono laurate)	Sigma
Tween 80 (Polysorbate 80: polyoxyethylene sorbitan mono oleate)	Sigma
Triton X-100 (polyoxyethylene tert-octylphenol)	Aldrich

Cationic surfactants

CTAB (cetyltrimethyl ammonium bromide)	BDH
TDAB (tetradodecylammonium bromide)	BDH

Anionic surfactants

SDS (sodium dodecyl sulphate: sodium lauryl sulphate)	Sigma

2.3 Procedures

2.3.1 Preparation of Glassware

Glassware was initially acid cleansed with concentrated nitric acid, then rinsed with water, and washed with 0.5% v/v Quadralene 3000 (Fisons, Loughborough, UK). It should be note that Quadralene showed no significant fluorescence in the region of interest (300 to 900 nm). This was very carefully rinsed with polished water and acetone. Before use, the glassware was rinsed with solvent or water whichever was appropriate.

Routinely cuvettes were emptied after use, rinsed repeatedly in the solvent, then with acetone or ethanol and dried in a warm current of air. The outside optical surfaces were polished with clean lens tissue. To removed stubborn stains the cuvettes were cleaned with with 0.5% v/v Quadralene 3000 detergent and/or 5% v/v nitric acid, the latter especially after the use of proteins. They were then very carefully rinsed with water, rinsed with acetone or ethanol and dried. The cuvettes were never allowed to soak in the cleaning solution for more than a half hour.

2.3.2 Buffer Reagents

These were prepared by diluting constituents in deionised water at room temperature, adjusting to the required pH and making up to volume. A few crystals of sodium azide were added as a preservative. The recipes were from Dawson et al. (1989) with originating paper referenced in brackets. The buffers are arranged in increasing pH. Tables 2.3 to 2.8 give the buffers used in the experiments for the characterisation of dyes as described in Chapter 3.

Table 2.3: *Clark and Lubs buffer (pH 1.0 to 2.2):* 25 ml of 0.2 molar potassium chloride (14.919 g/l) and x ml of 0.2 molar hydrochloric acid diluted to 100 ml with polished water (Bates and Bower, 1955).

pH at 25°C	x (ml)
1.00	67.0
1.50	20.7
2.00	6.5

Table 2.4: *Citric acid-disodium hydrogen phosphate (McIlvaine) buffer (pH 2.6 to 7.6)*

citric acid monohydrate: Mol. Wt. ($C_6H_8O_7 \cdot H_2O$) = 210.14

anhydrous disodium hydrogen phosphate Mol. Wt. (Na_2HPO_4) = 141.98

x ml of 0.1 molar citric acid (21.01 g/l) and y ml of 0.2 molar anhydrous disodium hydrogen phosphate (28.4 g/l) mixed. (McIlvaine, 1921)

pH at 25°C	x (ml)	y (ml)
3.0	89.10	10.90
4.0	61.45	38.55
5.0	48.50	51.50
6.0	36.85	63.15
6.6	27.25	72.75
7.0	17.65	82.35
7.4	9.15	90.85

Table 2.5: *Clark and Lubs Buffer (pH 8.0-10.2):* 50 ml of a mixture 0.1 molar with respect to both potassium chloride (7.455 g KCl per litre) and boric acid (6.184g H_3BO_3 per litre) and x ml of 0.1 molar sodium hydroxide. This was then diluted to 100 ml with water (Bates and Bower, 1955)

pH at 25°C	x (ml)
8.00	3.9
9.00	20.8
10.00	43.7

Table 2.6: *Sodium carbonate-sodium bicarbonate buffer (pH 9.2-10.8):*

sodium carbonate hexahydrate Mol. Wt. ($Na_2CO_3.10H_2O$) = 286.2

sodium hydrogen carbonate Mol. Wt. ($NaHCO_3$) = 84.0

x ml of 0.1 molar (28.62 g/l) sodium carbonate solution and y ml of 0.1 molar (8.4 g/l) of sodium hydrogen carbonate solution mixed together (Delory and King, 1945).

pH at 20°C	pH at 37°C	x (ml)	y (ml)
9.2	8.8	10	90
9.9	9.7	50	50
10.3	10.1	70	30
10.8	10.6	90	10

Table 2.7: *0.05 molar Phosphate buffer solutions (pH 11.0 to 11.9):*

anhydrous disodium hydrogen phosphate Mol.Wt. (Na_2HPO_4) = 141.98

50 ml of 50 millimolar disodium hydrogen phosphate (7.10 g/l) and x ml of 0.1 molar sodium hydroxide and then diluted to 100 ml with water (Bates and Bower, 1956).

pH at 25°C	x (ml)
11.00	4.1
11.50	11.1

Table 2.8: *Hydroxide-chloride buffer (12.0 to 13.0):* 25 ml of 0.2 molar potassium chloride (14.91 g/l) and x ml of 0.2 molar sodium hydroxide and then diluted to 100 ml with water (Bates and Bower, 1956).

pH at 25°C	x (ml)
12.00	6.0
13.00	66.0

The following buffers were used in the work with proteins:

50 mM Phosphate Buffer Saline (PBS) (pH 7.2)

2.9 g of disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$), 0.2 g potassium dihydrogen phosphate (KH_2PO_4), 8.0 g sodium chloride and 0.2 g potassium chloride were dissolved in 100 ml of polished water.

0.1 M Phosphate buffer saline (PBS) (pH 7.8)

2.9 g of hydrated disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$), 0.2 g anhydrous potassium dihydrogen phosphate (KH_2PO_4), 8.0 g sodium chloride and 0.2 g potassium chloride were dissolved in 100 ml of polished water.

0.1 M Potassium phosphate buffer (pH 8.8)

pH 8.8 potassium phosphate buffer was prepared from 6.2 ml of one molar anhydrous di-potassium hydrogen phosphate (K_2HPO_4) and 93.8 ml of one molar anhydrous potassium dihydrogen phosphate (KH_2PO_4) stock solution diluted to 1000 ml with polished water.

50 mM Tris Buffer Saline (pH 8.8)

3.94 g of 2-amino-2-(hydroxymethyl)propane-1,3-diol hydrochloric acid [Tris] and 14.63 g of sodium chloride were dissolved in 500 ml of polished water.

0.1 M Sodium carbonate-sodium bicarbonate saline (pH 9.2)

1.60 g of anhydrous sodium hydrogen carbonate, 7.56 g of anhydrous sodium hydrogen carbonate and 5.84 g of sodium chloride was dissolved in 100 ml of polished water.

2.3.3 Thin Layer Chromatography (TLC)

The separations were carried out in TLC tanks lined with filter paper (Whatman No. 1) thoroughly soaked in developing solvent. The tanks were left to equilibrate for about an hour. The plates used were either Merck fluorescent silica gel 60 F_{254} or silica gel 60 aluminium backed TLC plates. The plates were pre-run to remove contamination. The standards and samples were dissolved in either water or methanol. The sample was applied using five microlitre spotters about two centimetres from the bottom of the plate. The solvent front was allowed to run up to two centimetres from the top of the TLC plate. The plates were viewed under a ultraviolet viewer at 254 and 366 nm. All spots were marked and R_f values recorded.

2.3.4 Oxygen Flask

A 10 mg sample was weighed accurately onto a three square centimetres piece of Whatman No.540 ashless filter paper with a narrow three centimetres fuse. The paper was folded to enclose the sample and placed into the silica cradle with the fuse at the top. The flask was washed out three or four times with polished water. Ten millilitres of polished water and three drops of 20 volume hydrogen peroxide were added to the flask as the absorbent solution. The flask was flushed with oxygen. The flask was put into the fume cupboard behind the protective screen before the fuse was ignited and the stopper securely inserted. Combustion was complete after about fifteen seconds, and then the flask was shaken periodically over the next ten minutes.

The absorbent solution was quantitatively transferred to a Ehrlenmeyer flask and boiled for a few minutes to destroy the hydrogen peroxide. Three drops of Methyl Red (0.25 g per 100 ml of 90% ethanol) and three drops of Methylene Blue (0.166 g per 100 ml of 90% ethanol) were added to the cooled solution and

titrated with five millimolar sodium hydroxide. Then 10 ml of mercuric oxycyanide was added and titrated with the liberated alkali with five millimolar sulphuric acid.

Ion chromatography was also carried out on the combustion products, which confirmed the presence of chlorine This was carried out on the Dionex Series 4000i Ion Chromatograph fitted with a Conductivity Detector.

2.3.5 Dye Purification

Solvent extraction of dyes to remove impurities was carried out. The dye was recrystalised from the solvent by heating the beaker on the hotplate and letting it cool gradually. The precipitated dye was filtered using a Buchner Funnel.

The reactive dye was salted out from the reaction mixture by adding sufficient sodium chloride to make it 10% w/v saline solution. The dye was less soluble than sodium chloride and was precipitated leaving also the more soluble impurities.

The dye upon filtration was washed with ether or hexane to remove further impurities.

2.3.6 Protein Solution Preparation

Proteins were dissolved in the appropriate buffer and the concentration was determined by measuring the absorbance at 280 nm in conjunction with literature molar absorptivity, ε (Table 2.5). Protein solutions were never heated above room temperature and were always freshly prepared.

Table 2.9: Molecular weight and molar absorptivity at 280 nanometres in water of some proteins.

Protein	Mol. Wt. (daltons)	$\varepsilon \times 10^4$ $lmol^{-1}cm^{-1}$	Reference
albumin, human	68000	3.6	Phelps & Putman, 1960
albumin, bovine	69000	3.96	Weber, 1961
albumin, egg	43500		Fasman, 1989
α_1-acid glycoprotein	44100	3.9	Smith et al., 1950
β-lactoglobulin, bovine	35000	3.66	Gordon et al., 1961
IgA, human	160000		Fasman, 1989
IgD, human	184000		Fasman, 1989
IgG, human	156000		Schultze & Heremans, 1966
IgM, human	970000		Sober, 1970
insulin, bovine	5733	0.57	Glazer et al., 1961
ovomucoid	28800	1.19	Chatterbee et al., 1962
transferin, human	80000	9.23	Luk, 1971

2.3.7 Protein Conjugation

10 ml of 1-2 mg per ml of protein in buffer was incubated with a known amount of reactive dye for at least four hours and protected from light. The reactive dye was either added as a solid or in solution, usually in 30% v/v methanol/water. The dye solutions were always freshly prepared. The total amount of methanol never exceeded 1% v/v.

The molar ratio of dye to protein was estimated by solving the following simultaneous equations.

$$(\varepsilon_{280} \times c_{protein}) + (\varepsilon_{280} \times c_{dye}) = A_{280} \quad 2.3$$

$$(\varepsilon_{640} \times c_{protein}) + (\varepsilon_{640} \times c_{dye}) = A_{640} \quad 2.4$$

Where ε is the molar absorptivity, c in the concentration in moles per litre and A is the absorptivity. In Equation 2.3, A_{280} is the sum of the absorbance from the protein and the dye at 280 nm, the latter is assumed to be very small. In Equation 2.4, A_{640} is the sum of the absorbance from the protein (at this wavelength zero) and the dye at 640 nm. The 640 nm may be any wavelength that corresponds to the absorbance maximum for the dye in question. These equations assume that the dye does not change its spectroscopic characteristics upon binding to the protein. The molar absorptivities for the dyes came from Table 2.9.

2.3.8 Protein Conjugate Purification Methods

Gel chromatography was performed using a PD10 column packed with Sephadex G-25 (Pharmacia, UK). The exclusion limit for Sephadex G-25 (Pharmacia, UK) is a molecular weight of 5000.

Dialysis was undertaken using a thirty centimetre length of dialysis tubing (Medical International, London, UK). The tubing was boiled in polished water for about half hour to soften and remove the plasticisers. Dialysis was carried out with stirring stirred for over two days with the periodic changing of the buffer.

Protein concentration was carried out using a Centricon 30 microconcentrator (Amicon Ltd, Gloucestershire). Concentration is achieved by ultrafiltration of the sample through a membrane with a 30,000 daltons cutoff. Two millilitres of conjugation mixture was added to the sample reservoir. The device was centrifuged in a Wifug Studie (Germany) centrifuge at 4400 rpm for half hour. Centrifugation was repeated until the required volume of conjugate was obtained. Two millilitres of pH 7.0 potassium phosphate buffer was subsequently added to the sample reservoir to wash the concentrate followed by centrifugation for a further half hour

at 4400 rpm. This was repeated at least twice to ensure removal of the contaminating microsolute. The centrifugal force drive the solvent and the low molecular weight solutes through the membrane and the macrosolutes are retained on the membrane. The device was inverted and centrifuged at 1800 rpm for two minutes to transfer the concentrate into the retentate cup. The concentrate volume obtained was approximately fifty microlitres.

2.4 References

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Chapter 3

3.1 Near Infrared Dyes

A dye may be defined as an organic coloured mono-molecular substance. Dyes have traditionally been used as colouring materials for both natural and synthetic polymers in the textiles and plastics industry. In recent years, the focus in research on dye chemistry has been towards functional dyes for electro-optical applications. Griffiths (1986) described the exploitable properties of a dye as light absorption (colour), light emission (fluorescence and phosphorescence), light induced polarization, photoelectric properties (redox and photo-induced activity), chemical reactivity (colour change) and photochemical reactivity (photochromism and photosensitisation). Most of these attributes are related to the ability of the dye to interact strongly with visible electromagnetic radiation leading to colour, fluorescence, and various photochemical and photoelectrical processes. Infrared absorbing dyes have been applied to laser optical recording systems (Nakazumi, 1988), laser printing systems, laser thermal writing displays, infrared photography (Jones, 1989; Matsuoka, 1989), and medical or biological application (van der Bergh, 1986).

Several groups of compounds have been shown to exhibit emission in the 600-1000 nm range. In the following chapter, spectroscopic and physical properties of the azine, xanthene and polymethine dyes have been investigated to determine the best fluorophores in near infrared for further investigation as labels and probes. Fluorescein was used as the reference compound. The appendix gives a compilation of data from the literature of over five hundred dyes from the azine, polymethine, phthalocyanine and xanthene dye groups.

Most organic dyes have well defined structures, unfortunately, the purification of dyes is sometimes rather difficult. The literature on the purification and identification of dyes is not comprehensive. In this century, only three books have been published devoted completely to this subject. Both Green (1920) on the analysis of dyes and Brunner (1929) on the analysis of azo dyes are outdated. Venkataraman (1977) is the only recent book on the analytical chemistry of synthetic dyes. Current information on the chemical analysis of dyes can be found in the chapter by Sharples and Westwell in Ullmann's Encyclopedia of Industrial Chemistry (1987). The lack of literature is due to the complexity of the subject and the unwillingness of dye manufacturing companies to publish such information.

The selection of a particular dye to satisfy a specific spectroscopic or physical property is a problem because there is at present no directory that gives this information combined with the current supplier (Griffiths, 1988). Dyes with the same name may have a different structure or consist of a mixture of dyes, intermediates, by-products and other contaminants. In the textile industry, all that is required of a dye is that it has the correct hue rather than purity. Most commercial dyes are less than 40% pure with the major impurity being sodium chloride (Zollinger, 1987). The lineage of a dye can be best determined from the Colour Index Number (CI No.) or the Chemical Abstracts Service Number (CAS #) but even this is fraught with danger. For example, Horobin and Murgatroyd (1969) showed that of the four commercial Pyronine B (Figure 3.5 [XIV]) samples that were analysed, three were Rhodamine B (Figure 3.4 [IX]) and one was Rhodamine 6G (Figure 3.4 [VIII]).

3.1.1 Toxicology of dyes

It is important to have knowledge whether dyes are carcinogenic or have any serious toxic effect upon the user. There is very little information on the toxicology of dyes due to the expense of the methods of testing and that the exposure levels to dyes is normally low in normal laboratory use. Kues and Lutty (1985) screened 150 laser dyes and found that 28 were extremely toxic. The cyanines and carbocyanines were the most toxic. Dimethyl sulphoxide (DMSO) is the usual solvent used in laser spectroscopy and this facilitates the transport of toxic dyes through the skin. The hazards associated with the use of a toxic dye dissolved in a membrane-transferring solvent should not be underestimated.

The Ecological and Toxicological Association of the Dyestuff Manufacturing Industry (ETAD) was founded in 1974, in order to minimise the possible damage to man and environment from the production and application of dyes. ETAD has identified and assess risks of dyes and their intermediates according to their potential acute toxicity and their chronic effects on human health. A survey of acute oral toxicity, as measured by the LD_{50} (the lethal dose of the compound that would kill 50% of test animals, usually rats or mice, expressed in milligrams of compound per kilogram of animal) showed that out of 4461 colorants tested, only 44 had a LD_{50} less than 250 milligrams per kilograms and 3669 exhibited practically no toxicity (i.e. LD_{50} greater than 5 milligrams per kilograms). The most toxic colorants were found to be among the disazo and cationic dyes. For example, Oxazine 725 (Figure 3.17 [LI] also known as CI Basic Blue 3: CI 51004:

Oxazine 1), a cationic phenoxazine dye, has a LD_{50} of 100 milligrams per kilograms in rat and is toxic if swallowed (Anliker et al., 1988). Pigments and vat dyes have a very low acute toxicity due to their low solubility in water and in lipids.

ETAD regularly publishes papers on the toxicity of colorants and on the risks in the use and handling of colorants in the Journal of Dyers and Colourists. For example, Anliker and Steinle (1988) describe risk assessment in exposure and hazard posed by a given product.

The LD_{50} is outdated because of the large number of animals necessary to carry out such tests (see Zbinden, 1981). The European community (Anonymous, 1990) issued a statement on its intention to replace the LD_{50} test by the Fixed-Dose Procedure. Fairhurst in 1989 gave a good outline of the classification and interpretation toxicological data with special application to the dye industry.

3.2 Methods

The following experiments were carried out on the xanthenes (fluoresceins, rhodamines and pyronines), polymethines (cyanines and merocyanines) and azines (phenoxazines, phenoxazones and thiazines) and the results are discussed in section 3.3, 3.4 and 3.5 respectively.

3.2.1 Solubility (Tables 3.1, 3.9 & 3.17)

The solubility of dyes was tested in various solvents in order to determine the best solvents to use for further investigation of their chemical and physical properties. Approximately 5 mg of dye was added to about 0.5 ml of solvent and was sonicated for 10 minutes. Certain aqueous dye solutions, principally the azines, were passed through a 0.8 micrometer Acrodix syringe filter (Gelman Science) in order to determine that they were true solutions rather than colloidal suspensions. Being that a dye is a coloured compound, the solubility was subjectively defined as follows.

i	insoluble: no colour imparted to the solvent.
vss	very slightly soluble: some colour imparted to the solution.
ss	slightly soluble (under 1%): not all of the dye dissolved.
s	soluble (around 1%)
vs	very soluble (over 10%): Dye dissolved without sonication.

Literature values from Lillee (1969) and Green (1990) were used where appropriate. The solubility of xanthenes, polymethines and azines are shown in Tables 3.1, 3.9 and 3.17 respectively.

3.2.2 Influence of pH on fluorescence (Figures 3.5, 3.6, 3.12, 3.18)

pH exerts more influence on the fluorescence than absorbance because the protolytic dissociation reaction is faster than the time taken for fluorescence decay to occur in about ten nanoseconds. If a substance undergoes a sudden change of fluorescence at a specific pH, it may be used as a fluorescent indicator (Kolthoff and Stenger, 1957; Ross et al., 1989).

The fluorescence of approximately 50 nanomolar dye solutions was measured in 50% methanol/buffer solution (pH 1 to 13) because of the problems with the low solubility of many of the dyes in water and fluorescence intensities were severely quenched in water. The pH(R) was measured using a pH meter and was then corrected to pH* as defined in Section 2.1.4.1. It should be noted that this

is not a direct measure of pH as this is only correct in aqueous medium (Bates, 1964) but it gives an indication of the influence of hydrogen ion concentration on the fluorescence of the dyes. pKa values for the xanthenes, cyanines and azines are shown in Tables 3.2, 3.10 and 3.18 respectively. Note that most of these pKa values are pKa* values determined in partially aqueous solvents due to their low solubility in water.

The fluorescence was measured on a Perkin Elmer (Beaconsfield, Buckinghamshire, UK) MPF-44B Spectrofluorimeter at 25 °C. The fluorescence intensity was plotted against the pH* of the 50% methanol/buffer system for the fluoresceins, xanthenes, cyanines and azines can be seen in Figures 3.5, 3.6, 3.12 and 3.18 respectively.

3.2.3 Chemical Stability and Properties (Tables 3.3, 3.11-3.13, 3.19-3.21)

The chemical properties of dyes were tested to determine their suitability for derivatisation where extremes of pH could be used to isolate the derivatives. A known quantity from the stock solution was pipetted into sample tubes containing 2 ml of concentrated sulphuric acid, concentrated hydrochloric acid, five molar hydrochloric acid, aqueous pH 4.0 buffer, distilled water (about pH 6), aqueous pH 10 buffer, aqueous pH 11.5 sodium carbonate buffer and one molar sodium hydroxide respectively. The colour was noted and the absorption spectrum was taken for each of the sample tubes. Tables 3.3, 3.11-3.13, and 3.19-3.21 give the results for the xanthenes, cyanines and azines respectively. Concentrated nitric acid decomposed all the dyes giving a pale yellow or colourless solution.

Dyes may encounter a reducing environment and the knowledge of what happens to the dye when it is reduced and subsequently oxidised is important. An aqueous dye solution was reduced by either neutral sodium dithionite or alkaline sodium dithionite (5% w/v sodium hydroxide). The resulting solutions were re-oxidised by exposure to air or by the addition 2% w/v potassium persulphate in 2% w/v sulphuric acid. Absorbance spectra was taken prior to reduction, after the dye was reduced and when the dye was re-oxidised.

3.2.4 Spectroscopy (Tables 3.4/3.5, 3.14/3.15, 3.22/3.23)

Tables 3.4, 3.14 and 3.22 show the absorption and the fluorescence emission maxima of xanthenes, polymethines and azines respectively in water. Tables 3.5, 3.15 and 3.23 give the absorption and fluorescence maxima for these dye groups in methanol. Wherever possible the absorption maximum was used to

excite the fluorescence. In some cases where the Stoke's shift was small (less than twenty nanometres) the excitation wavelength used had to be reduced by ten to twenty nanometres, in order to resolve the fluorescence band from the Rayleigh scatter peak. The molar absorptivity (ε), fluorescence quantum efficiency (ϕ_f) and fluorescence lifetime (τ_f) are literature values from Birge and Bohwon (1986), Brackmann (1986), and Haugmann (1989, 1992). The limit of detection was determined in methanol on a Perkin Elmer (Beaconsfield, Buckinghamshire, UK) MPF-44B Spectrofluorimeter and was calculated in the usual manner.

3.2.5 **Solvatochromism** (Tables 3.6, 3.16, 3.24: Figures 3.13, 3.20)

A change of solvent may cause a shift in absorbance and fluorescence by changing the electronic energy levels as described in Section 1.5.1.

Absorbance spectra of dyes in both low polar and high polar solvents were recorded and the wavelengths recorded as shown in Tables 3.6, 3.16 and 3.24. The excitation and emission maxima, and fluorescence intensity of 50 nanomolar dye solutions were plotted against the E_T^N value (Table 1.2) as shown in Figures 3.13 and 3.20.

3.2.6 **Temperature Effect**

A decrease in temperature produces an increase in fluorescence intensity (Udenfriend, 1962). A 50 nanomolar aqueous solution of dye was cooled from 45 °C to 15 °C using a temperature regulating cuvette holder in a Perkin Elmer (Beaconsfield, Buckinghamshire, UK) MPF-44B Spectrofluorimeter over a period of several hours. Emission spectra were taken at regular temperature intervals.

3.3 Xanthenes

The xanthenes (Figure 3.1) include the fluoresceins [I], rhodamines [II] and pyronines [III], and have a dibenzo-1,4-pyran structure with either amino or hydroxyl groups meta position to the oxygen bridge. These dyes are widely used as colorants for textiles, paper, cosmetics, and food (Green, 1990). In photochemistry the xanthenes are used as laser dyes (Birge and Bohwon, 1986; Schäfer, 1973; Steppel, 1982; Maeda, 1984; Brackmann, 1986; Raue, 1990a; Schwander and Hendrix, 1988), as photosensitising dyes (Grossweiner, 1970; Lamberts and Neckers, 1984; Bellin and Ronayne, 1966) and as a source of singlet oxygen (Gollnick and Schenck, 1964; Gandin et al., 1983). In biotechnology, they are widely used as probes, stains and immunoassay labels (Haugmann, 1989 and 1992).

Fluoresceins (Figure 3.1 [I]) have both 2- and 7-positions in the xanthene ring substituted by hydroxyl groups. Fluoresceins absorb at shorter wavelength than rhodamines and pyronines. Fluorescein (Figure 3.2 [IV]) and its derivatives (e.g. Fluorescein isothiocyanate (FITC) [VI]) form the foundation for the molecular probe industry so important to medical diagnostics, which encompass immunoassay and biological staining. Uranin (the sodium salt of Fluorescein [IV]) is the most common fluorescent marker in hydrology (Viriot and Andre, 1989) and was used during the second world war as a marker for sea rescue of pilots (Gurr, 1971). The fluoresceins have also been used as both fluorescent and colorimetric indicators (Bishop, 1972). The halo-fluoresceins (e.g. Rose Bengal Figure 3.2 [VII]) have low fluorescent quantum yields due to intersystem crossing and so can show phosphorescence even at room temperature (Neckers, 1987). The longest wavelength fluorescein is Naphthofluorescein (Figure 3.2 [V]), which at absorbs at 600 nm in methanol (Haugmann, 1992).

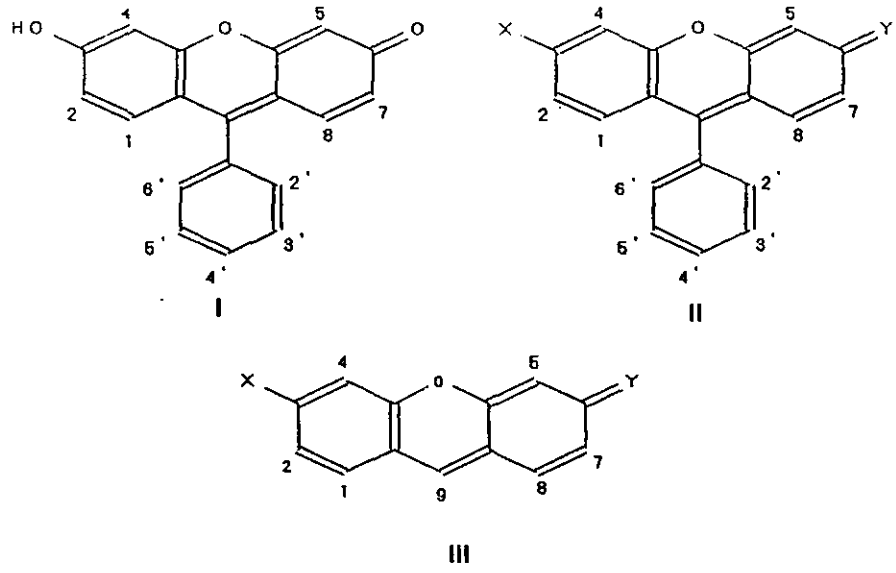


Figure 3.1: General structural formulae for the Xanthenes: fluoresceins (I), rhodamines (II), and pyronines (III). Where X and Y are either primary, secondary or tertiary amines.

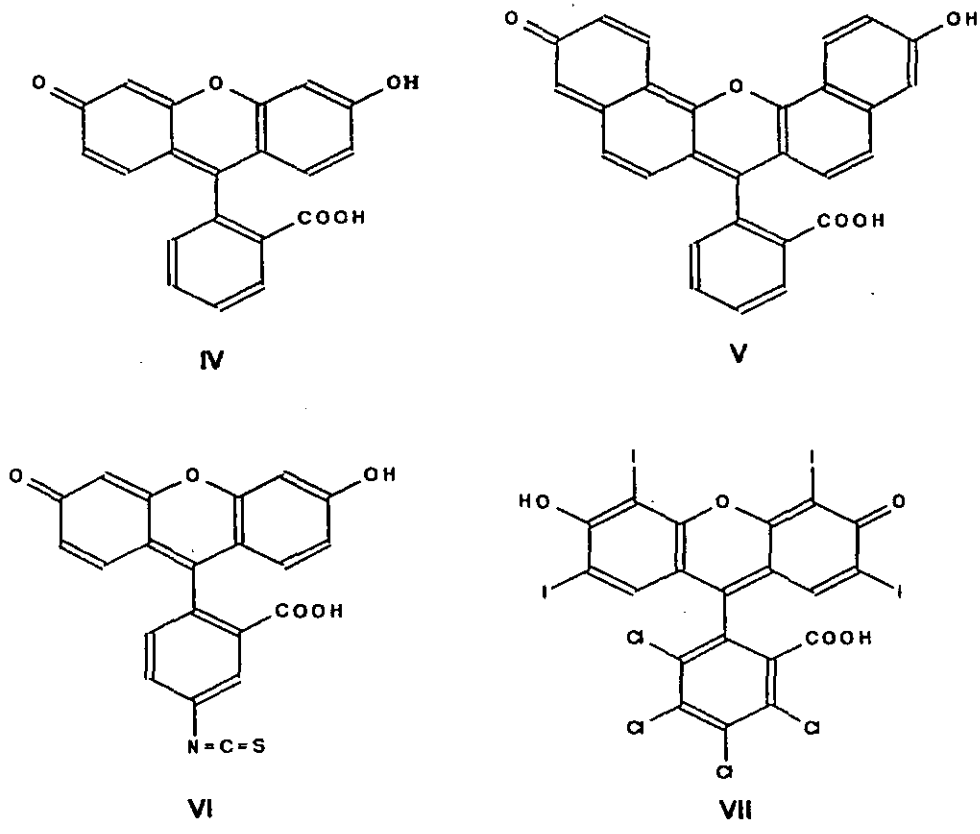
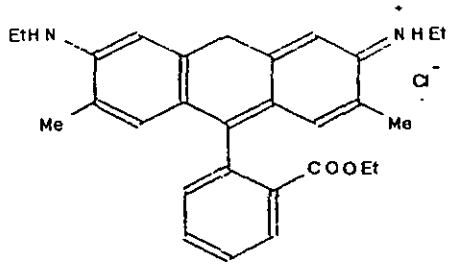


Figure 3.2: Fluoresceins: excitation and emission wavelengths in methanol.

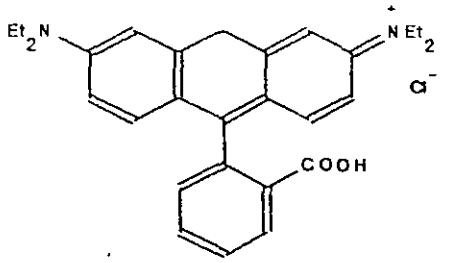
		Ex (nm)	Em (nm)
IV	Fluorescein	491	521
V	Naphthofluorescein	600	630
VI	Fluorescein isothiocyanate [FITC]	488	514
VII	Rose Bengal	564	581

Rhodamines (Figure 3.1 [II]) have the 2- and 7- positions in the xanthene ring substituted by either amine, alkyl amine or di-alkyl amines groups and usually a carboxy phenyl group in the 9-position that is not part of the chromophore. Esterification of this carboxylic acid group yields a more strongly basic dye with virtually identical absorption and fluorescence wavelengths. Texas Red (Figure 3.3 [XIII] is an example of the rhodamine reactive dyes developed for labelling amine groups on proteins for flow cytometry and immunoassay. The rhodamines have been used as laser dyes, photon counters (e.g. Rhodamines 6G (Figure 3.3 [VIII]) and Rhodamine B [IX]; Demas and Crosby, 1971) and photosensitisers (e.g. Rhodamine B (Figure 3.3 [IX]); Kramer and Mante, 1972). The highest absorbing member being Rhodamine 800 [XI] which absorbs at 685 nm in water.

Pyronines (Figure 3.1 [III]) have primary, secondary or tertiary amines substituted in the 2- and 7-position in the xanthene ring and hydrogen in the 9-position. These have similar absorbance maxima to rhodamines because the latter's carboxyphenyl substituent is not part of the chromophore. For example Pyronine 20 (Figure 3.4 [XV]) absorbs at 527 nm, compared to the corresponding rhodamine, Rhodamine B (Figure 3.3 [IX]) at 524 nm (Drexhage, 1973b).



VIII



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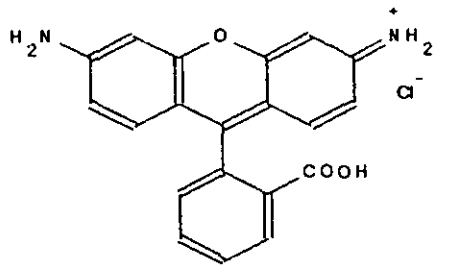

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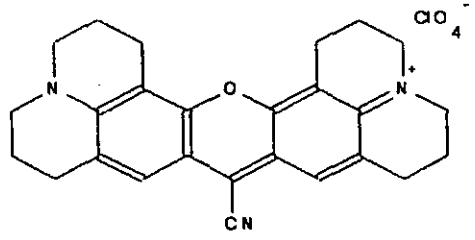

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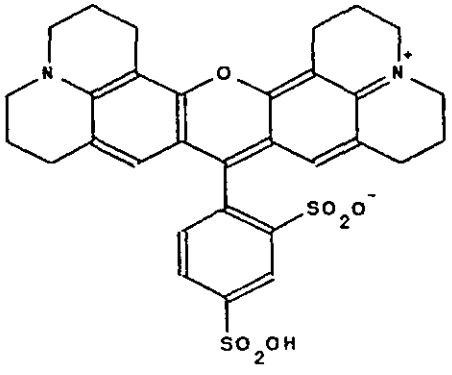

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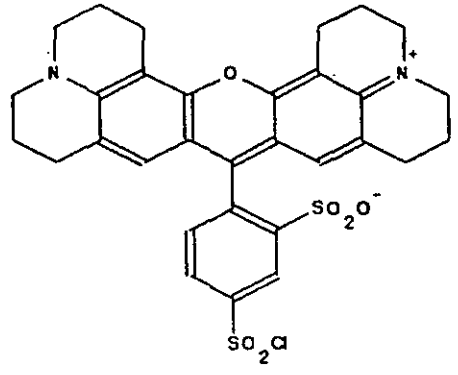
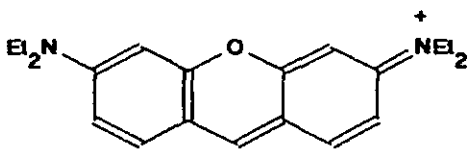


XIII

Figure 3.3: Rhodamines: excitation and emission wavelength in methanol

		Ex (nm)	Em (nm)
VIII	Rhodamine 6G [Rhodamine 590]	524	550
IX	Rhodamine B [Rhodamine 610]	552	550
X	Rhodamine 110	496	512
XI	Rhodamine 800	660	700
XII	Sulphohodamine 101	578	605
XIII	Texas Red	580	606



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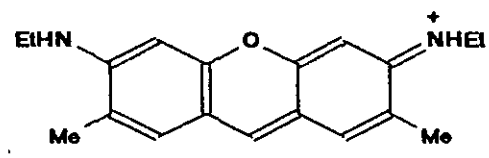


XV

Figure 3.4: Pyronines: excitation and emission wavelngths in methanol

		Ex (nm)	Em (nm)
XIV	Pyronine B	552	562
XV	Pyronine 20	527	540

3.3.1 Results

Table 3.1: The solubility of the xanthenes in distilled water (pH~6), 96% ethanol, diethyl ether, xylene, and dimethyl sulphoxide (DMSO). vs = very soluble (>10% w/v), s = soluble (~1% w/v), ss = slightly soluble and i = insoluble.

	Solubility				
	water	ethanol	ether	xylene	DMSO
Fluoresceins					
Fluorescein [IV]	0.03%[1]	2.21%[1]	s	s	
Fluorescein sodium [IV][2]	50.03%[3]	7.2%[2]	i	ss	10.0%[1]
FITC [VI]	<0.01%[1]	2.0%[1]	s	s	
Rose Bengal [VII]	36.3%[3]	7.5%[3]	i	i	
Rhodamines					
Rhodamine 6G [VIII]	5.4%[3]	5.0%[1]	i	i	
Rhodamine B [IX]	1.2%[3]	1.5%[3]	ss	ss	
Rhodamine 800 [XI]	ss	s	i	i	s
Sulphorhodamine 101 [XII]	2.0%[1]	3.0%[1]	i	i	s
Pyronines					
Pyronine B [XIV]	2.0%[1]	0.7%[1]	ss	ss	vs

[1] Green (1990).
[2] The sodium salt of fluorescein, also known as Uranin.
[3] Lillee (1969).

Table 3.2: pKa for the xanthenes.

	pKa_1	pKa_2	pKa_3
Fluoresceins			
Fluorescein [IV]	2.2[1]	4.4[1]	6.7[1]
Naphthofluorescein [V]	7.99[2]		
Rose Bengal [VII]	3.5[3]		
Rhodamines			
Rhodamine B [IX]	4.6[4](3.1[5])		
Rhodamine 800 [XI]	11.0[3]		
Pyronines			
Pyronine B	7.1[4]		

[1] pKa value: Lee et al. (1989), Bishop (1972) and Haugmann (1989, 1992)
[2] pKa value: Lee et al. (1989)
[3] pKa^* value: buffered 50% methanol (Summerfield, unpublished).
[4] pKa^* value: buffered 50% ethanol (Woislawski, 1953)
[5] pKa value: Andre and Molinari (1976)

Table 3.3: The chemical stability of fluoresceins (Fluorescein [IV] and Rose Bengal [VII]) and rhodamines (Rhodamine B [IX] and Rhodamine 800 [IX]) to acids and alkalis.

	Fluorescein		Rose Bengal	
	Abs./nm	colour	Abs./nm	colour
conc. H_2SO_4		yellow		brown
5M HCl			220	colourless, on dilution deep pink
1M HCl	220	colourless on dil. pink then yellow	220	colourless, on dilution deep pink
pH 4	435	pink with green fluorescence	550	deep pink
Water	490	yellowish red with green fluorescence	550	deep pink
pH 10	490	yellowish red with green fluorescence	550	deep pink
pH 11.5			550	deep pink
1M NaOH		dark yellow solution with dark green fluorescence		crimson red precipitate

	Rhodamine B		Rhodamine 800	
	Abs./nm	colour	Abs./nm	colour
Conc. H_2SO_4		yellow-brown with green fluorescence	526	mauve, on dil. orange then blue
5M HCl			462/495/526	orange
pH 4	558	deep pink	605/668	turquoise
Water	555	deep pink	605/668	turquoise
pH 10	555	deep pink	605/668	turquoise
pH11.5			605/668	turquoise
1M NaOH		rose red precipitate	395	pale yellow

Table 3.4: Spectroscopic properties of the xanthenes in distilled water (pH ~6). The molar absorptivity (ε), fluorescence quantum efficiency (ϕ_f) and fluorescence lifetime (τ_f) are all literature values from Birge and Bohwon (1986), Brackmann (1986) and Haugmann (1989, 1992).

	Abs. (nm)	$\varepsilon \times 10^4$ $M^{-1}cm^{-1}$	Em. (nm)	ϕ_f	τ_f (ns)
Fluoresceins					
Fluorescein [IV]	491	6.6	521		4.5
Fluorescein sodium [IV][1]	488	8.6	514	0.81	4.5
Rose Bengal [VII]	549	7.76	590		
Rhodamines					
Rhodamine 6G [VIII]	524	19.9	550	0.23	
Rhodamine B [IX]	552	1.00	576	0.70	3.0
Rhodamine 800 [XI]	685		700	0.90	
Sulphorhodamine 101 [XII]	595	8.5	615	0.30	
Pyronines					
Pyronine B [XIV]	550		560		

[1] The sodium salt of fluorescein is also known as Uranin.

Table 3.5: The spectroscopic properties of xanthenes in methanol. The molar absorptivity (ε), fluorescence quantum efficiency (ϕ_f) and fluorescence lifetime (τ_f) are literature values from Birge and Bohwon (1986), Brackmann (1986) and Haugmann (1989, 1992). The limit of detection (LOD) was calculated in the usual manner.

	Abs. (nm)	$\varepsilon \times 10^4$ $M^{-1}cm^{-1}$	Em. (nm)	ϕ_f	LOD (g/l)
Fluoresceins					
Fluorescein [IV]	491		530		
Fluorescein sodium [IV][1]	498	6.39	518	0.90[e]	2.39×10^{-10}
Rose Bengal [VII]	554		581		
Rhodamines					
Rhodamine 6G [VIII]	528	10.4	553	0.95[e]	
Rhodamine 800 [XI]	674	8.95[e]	708		8.11×10^{-10}
Rhodamine B [IX]	545	10.2	565	0.60	
Sulphorhodamine 101 [XII]	578	13.9	605	0.90	
Pyronines					
Pyronine B [XIV]	552		562		

[1] The sodium salt of fluorescein, also known as Uranin
[e] ethanol

Table 3.6: The influence of a nonpolar solvent, (diethyl ether or chloroform) and a polar solvent (distilled water or methanol), on the absorbance wavelength of various xanthenes. A positive $\Delta\lambda_{abs}$ represents a bathochromic shift in polar solvents and a negative $\Delta\lambda_{abs}$ represents a hypsochromic shift.

	λ_{abs}/nm (nonpolar solvent)	λ_{abs}/nm (polar solvent)	$\Delta\lambda_{abs}$/nm
Fluoresceins			
Rose Bengal [VII]	565 (ether)	550 (water)	15
Rhodamines			
Rhodamine 6G [VIII]	550 ($CHCl_3$)	524 (water)	26
Rhodamine B [IX]	562 ($CHCl_3$)	552 (water)	10
hodamine 110 [X]	506[1] ($CHCl_3$)	498[1] (MeOH)	8
Rhodamine 800 [XI]	672 (ether)	685 (water)	-13
Sulphorhodamine 101 [XII]	589 (ether)	587 (water)	2

[1] Zollinger, 1990.

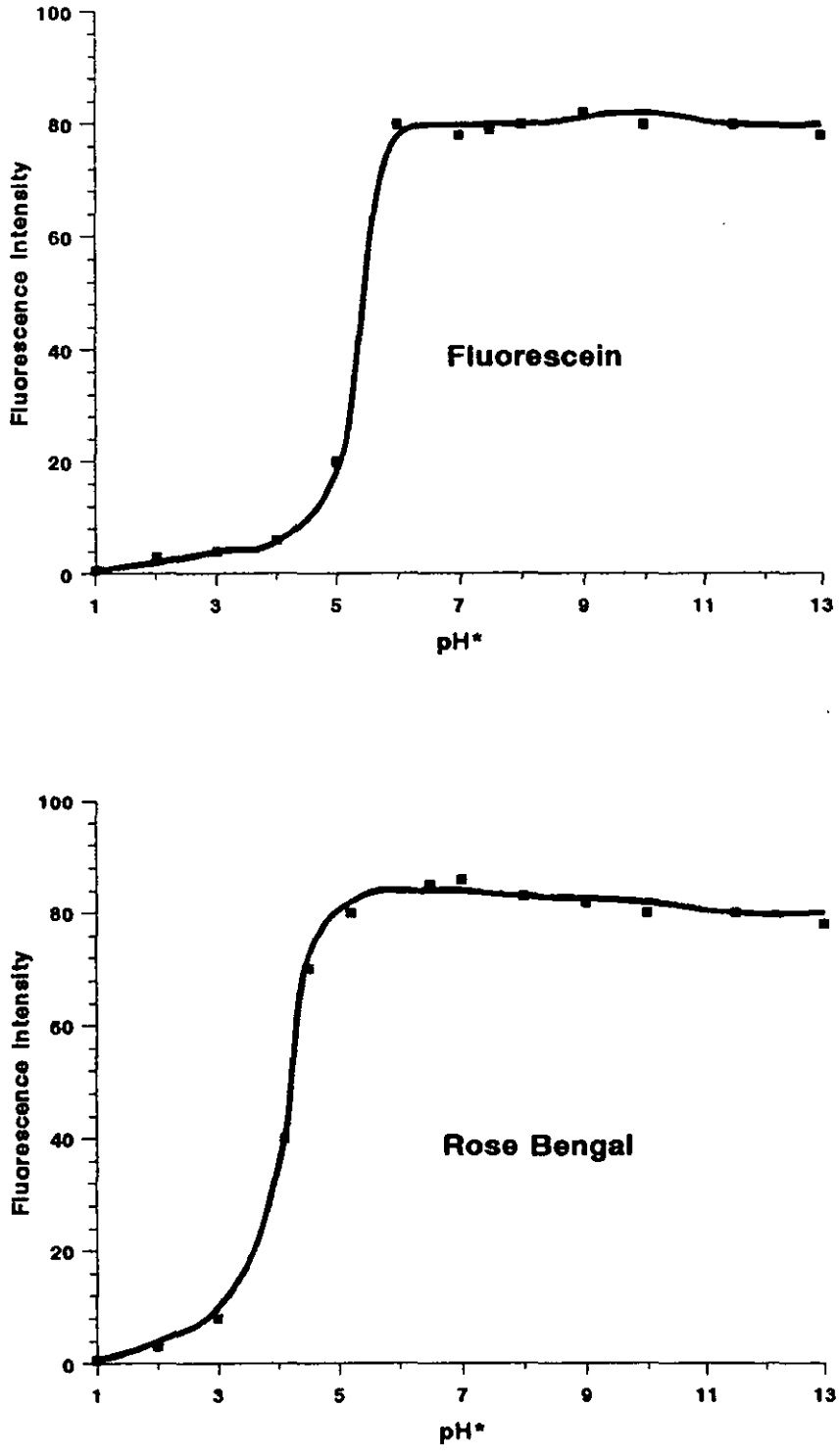


Figure 3.5: Influence of pH* on the fluorescence of Fluorescein [IV] and Rose Bengal [VII] in 50% methanol.

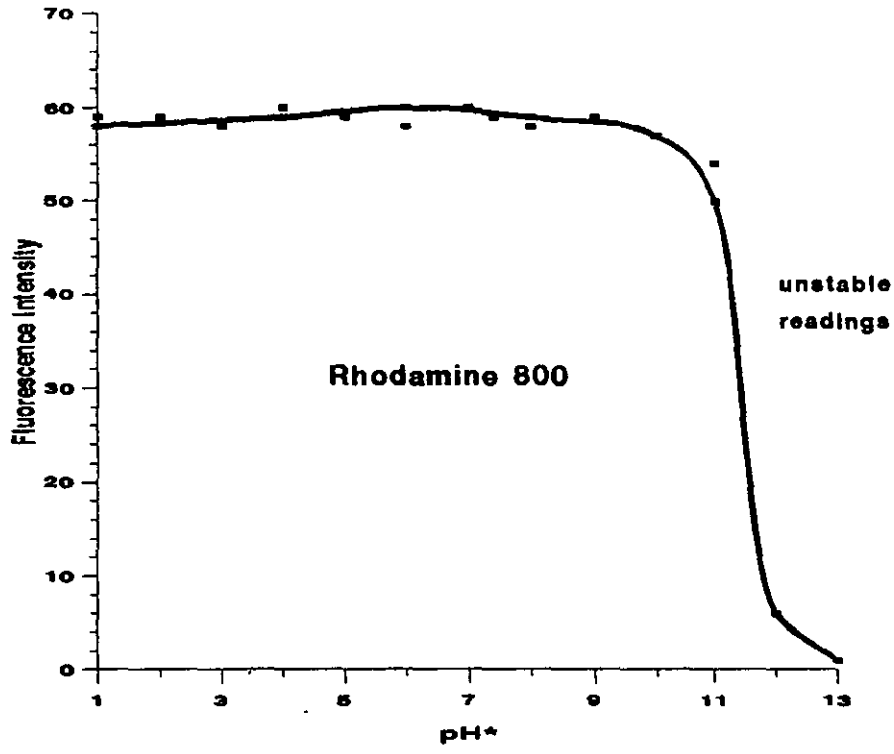


Figure 3.6: The influence of apparent pH* on the fluorescence intensity of Rhodamine 800 in 50% methanol.

3.3.2 Discussion

The solubility of the xanthenes was found to be regulated by their ionic form. Xanthenes with a non esterified carboxylic acid (e.g. Fluorescein sodium, Rose Bengal (Figure 3.2 [VIII]), Rhodamine B (Figure 3.3 [IX])) were very soluble in water as the sodium salt but only slightly soluble as the free acid.

Fluorescein (Figure 3.2 [IV]) and Rhodamine B [IX], xanthenes with free carboxylic groups, were protonated at the 2'-position and lactonisation occurred at the 9-position at low pH. For example, Fluorescein [IV] was in the lactone (cationic) form below pH 2.2 (colourless and not fluorescent); between 2.2 and 4.4 (neutral with the carboxylic acid protonated); between pH 4.4 and 6.7, Fluorescein was mono-anionic (pink with green fluorescence) and above pH 6.7 Fluorescein was di-anionic (yellowish red with green fluorescence). The fluorescence intensity for Fluorescein [IV] was zero below pH* 6 as shown in Figure 3.5. The fluorescence of Rhodamine 800 (Figure 3.3 [XI]) was drastically reduced above pH* 10, as shown in 3.6, by the formation of the neutral salt which was characterised also by a large hypsochromic shift of absorbance.

The xanthenes were decomposed by concentrated nitric acid. The reaction in other concentrated mineral acids was slow (Table 3.3). Xanthene dyes were more chemically stable than the polymethines (see section 3.4).

Xanthenes were only partially de-coloured by neutral sodium dithionite but were colourless in alkaline sodium dithionite. The colour returned on the exposure to air or by the addition of mild oxidising agent (for example, 2% potassium persulphate and 2% sulphuric acid).

An increase in temperature reduced the fluorescence of Fluorescein [IV] and Rhodamine B [IX] by 1.2% per °C and 2.5% per °C respectively. Drexhage (1973a,b) reported the fluorescence quantum efficiency of Rhodamine B in ethanol as 40% at room temperature which was increased to almost 100% by reducing the temperature and only a couple of percent at the boiling point of ethanol.

Pyronine B had the characteristic small Stokes' shift of 10 nm of the pyronines. The fluoresceins and rhodamines had Stokes' shift of between 20 and 30 nanometres (Table 3.5 and 3.6). The absorbance and fluorescence wavelengths of the xanthenes were bathochromically shifted according to the substitution of substituents in the 2, 7-position in the xanthene ring as shown in Table 3.7. This substituent also influenced the fluorescence quantum yield as indicated by Reynolds and Drexhage (1975), who reported that the coupling of two alkyl groups to the aromatic ring as in Rhodamine 101 and Sulphorhodamine 101 [XII] reduced the

mobility of the amino groups so the fluorescence quantum efficiency reached almost 100% at room temperature.

Table 3.7: The influence of substituents in the 2,7-position in the xanthene ring on the absorbance and fluorescence wavelengths in methanol for a homologous series of xanthenes.

Substituent		Dye	Abs. (nm)	Fl. (nm)
hydroxyl	OH	Fluorescein [IV]	491	521
1° amine	NH_2	Rhodamine 110 [X]	496	512
2° amine	NH-alkyl	Rhodamine 6G [VIII]	524	553
3° amine	$N(alkyl)_2$	Rhodamine B [IX]	545	565
cyclised 3° amine		Sulphorhodamine 101 [XII]	578	605

A further bathochromic shift was caused by changing the group in the 9-position (Table 3.8).

Table 3.8: The influence of substituents in the 9-position in the xanthene ring on the absorbance and fluorescence wavelengths in ethanol for a homologous series of xanthenes.

Substituent		Dye	Abs. (nm)	Fl. (nm)
carboxyphenyl		Rhodamine 101	568[1]	583[1]
sulphoxyphenyl		Sulphorhodamine 101 [XII]	578	605
trifluoromethyl	$-CF_3$	Rhodamine 700	643[1]	666[1]
cyano	-CN	Rhodamine 800 [XI]	674	708

[1] Absorbance and fluorescence values from Brackmann (1986).

The absorbance of the xanthenes displayed a small solvatochromic shifts in wavelength with increasing solvent polarity (Table 3.6). The tertiary amines of Rhodamine B (Figure 3.3 [IX]), Rhodamine 800 [XI] and Sulphorhodamine 101 [XII] showed more solvent dependence than Rhodamine 110 [X] with primary amines and Rhodamine 6G [VIII] with secondary amines. The zwitterionic Rhodamine B [IX] was bathochromically shifted by about 10 nm from that of the acid form. Polar solvents, high concentrations and low temperature displaced the equilibrium towards

the zwitterion and hence, the Rhodamine B [IX] in polar solvents is bathochromically shifted. In non-polar solvents the zwitterionic form was unstable and the colourless lactone was formed, which photochemically reverted to the coloured species.

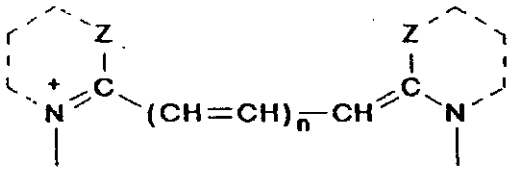
3.4 Polymethines

Polymethines absorb between 340 to 1400 nanometres and were first discovered in 1856 by Greville Williams, they are important in the photographic industry because of their ability to sensitise silver halide to longer wavelength radiation. Polymethines have also been used as dyes for xerography (Jones, 1989), lasers (Birge and Bohwon, 1986; Schäfer, 1973; Maeda, 1984; Brackmann, 1986; Steppel 1982), for potential-sensitive measurements (Haugmann, 1989, 1992), as photo-, thermo-, and piezochromic dyes (Matsuoka, 1990), in liquid crystal displays, as infrared absorbing dyes for optical storage (Nakazumi, 1988; Emmelius et al., 1989), as cosmetic ingredients and as quasi-drugs (Matsuoka, 1990). The chemistry of the polymethines has been reviewed by Venkataraman (1952), Hamer (1964), Ficken (1971), Sturmer (1977, 1979a,b) and Raue (1990b).

Systematic nomenclature of the polymethines is somewhat confusing due to the number of classification systems in use. The names carbo-, dicarbo-, tricarbo-(etc.)cyanines refer to the number of ethylene units, n = 1, 2, and 3 respectively. The length of the ethylene unit in the conjugation bridge strongly influences the position of the absorbance band, and near-infrared absorption generally can be obtained with more than three ethylene units.

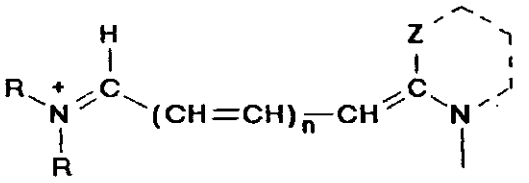
Cationic polymethines (Figure 3.7) are called cyanines [XVI], styryls (hemicyanines) [XVII] or streptocyanines [XVIII] respectively according to whether both, one or none of the nitrogens are components of the ring. The cyanines where n is zero, are non-fluorescent with the exception of 3,3'-diethyloxacyanine. Hofer et al. (1950) explained this was due a steric effect of the end-groups of the molecule. The structure of the terminal group influences the the position of the absorbance band and the fluorescence quantum yield, benzoxazole (Figure 3.11 [XXXIII]) and benzothiazole (Figure 3.11 [XXXV]) have the highest. The carbocyanines such as DTTC (Figure 3.8 [XXI]) and HITC [XXII] have been determined in picomolar amounts using a pulsed semiconductor laser fluorimeter, (Imasaka et al., 1985). Ernst et al. (1989) used cyanines and merocyanines as fluorescent labels for the thiol groups of proteins. Mujumbar et al. (1989) used isothiocyanate derivatives of cyanines as covalent labelling reagent for proteins. Indocyanine Green (IR125) (Figure 3.8 [XXIII]) has achieved some importance in the measurement of cardiac output (Heseltine, Broocker & Eastman Kodak USP 2,895,955).

Cyanines



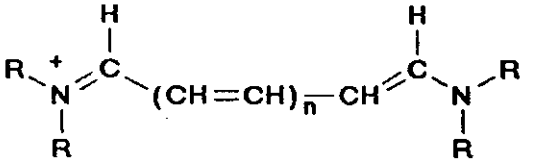
XVI

Hemicyanines



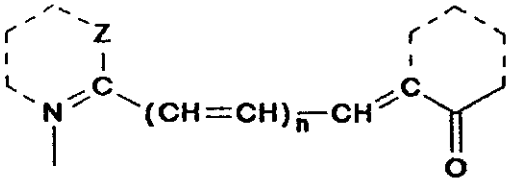
XVII

Streptocyanines



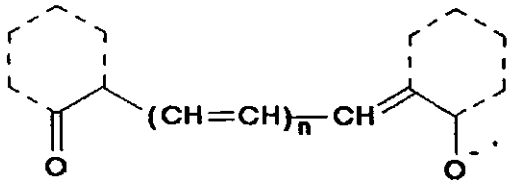
XVIII

Merocyanines



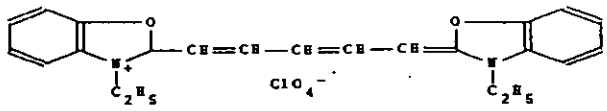
XIX

Oxonols

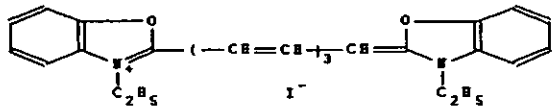


XX

Figure 3.7: General structures for the polymethines.
n is the number of ethylene groups;
R is either a hydrogen atom, alkyl or aryl group;
Z is either a heteroatom (O, S, Se, NR_2), CH or CR_2.



XXI



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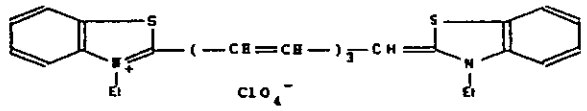
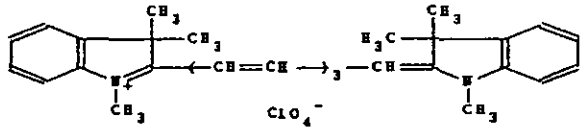
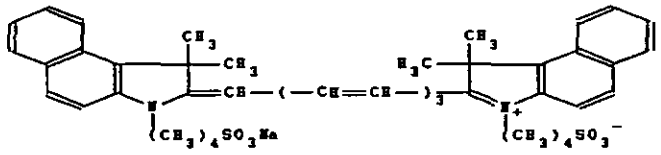


XXIII



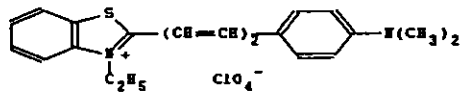
XXIV



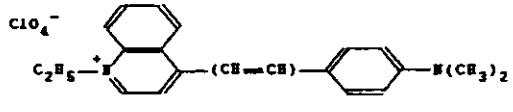
XXV

Figure 3.8: Symmetrical polymethines: excitation and emission wavelengths in methanol.

		Ex (nm)	Em (nm)
XXI	DODC perchlorate	579	603
XXII	DOTC iodide	695	705
XXIII	DTTC perchlorate	755	788
XXIV	HITC perchlorate	739	775
XXV	IR125 (Indocyanine Green)	780	830



XXVI



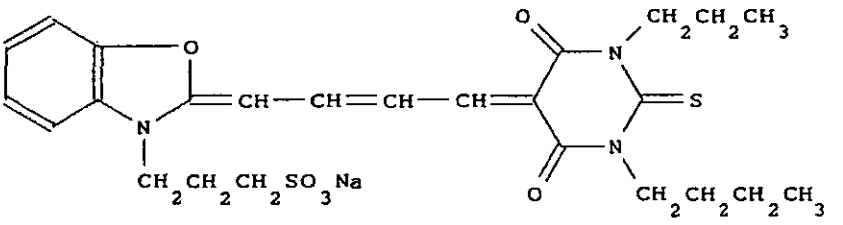
XXVII

Figure 3.9: Hemicyanines (styryl): excitation and emission wavelngths in methanol.

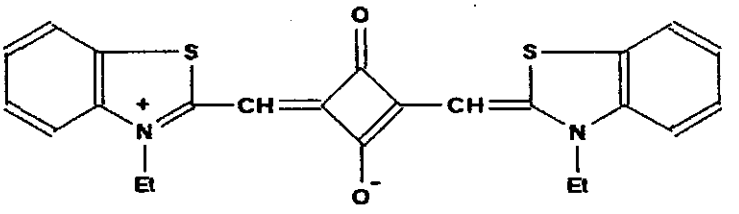
		Ex (nm)	Em (nm)
XXVI	Styryl 7	565	704
XXVII	Styryl 11	558	638

Merocyanines [XIX] (Figure 3.7) are neutral polymethines and were first developed as photographic sensitizers (Hamer, 1982; Broocker et al. 1965). Merocyanine 540 (Figure 3.10 [XVI]) has been used for recording the intra-cellular potential (Dragston and Webb, 1978; Ross et al., 1974; Waggoner, 1976 and 1979) and for binding to leukaemia cells (Easton et al., 1978; Schleger, 1980). Merocyanines have also been examined for anti-tumour activity but their activity in this respect was rather low (Peterson, 1964).

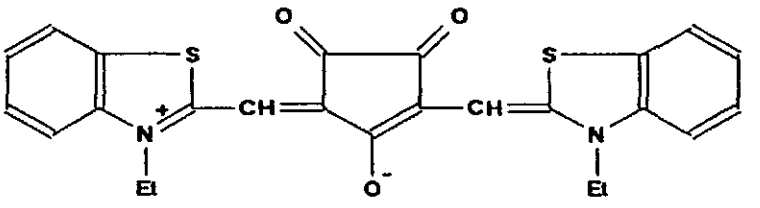
Oxonols [XX] (Figure 3.7) are anionic polymethines and are the most unstable of the polymethines. The stability decreases rapidly as the number of ethylene groups increases. These are not widely used due to their lack of stability and were not investigated.



XXVIII



XXIX



XXX

Figure 3.10: Merocyanines and rigidised polymethines: excitation and emission in methanol

		Ex (nm)	Em (nm)
XXVIII	Merocyanine 540	540	560
XXIX	a thiazolium squarylium	663	ND
XXX	a thiazolium croconium	771	ND

3.4.1 Extending the wavelength of the polymethines

1. The absorption and the corresponding fluorescence bands are bathochromically shifted by about 100nm for symmetrical polymethines by lengthening the conjugation bridge by one ethylene group (Figure 3.11). A smaller bathochromic shift is observed for the asymmetric polymethines (Broocker et al., 1951). The extinction coefficients initially increase with increasing chain length until n=4 when the molar absorptivity decreases sharply and is accompanied by a flattening of the absorption curve. The stability is reduced with increasing chain length caused by the oxidative attack of the long chain.

2. Rigidisation of the bridge by adding a cyclic group (such as the squaryliums, Figure 3.10 [XXIX] and the croconiums, Figure 3.10 [XXX]), to the conjugation bridge bathochromically shifts the wavelength and increases the photo-stability of the dye. The introduction of the croconic moiety into the methine chains of cyanines produces a 120-126 nm bathochromic shift; introduction of the squaric moiety produces only a 12 to 18 nm bathochromic shift (Matsuoka, 1990).

3. Increasing the basicity of the heteroatom causes a bathochromic shift. This is shown in figure 3.11 with the end-groups arranged with increasing basicity.

   a. heterocyclic termini (e.g. benzothiazole, Figure 3.11 [XXXV]) give a greater bathochromic shifts than acyclic termini.

   b. unsaturated terminal groups (e.g. benzoxazole, Figure 3.11 [XXXII], benzothiazole [XXXIII] and indole [XXXIV] are more bathochromic than the corresponding dyes with saturated terminal groups (e.g. thiazole [XXXII] and N-diethyl [XXXI]).

   c additional conjugation at the terminal groups can also cause a further bathochromic shift (cf. indole (Figure 3.11 [XXXIV]) and benzoindole [XXXVII]).

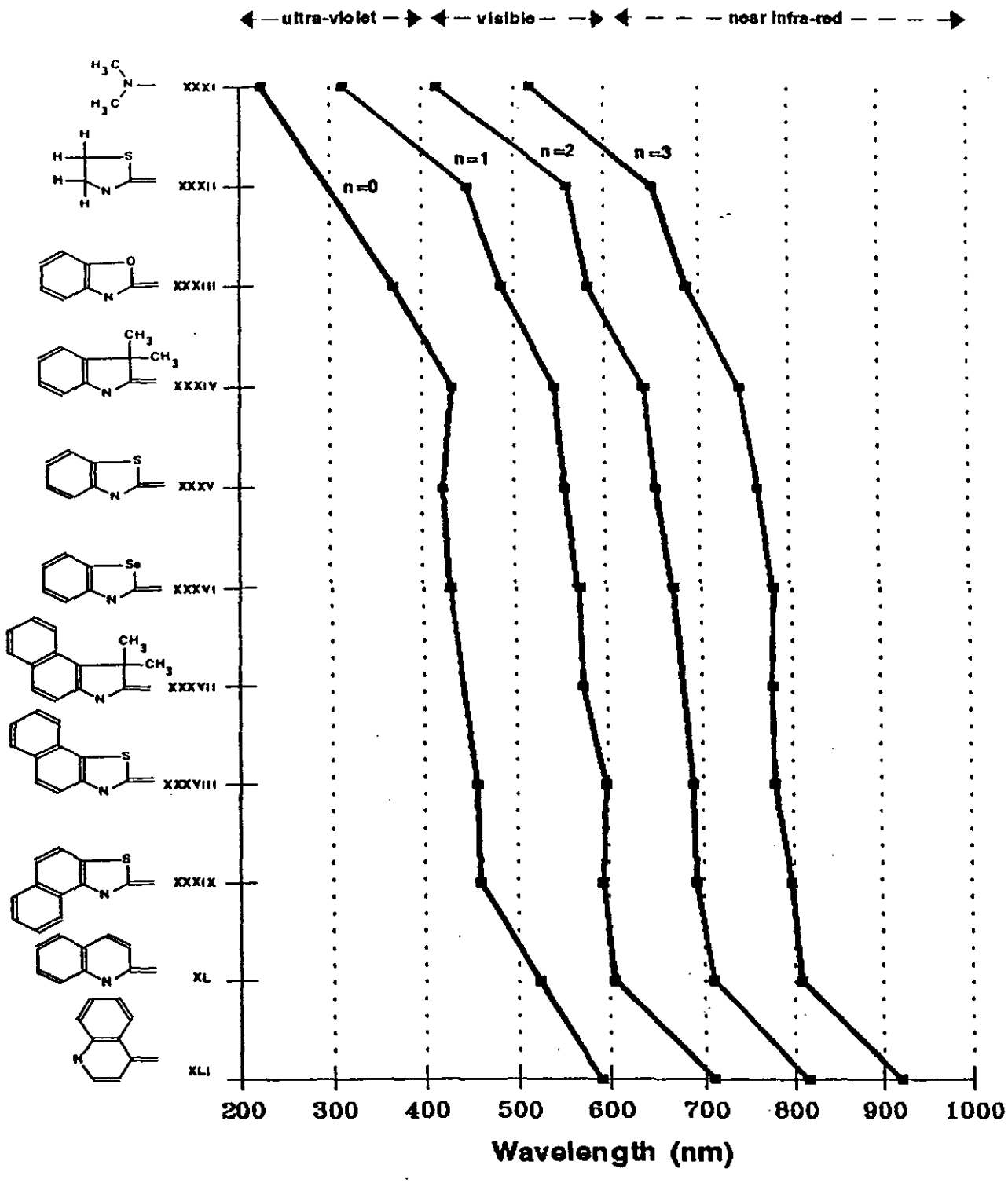


Figure 3.11: The influence of changing end groups and the number of ethylene groups (n) on the absorbance maxima of homologous series of symetrical carbocyanines in methanol. After Brooker et al. (1945, 1951), Brackmann (1986), Okawara et al. (1988), Venkataraman (1952) and Summerfield (unpublished).

XXXI	N,N-dimethyl	XXXVII	benzoindole
XXXII	thiazole	XXXVIII	alpha-naphthothiazole
XXXIII	benzoxazole	XXXIX	beta-naphthothiazole
XXXIV	indole	XL	2-quinoline
XXXV	benzothiazole	XLI	4-quinoline
XXXVI	benzoselenazole		

3.4.2 Results

Table 3.9: The solubility of the polymethines in distilled water (pH~6), methanol, diethyl ether, and dimethyl sulphoxide. (vs = very soluble (>10% w/v), s = soluble (~1% w/v), ss = slightly soluble and i = insoluble).

	Solubility			
	water	methanol	ether	DMSO
symmetrical carbocyanines				
DODC [XXI]	s	vs	i	s
DOTC [XXII]	s	vs	i	s
DTTC [XXIII]	ss	s	i	s
HITC [XXIV]	s	s	i	s
IR125 [XXV]	s	s	i	vs
asymmetrical carbocyanines				
Styryl 7 [XXVI]	s	s	i	s
Styryl 11 [XXVII]	s	s	i	s
merocyanines				
Merocyanine 540 [XXVIII]	s	vs	i	vs

Table 3.10: Approximate pKa* values for the carbocyanines in 50% methanol.

	pKa_1*	pKa_2*
Carbocyanines		
DODC	2.0	10.1
DOTC	1.5	9.2
DTTC	1.8	
IR125	3.1	

Table 3.11: The chemical stability of symmetrical dicarbocyanine DODC [XXI] to alkalis and acids.

	Abs. (nm)	colour
Conc. H_2SO_4	220	instantly discoloured exothermically, not as violently as DOTC
5M HCl	360/460	instantly discoloured & a golden yellow solution developed
1M HCl	345/460	instantly discoloured
pH 4	576	deep pink
Water	577	deep pink
pH 10	577	deep pink
pH 11.5	580	violet
1M NaOH	435	pink solution quickly turned to orange and then finally to a yellow solution.

Table 3.12: The chemical stability of asymmetrical dicarbocyanine Styryl 7 [XXVI] to alkalis and acids.

	Abs. (nm)	colour
Conc. H_2SO_4	220	instantly discoloured with some heat evolved
5M HCl	350/450	instantly discoloured & a golden yellow solution developed
1M HCl	345/460/560	slowly discoloured
pH 4	560	deep pink
Water	560	deep pink
pH 10	560	deep pink
pH 11.5	575	violet
1M NaOH	420	pink solution turned quickly orange and then finally to a pale yellow solution.

Table 3.13: The chemical stability of symmetrical tricarbocyanines DOTC [XXII], DTTC [XXIII] and IR125 [XXV] to alkalis and acids.

	DOTC Abs. (nm)	colour
Conc. H_2SO_4		immediate decomposed to leave a pale yellow solution
5M HCl	360	colourless then a pale pink colour develops (decomposed)
1M HCl	374	pale yellow on dilution blue colour returns.
pH 4	678	blue
Water	678	blue
pH 10	678	blue
pH 11.5	620/678	dark blue
1M NaOH	295/452	yellow (decomposed)

	DTTC Abs. (nm)	colour
Conc.. H_2SO_4		violent reaction, decomposed to pale yellow solution
5M HCl	384	decomposed to a yellow solution
1M HCl	390	pale yellow green then yellow (decomposed slowly)
pH 4	605/745	deep green blue
Water	650/750	turquoise
pH 10	605	turquoise
pH 11.5	615/740	greenish blue
1M NaOH		slowly turned purple from turquoise (decomposed)

	IR125 Abs. (nm)	colour
Conc. H_2SO_4		decomposed violently to a bright golden yellow solution
5M HCl	460	yellow that is slowly de-coloured
1M HCl	460	yellow
pH 4	705/788	black green
Water	788	dark green
pH 10	705/788	blue green
pH 12	700	turquoise
1M NaOH	400/715	grass green, then slowly becomes more yellow

Table 3.14: Absorption and emission wavelengths of polymethines in distilled water (pH~6).

	Abs. (nm)	Em. (nm)
Symmetrical Carbocyanines		
DODC [XXI]	577	597
DOTC [XXII]	678	698
DTTC [XXIII]	750	770
HITC [XXIV]	732	753
IR125 [XXV]	788	810
Asymmetrical Carbocyanines		
Styryl 7 [XXVI]	560	690
Styryl 11 [XXVII]	555	685
Merocyanines		
Merocyanine [XXVIII]	533	572

Table 3.15: Spectroscopic properties of polymethines in methanol. The molar absorptivity (ε), fluorescence quantum efficiency (ϕ_f) and fluorescence lifetime (τ_f) are literature values from Birge and Bohwon (1986) and Brackmann (1986). The limit of detection (LOD) was determined in methanol and calculated in the usual manner.

	Abs. (nm)	$\varepsilon \times 10^4$ $M^{-1}cm^{-1}$	Em (nm)	ϕ_f	τ_f (ns)	LOD (g/l)
Symmetrical Carbocyanines						
DODC [XXI]	579	24.0	603	0.44[e]	0.42[e]	
DOTC [XXII]	695	25.1[e]	705	0.49[e]	1.15[e]	1.40×10^{-10}
DTTC [XXIII]	755	20.9[e]	788			5.10×10^{-10}
HITC [XXIV]	739	24.2	775	0.50[a]	1.2[a]	2.56×10^{-10}
IR125 [XXV]	780		830			5.83×10^{-10}
Asymmetrical Carbocyanines						
Styryl 7 [XXVI]	565	6.15	704			
Styryl 11 [XXVII]	558	4.77[e]	638			
Merocyanines						
Merocyanine 540	540		560			

[e] = ethanol; [a] = acetone.

Table 3.16: The influence of a nonpolar solvent (chloroform or dichloromethane) and a polar solvent (distilled water or methanol) on the absorbance wavelength of various tricarbocyanines.

	λ_{abs}/nm (nonpolar solvent)	λ_{abs}/nm (polar solvent)	$\Delta\ \lambda_{abs}$/nm
Tricarbocyanines			
DOTC [XXII]	705 (CH_2Cl_2)	695 (MeOH)	10
DTTC [XXIII]	785 ($CHCl_3$)	758 (water)	27
HITC [XXIV]	757 ($CHCl_3$)	740 (MeOH)	17
IR125 [XXV]	800 ($CHCl_3$)	780 (water)	20

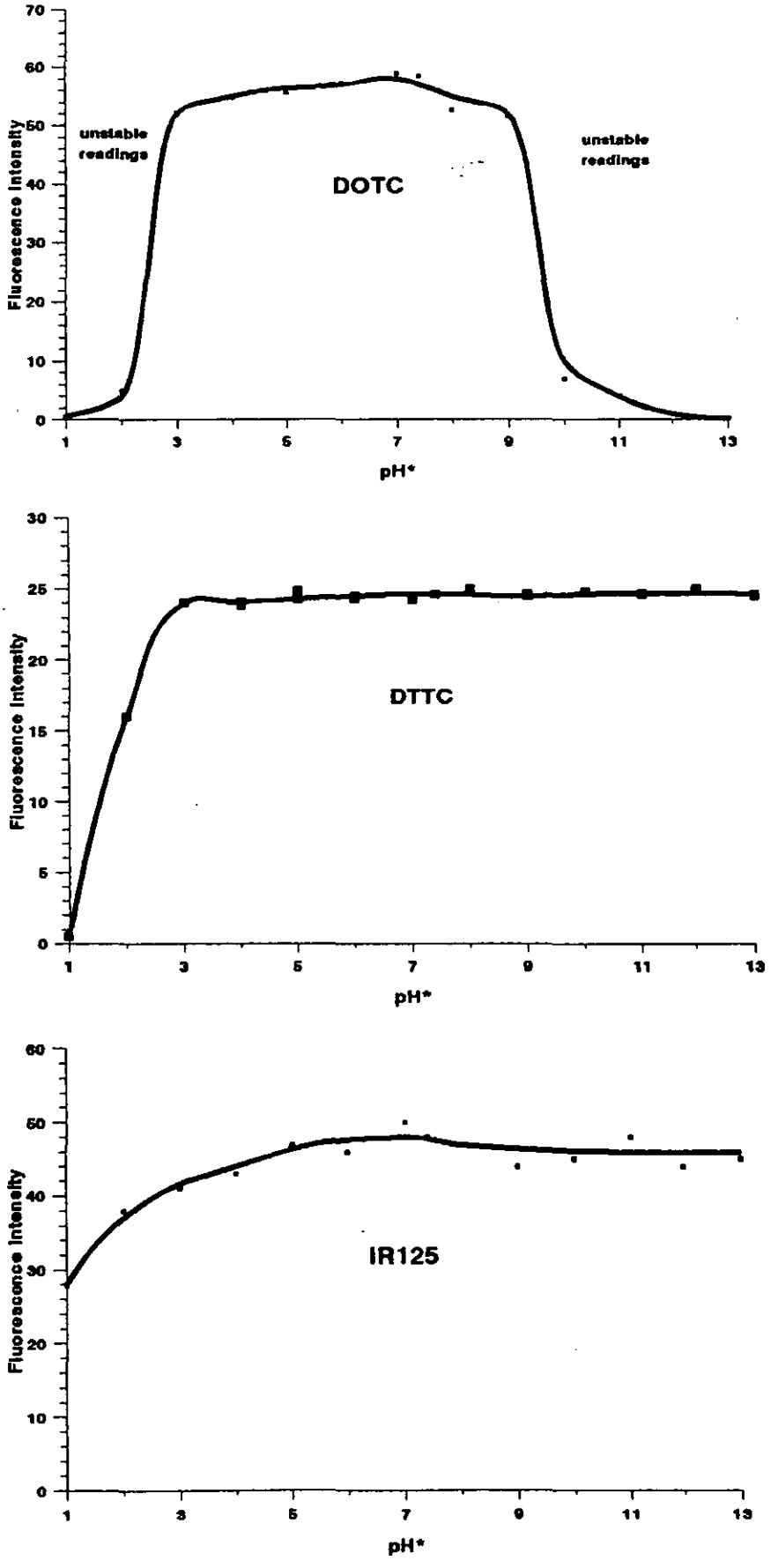


Figure 3.12: Influence of pH* on the fluorescence of DOTC [XXII], DTTC [XXIII] and IR125 (Indocyanine Green) [XXV] in 50% methanol.

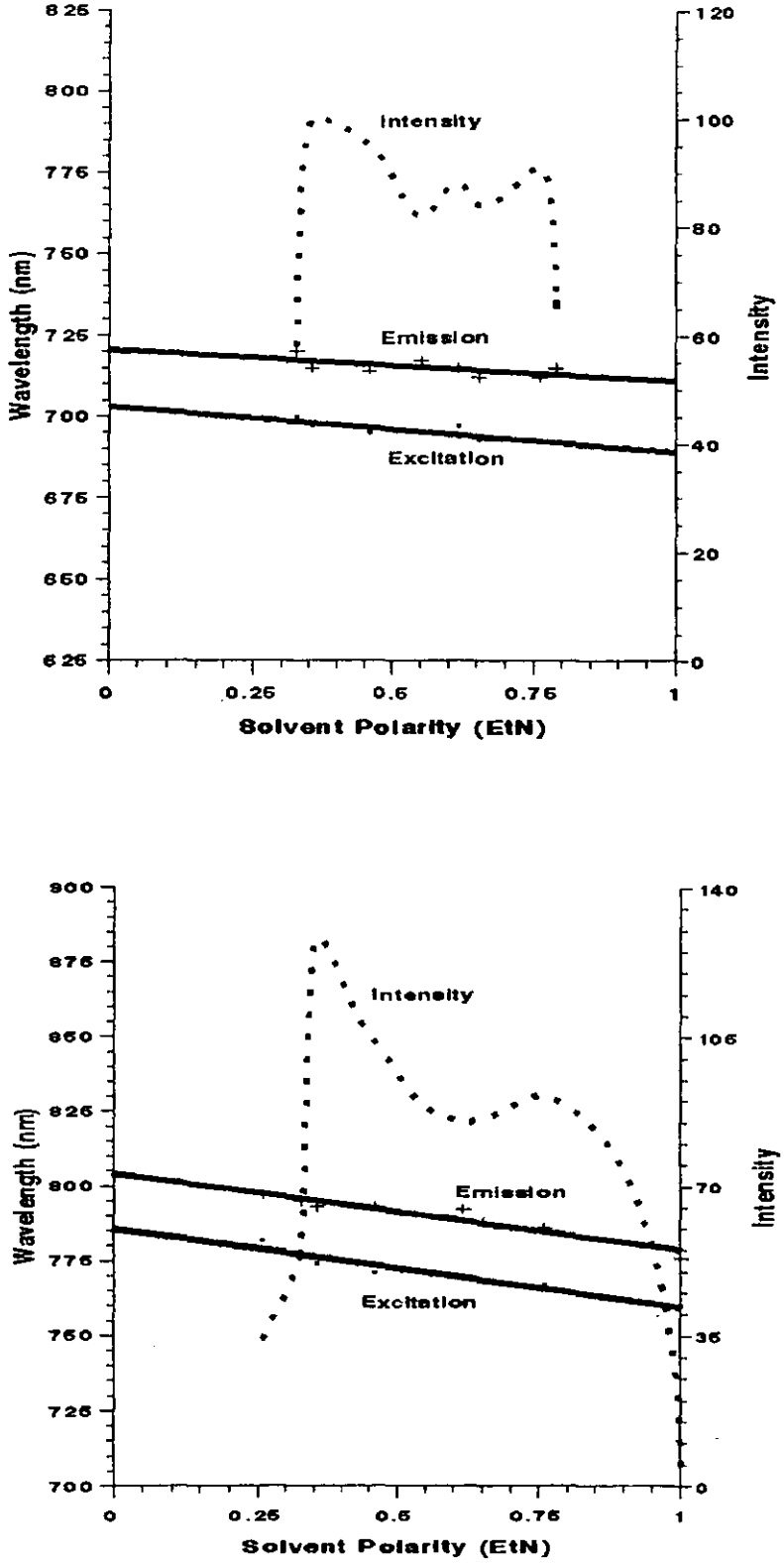


Figure 3.13: The influence of solvent polarity on the fluorescence intensity, excitation and emission wavelength of DOTC [XXII] (top) and DTTC [XXIII] (bottom). Both DOTC and DTTC shows a small hypsochromic shift in polar solvents.

3.4.3 Discussion

All the polymethines tested (Table 3.9) were soluble in water and other hydrophilic organic solvents (i.e. acetonitrile, ethanol, methanol etc.) but had low solubility in hydrophobic solvents (such as diethyl ether and n-hexane). The indolenines, HITC (Figure 3.8 [XXIV]) and IR125 [XXV]) and benzoxazoles (DOTC [XXI] and DOTC [XXII]) were more soluble than the benzothiazole (DTTC [XXIII]). Their solubility in organic solvents was strongly influenced by the combination of the nature of the N-alkyl substituent, the heteroatom in the aromatic ring, and the counter anion (e.g. iodide, chloride, perchlorate etc.). Matsuoka (1990) stated that the perchlorates were more soluble in non-polar solvents, such as methylene chloride, than the iodides, which were more soluble in polar solvents, such as methanol and water.

The polymethines were more chemically unstable than any of the dye groups tested. All polymethines were completely decomposed by concentrated nitric, sulphuric acid, 5 molar hydrochloric acid, and one molar sodium hydroxide (Tables 3.11 to 3.13). The stability of the polymethines was significantly reduced with increasing chain length. This was also caused by the keener susceptibility to oxidative attack of the longer conjugation bridge. This was shown by the less violent reaction of dicarbocyanines (DODC (Figure 3.8 [XXI]) and Styryl 7 (Figure 3.9 [XXVI])) to concentrated sulphuric acid than that of the tricarbocyanines (DOTC [XXII], DTTC [XXIII] and IR125 [XXV]).

Polymethines were not de-coloured by neutral sodium dithionite but were de-coloured by either alkaline sodium dithionite or boiling with glacial acetic acid and zinc dust. Upon oxidation by the air, the colour was returned. The mild oxidizing agent, potassium persulphate and 2% sulphuric acid destroyed the chromophore.

Cyanines were reversibly protonated in acid solution with the loss of the visible absorption band and subsequent reduction in fluorescence intensity as shown in Figure 3.12. Various authors have used this property to measure the basicity of cyanines (Broocker et al., 1965; Ficken, 1971).

The molar absorptivity and fluorescence quantum efficiency was a function of the counter-ion. Molar absorptivities and fluorescence quantum efficiency were best in the following order; perchlorate, chloride and iodide as the anion. Styryl 11 [XXVII] and DTTC [XXIII] showed very poor fluorescence in water. The structural relationship of the polymethines has already been discussed in Section 3.4.1.

An increase in temperature caused the fluorescence of DTTC [XXIII] in methanol to be reduced by less than 0.4% per °C and was comparable to that

observed in the ultraviolet-visible region, for example, the fluorescence of Fluorescein (Figure 3.2 [IV]) was reduced by 1.2% per °C.

There was an 11% and 8% increase in fluorescence intensity on the addition of anhydrous sodium sulphite to a dilute solution of DOTC [XXII] and DTTC [XXIII] in methanol respectively. This showed that the fluorescence intensity depended on the concentration of dissolved oxygen in the solvent. Cyclooctatetraene (COT), a triplet quencher, increased both the fluorescence intensity and lifetime of DOTC (Hirth et al., 1973).

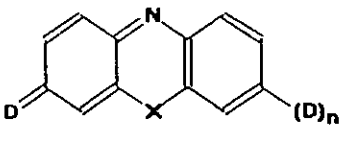
The cyanines (e.g. DTTC [XXIII], DOTC [XXII] and HITC [XXIV]) exhibited a hypsochromic shift in polar solvents (Table 3.16 and Figure 3.13). This was caused by decrease in the dipole moment of the ground state in the more solvating polar solvent. The usefulness of this phenomena is probably limited because the hypsochromic shift is only 10nm as compared to Nile Red's (Figure 3.16 [LIII]) 95 nm. The small Stoke's shift means that a lower excitation wavelength than absorption maxima is required to avoid problems with Rayleigh Scatter. Strongly polar Merocyanine 540 (Figure 3.10 [XXVIII]) shifted its wavelength to shorter wavelength with an increase in solvent polarity. This solvatochromic change was much larger than for the carbocyanines (apart from those caused by aggregation).

3.5 Azines

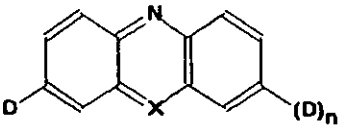
The azines are formed by the replacement of the -CH= group in xanthene by nitrogen, oxygen or sulphur leading to the phenazines, the phenoxazines, and the thiazines respectively (Figure 3.14). According to Kuhn's (1959) gas model of methine dyes, replacing the central methine group with one of greater electron affinity produces a marked bathochromic shift (figure 3.15). The azine dyes include cationic, anionic, and neutral dyes, depending on the class of substituent groups. These are normally amino, arylamino, dialkylamino or hydroxyl groups located in the 3- and 7- positions. The azines show excellent chemical and photo-stability. The chemistry of the azines has been outlined by Venkataraman (1952), McKee (1963), Nursten (1963), Coffey (1978), Conger (1978) and Raue (1985).

Phenazines have the 1,3 diazine as the chromophore, e.g. structure XLV. These are predominately red dyes absorbing between 530 and 590 nanometres and so were not investigated because their absorbance wavelengths were too low to be of interest.

Phenoxazines (Figure 3.16) have a heterocyclic ring with both a nitrogen and an oxygen bridge in the 1,3 position and amine groups at the 3- and/or 7- positions. The phenoxazines are widely used for the dying of acid modified synthetic fibres (Colour Index, 1971; Venkataraman, 1952, 1971), laser dyes, biological stains (Haugmann, 1989, 1992), as photosensitisers for the destruction of carcinoma cells after irradiation (Cinotta et al., 1987) and as inhibitors to tumour growth in chemotherapy. The reduced phenoxazines (the leuco-phenoxazines) are colourless and have been used as dyes for pressure-sensitised copying paper, as highly sensitive oxygen detectors and for the analytical determination of ascorbic acid using the ease with which the leuco-phenoxazine is oxidised to the intensely coloured phenoxazine by exposure to the air. Oxazine 725 [LI] was suggested as a near infrared quantum counter by Demas et al. (1985), and Kopf and Heinze (1984)



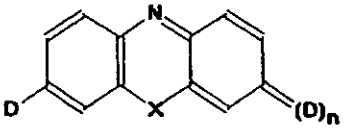
XLII



XLIII

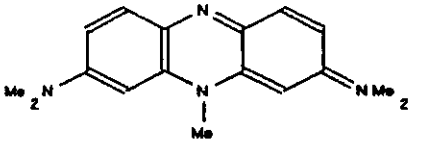
D = primary, secondary or tertiary amine, or hydroxyl group

X = -NR- phenazine (R=H, aryl or alkyl)
or -O- phenoxazine
or -S- thiazine



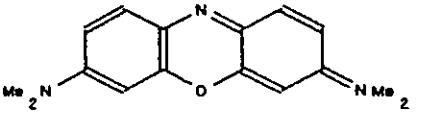
XLIV

Figure 3.14: The resonance structures of the azines. The resonance structure XLIV is only possible when n=1 and this is the predominant structure of the phenoxazones and thiazones.



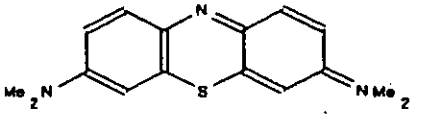
XLV

phenazine 567 nm



XLVI

phenoxazine 645 nm
(Methyl Capri Blue)



XLVII

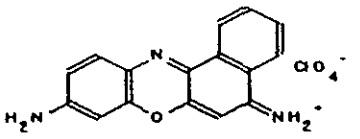
thiazine 653 nm
(Methylene Blue)

Figure 3.15: Absorbance maxima for a homologous series of azine dyes in methanol (Zollinger, 1990 and Summerfield, unpublished).

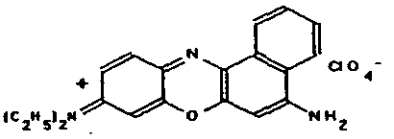
Phenoxazones are related to the phenoxazines but have in the 3-position a hydroxyl group instead of a amine group and occur in nature as orcein dyes in lichens (Schäfer, 1964). These dyes have been used for the dying of textiles made from polypropylene. Nile Red [Figure 3.16; LIII] has been used as a polarity probe (Deye and Berger, 1990), protein probe (Sacket and Wolff, 1987), as an intra-cellular lipid stain (Greenspan et al., 1985a,b), as a laser dye and as a stain for whole tissues (Fowler and Jamieson, 1985; Fowler and Greenspan, 1985).

Thiazines (Figure 3.17) have a heterocyclic ring with both a nitrogen and a sulphur bridge. The introduction of sulphur into the ring causes a bathochromic shift larger than for either the phenazines or the phenoxazines (Figure 3.15). The thiazines are only of minor importance in the textile industry (Zollinger, 1990). They have been used in photochemical applications as sensitizers in photo-polymerisation, in dyeing of paper and as a biological stain (Gurr, 1972). The most important member of this group is Methylene Blue [XLVII]. Toluidine Blue [XXXV] is a RNA stain (Haugmann, 1990). Thiazines have served as the basis of drugs for over a half of a century (Gupta, 1988). From 1911-30 Methylene Blue [XLVII] and its derivatives were researched as antimalarial drugs, but their potential was prevented by their dye character. However, its antimalarial potential lead scientists to the design of analogous heterocyclic systems and served as the basis for the discovery of Plasmoquine as a potential antimalarial. However, it was not until Chloroprimazine was discovered in the 1950's that the real medical potential of the thiazines was realised. This has been shown to have analgesic, anti-shock, anti-convulsive, anti-inflammatory, antipyretic and adrenolylic qualities. Over four thousand thiazines have been prepared and many have been tested for biological activity. In addition, some of them posses significant insecticidal and pesticidal properties.

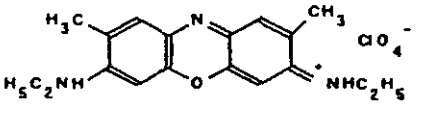
Thiazones are produced as the oxidation products of the thiazines (Dean et al., 1976). Methylene Violet (Figure 3.25) [LVIII] is a degradation product of Methylene Blue [XLVII].



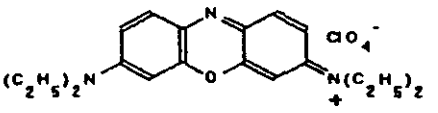
XLVIII



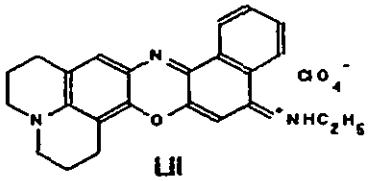
XLIX



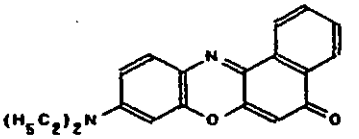
L



LI



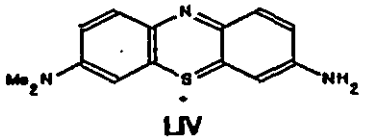
LII



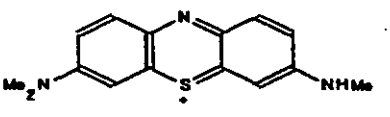
LIII

Figure 3.16: Phenoxazines and Phenoxazones: excitation and emission wavelengths in methanol

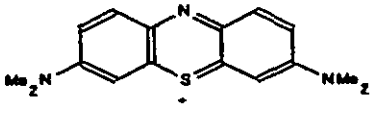
		Ex (nm)	Em (nm)
XLVIII	Cresyl Violet (Oxazine 9)	593	615
XLIX	Nile Blue A	627	660
L	Oxazine 4 (LD690)	615	633
LI	Oxazine 725 (Oxazine 1)	643	658
LII	Oxazine 750	662	680
LIII	Nile Red	565	638



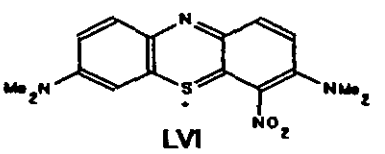
LIV



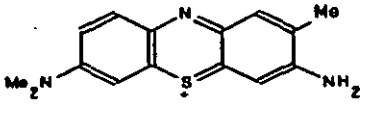
LV



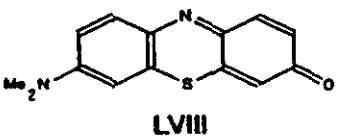
XLVII



LVI



LVII



LVIII

Figure 3.17: Thiazines and thiazones: excitation and emission wavelengths in methanol

		Ex (nm)	Em (nm)
LIV	Azur A	628	653
LV	Azur B	638	665
XLVII	Methylene Blue	653	680
LVI	Methylene Green	650	675
LVII	Toluidine Blue	629	658
LVIII	Methylene Violet	580	610

3.5.1 Results

Table 3.17: The solubility of phenoxazines, phenoxazones and thiazines in distilled water (pH~6), 96% ethanol, diethyl ether, xylene, and dimethyl sulphoxide. [vs = very soluble (>10% w/v), s = soluble (~1% w/v), ss = slightly soluble and i = insoluble.]

	Solubility				
	water	ethanol	ether	xylene	DMSO
Phenoxazines					
Cresyl Violet [XLVIII]	0.38%[1]	0.25%[1]	i	i	s
Nile Blue A [XLIX]	0.2%[2]	0.2%[2]	i	i	s
Oxazine 4 [L]	s	s	i	i	s
Oxazine 725 [LI]	s	s	i	i	s
Oxazine 750 [LII]	s	s	i	i	s
Phenoxazones					
Nile Red [LIII]	0.02%[2]	0.1%[2]	s	s	vs
Thiazines					
Azur A [LIV]	4.0%[2]	1.0%[2]	i	vss	
Azur B [LV]	4.15%[1]	0.68%[1]	i	vss	
Methylene Blue [XLVII]	2.5%[1]	2.5%[1]	i	i	s
Methylene Green [LVI]	1.5%[1]	0.12%[1]	i	i	s
Methylene Violet [LVIII]	0.06%[2]	0.4%[2]	s	s	
Toluidine Blue [LVII]	3.82%[1]	0.57%[1]	i	i	

[1] Lillee, 1969
[2] Green, 1990.

Table 3.18: pKa values for the azines.

	pKa_1	pKa_2
phenoxazines		
Brilliant Cresyl Blue [XLV]	9.9[1]	
Nile Blue A [XLIX]	1.6[1]	9.7[1]
phenoxazones		
Nile Red [LIII]	1.0[2]	
thiazines		
Methylene Blue [XLVII]	3.8[1]	
Methylene Green [LVI]	9.7[1]	
Thionine [LV??]	11.2[3]	
Toluidine Blue [LVII]	10.8[1]	

[1] buffered 50% ethanol (Woislawski, 1953)
[3] Deye and Berger, 1990
[2] unbuffered 50% ethanol (Woislawski, 1953)

Table 3.19: Chemical stability of four phenoxazines (Nile Blue A [XLIX], Cresyl Violet [XLVIII], Oxazine 750 [LII] and Oxazine 4 [L]) to acids and alkalis. (ND is not determined.)

	Nile Blue A		Cresyl Violet	
	Abs. (nm)	colour	Abs. (nm)	colour
Conc. H_2SO_4	430/519	orange	432/508	orange
5M HCl	430	green	332/415/470	golden yellow
pH 4	638	blue	588	violet with red fluorescence
Water	638	blue	588	violet with red fluorescence
pH 10	505	purple-red	480/588	deep crimson
pH 11.5	505	purple-red, brownish red ppt.	470	crimson
1M NaOH		brownish-red ppt	470	golden orange

	Oxazine 750		Oxazine 4	
	Abs. (nm)	colour	Abs. (nm)	colour
Conc. H_2SO_4	522	pink	432/510/580	olive green
5M HCl	518	pink	395/490/615/684	red orange
pH 4	674	turquoise	ND	ND
Water	674	turquoise	615	blue with red fluorescence
pH 10	ND	turquoise	615	blue with red fluorescence
pH 11.5	540/670	pink	615	blue with red fluorescence
1M NaOH	510	pink	460	deep pink

Table 3.20: Chemical stability of Nile Red [LIII] (a phenoxazone) to acids and alkalis.

	Nile Red	
	Abs. (nm)	colour
Conc. H_2SO_4	491	golden yellow
Conc. HCl	ND	yellow
5M HCl	595/637	blue green
pH 4	580	red
Water	580	red
pH 10	580	red
pH 11.5	585	purple
1M NaOH	590	purple-red

Table 3.21: Chemical stability of four thiazines (Azur A [LIV], Azur B [LV], Toluidine Blue [LVIII] and Methylene Blue [XLVIII]) to acids and alkalis.

	Azure A		Azure B	
	Abs. (nm)	colour	Abs. (nm)	colour
Conc. H_2SO_4	437/705	green	655	yellow green
5M HCl	712	blue	657/728	blue green
pH 4	625	blue	648	blue
Water	625	blue	648	blue
pH 10	625	blue	648	blue
pH 11.5	632	blue	648	blue
1M NaOH	530/665	purple solution with dull pink ppt	545	pink solution with violet ppt

	Toluidine Blue		Methylene Blue	
	Abs. (nm)	colour	Abs. (nm)	colour
Conc. H_2SO_4		dark green on on dilution blue		yellow green
5M HCl		blue		
pH 4		blue	663	blue
Water	632	blue with red fluorescence	663	blue
pH 10	632	blue with red fluorescence		
pH 11.5	632	blue with red fluorescence		violet
1M NaOH	ND	pink solution with dull violet ppt	ND	dull pink ppt

Table 3.22: Spectroscopic properties of the phenoxazines, Nile Red and thiazines in water. The molar absorptivity (ε) and fluorescence lifetime (τ_f) are literature values from Birge and Bohwon (1986) and Brackmann (1986).

	Abs. (nm)	$\varepsilon \times 10^4$ $M^{-1}cm^{-1}$	Em (nm)	τ_f (ns)
Phenoxazines				
Cresyl Violet [XLVIII]	590		628	2.32
Nile Blue A [XLIX]	635	3.98	670	0.38
Oxazine 4 [L]	612		635	
Oxazine 725 [LI]	649		667	
Oxazine 750 [LII]	674		692	
Phenoxazones				
Nile Red [LIII]	580		665	
Thiazines				
Azure A [LIV]	625		645	
Azure B [LV]	637		670	
Methylene Blue [XLVII]	663	5.01	682	
Methylene Green [LVI]	655		678	
Methylene Violet [LVIII]	590		620	
Toluidine Blue [LVII]	632		674	

Table 3.23: Spectroscopic properties of the phenoxazines, Nile Red and thiazines in water. The molar absorptivity (ε), fluorescence quantum efficiency (ϕ_f) and fluorescence lifetime (τ_f) are literature values from Birge and Bohwon (1986) and Brackmann (1986). LOD in methanol was calculated in the usual manner.

	Abs. nm	$\varepsilon \times 10^4$ $lM^{-1}cm^{-1}$	Em nm	ϕ_f	τ_f ns	LOD g/l
Phenoxazines						
Cresyl Violet	593	8.30	615	0.70	3.23[1]	
Nile Blue A	627	7.75	660	0.47	1.19[1]	7.0x10-11
Oxazine 4	615	10.3	633		1.13[1]	4.0x10-11
Oxazine 725	643	12.3	658	0.15[e]	1.02[e]	
Oxazine 750	662	8.25[e]	680			
Phenoxazones						
Nile Red	565	4.00	638		2.79[1]	3.0x10-11
Thiazines						
Azure A	628		653			
Azure B	638		665			
Methylene Blue	653		680			
Methylene Green	650		675			
Methylene Violet	580		610			
Toluidine Blue	629		658			8.2x10-11

[e] in ethanol: [1] Dutt et al. (1990)

Table 3.24: The influence of nonpolar solvent (n-hexane or diethyl ether) and polar solvent (water) on the absorbance wavelength of various phenoxazines.

	λ_{abs}/nm (nonpolar solvent)	λ_{abs}/nm (polar solvent)	$\Delta \lambda_{abs}$/nm
Phenoxazines			
Cresyl Violet [XLVIII]	581 ether	690 water	9
Nile Blue [XLIX]	626 $CHCl_3$	635 water	9
Oxazine 4 [L]	610 ether	615 water	5
Oxazine 725 [LI]	642 ether	649 water	6
Oxazine 750 [LII]	630 ether	665 water	35
Phenoxazones			
Nile Red [LIII]	485 n-hexane	580 water	95

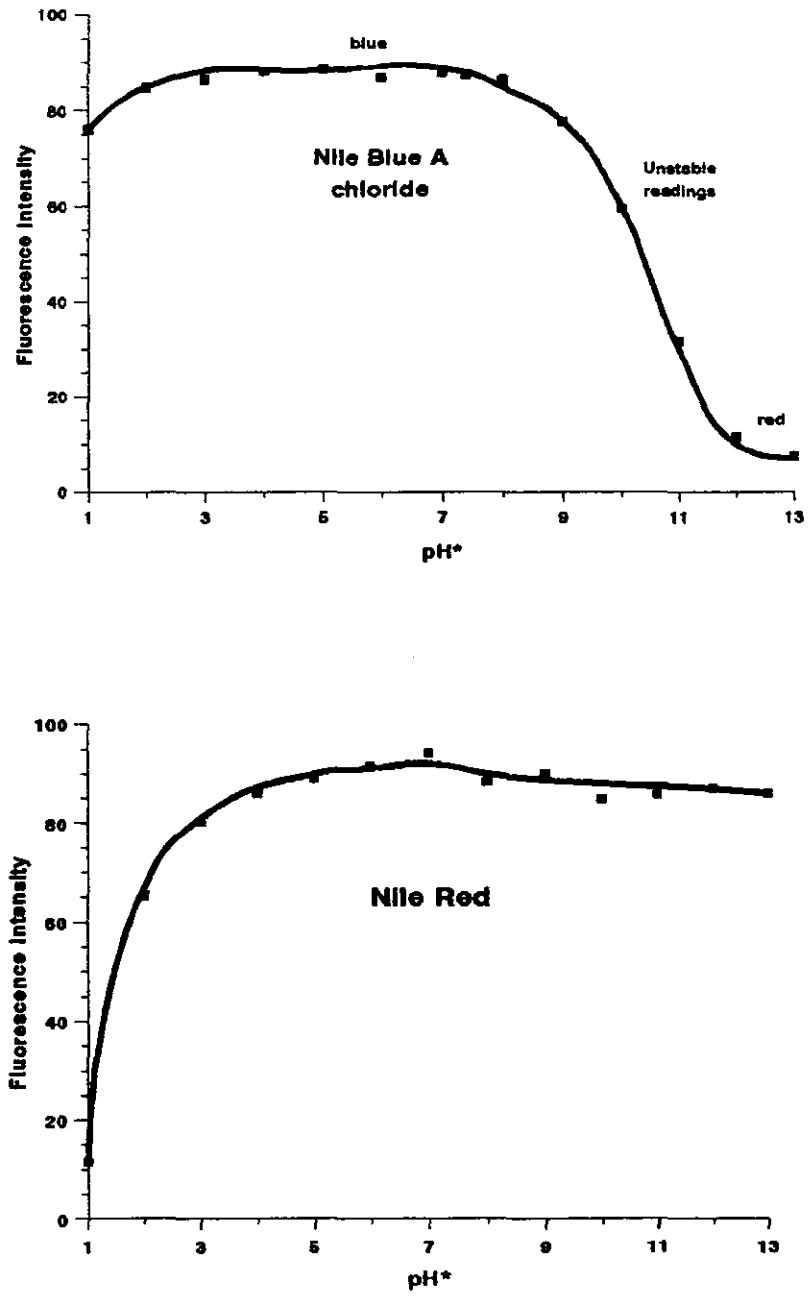
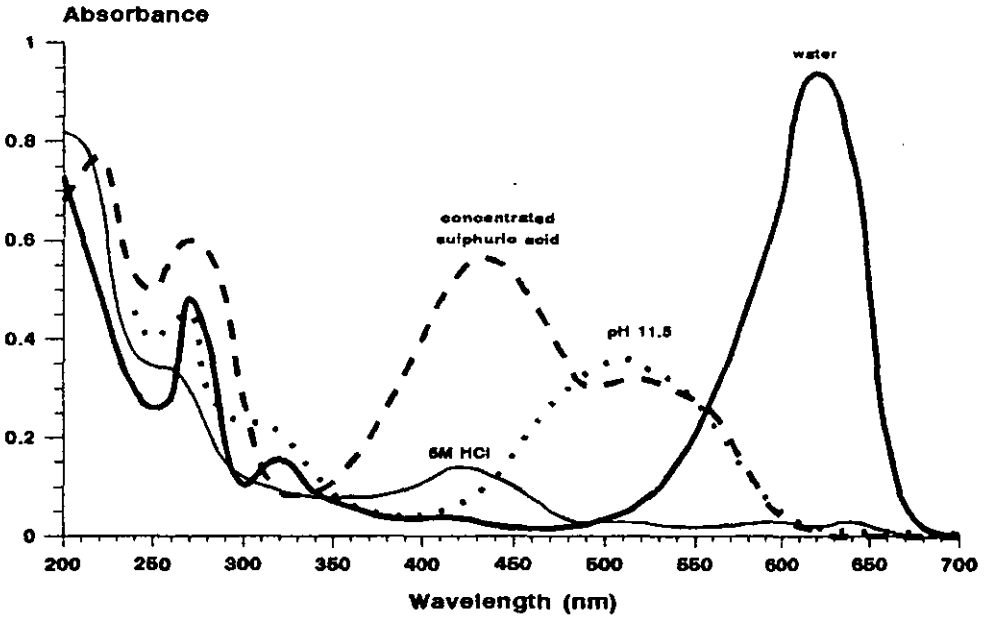


Figure 3.18: Influence of pH* on the fluorescence of Nile Blue A chloride [XLIX] and Nile Red [LIII] in 50% methanol.



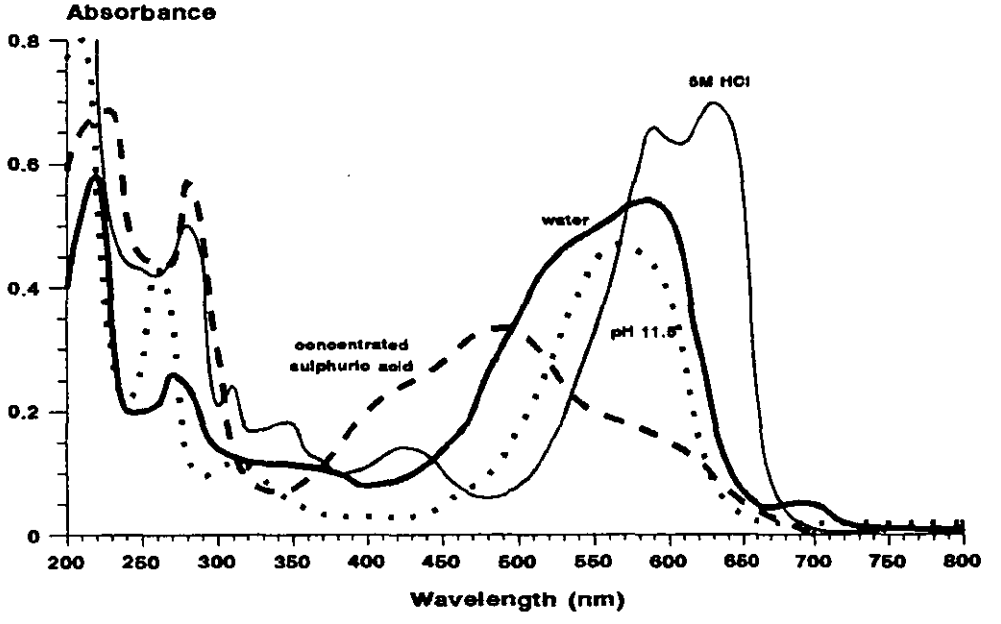


Figure 3.19: Nile Blue A chloride [XLIX] (top) and Nile Red [LIII] (bottom) in concentrated sulphuric acid, 5 molar hydrochloric acid, water and pH 11.5 buffer.

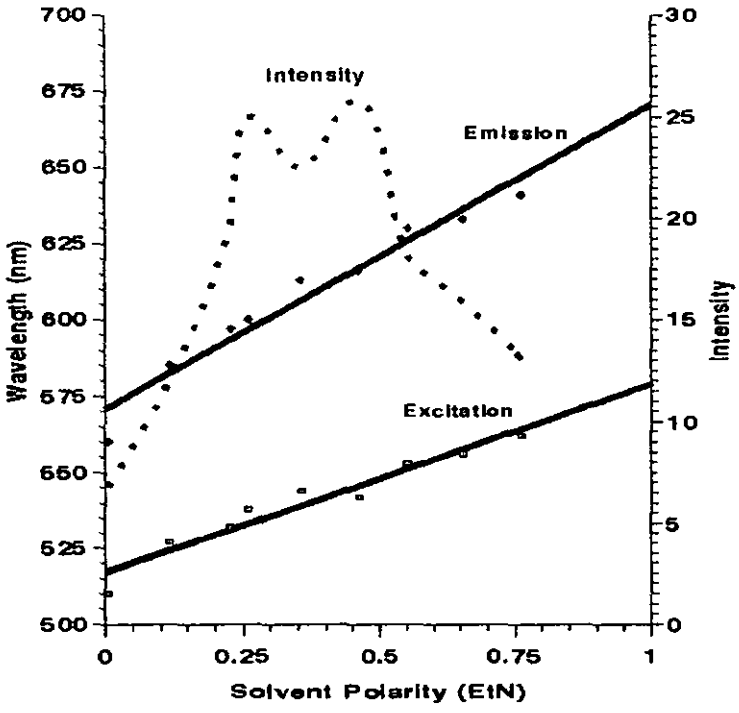


Figure 3.20: The influence of solvent on the excitation, emission and fluorescence intensity of Nile Red [LIII].

3.5.2 Discussion

Nile Blue A (Figure 3.15 [XLIX]) formed solutions in water of colloidal nature above 0.1 micromolar and exhibited dichroism. The fluorescence of Nile Blue A [XLIX] in water was stable over time unlike Nile Red [LIII].

Nile Red [LIII] was soluble in most organic solvents. Nile Red showed halochromic shifts in strong mineral acids. Nile Red formed a stable blue green salt with hydrochloric acid containing one equivalent of the acid that dissociated by adding water. Nile Red was precipitated from glacial acetic acid when the volume of water exceeded 20% of the total. Nile Red was precipitated when an equal volume of water was added to solution of twenty micromolar Nile Red in acetonitrile.

The azines were decomposed in concentrated nitric acid and this was therefore used to clean glassware that had come into contact with the dyes. The azines underwent very slow hydrolysis in the other mineral acids. Nile Red [LIII] was stable in strong acids and strong alkalis. Nile Red, which was discovered as an impurity of Nile Blue A [XLIX], could be extracted from acidified aqueous solution of Nile Blue A [XLIX] and extracted with xylene or diethyl ether. Thorpe (1907) prepared Nile Red [LIII] by boiling Nile Blue A [XLIX] in dilute sulphuric acid overlaid with toluene. The hydrolysis product was extracted into the toluene layer. The phenoxazines and thiazines were destroyed by strong oxidising agents such as chlorates.

The phenoxazines formed insoluble anhydro bases in alkaline solution (Table 3.19). For example, Nile Blue A (Figure 3.21 [XLIX]) formed the insoluble brownish-red anhydro-base [LX] on addition of aqueous sodium hydroxide. Nile Blue did not undergo alkaline hydrolysis. The absorbance band of Nile Blue A [XLIX] was halochromically shifted to 470 nanometres in 5 molar hydrochloric acid (Figure 3.19) by the protonation of both amino groups (Figure 3.21 [LIX]). This was the reason for the reduction in fluorescence shown in Figure 3.18. Above pH 10 the anhydro base [LX] was formed this was not fluorescent. Nile Red [LIII] was protonated in acidic conditions giving a blue product (Figure 3.22 [LXI]) and this caused the shift in absorbance band (Figure 3.19) and reduction of fluorescence as shown in Figure 3.18.

All azines were reduced by both neutral and alkaline sodium dithionite to the pale yellow or colourless leuco form, for example Nile Blue A [XLIX] was reduced to the colourless leuco-compound (Figure 3.23 [LXII]). The colour returned when the solution was oxidised on exposure to the air and on addition of mild oxidizing

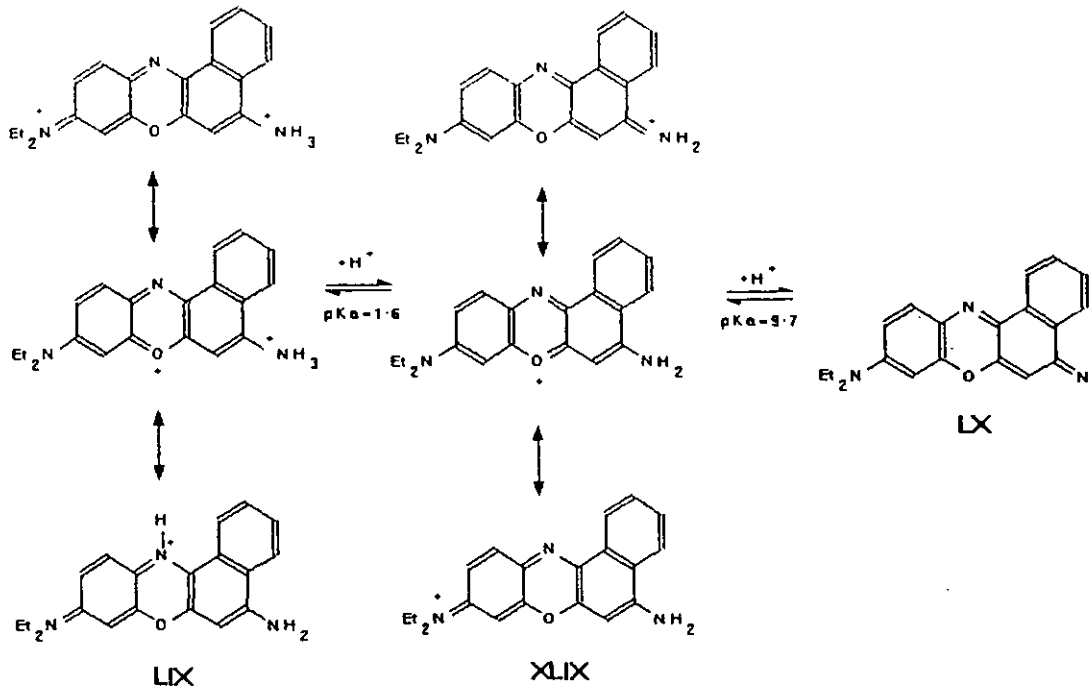


Figure 3.21: Nile Blue A [XLIX] is protonated in acidic conditions to LIX and in alkali the anhydro base [LX] is formed.

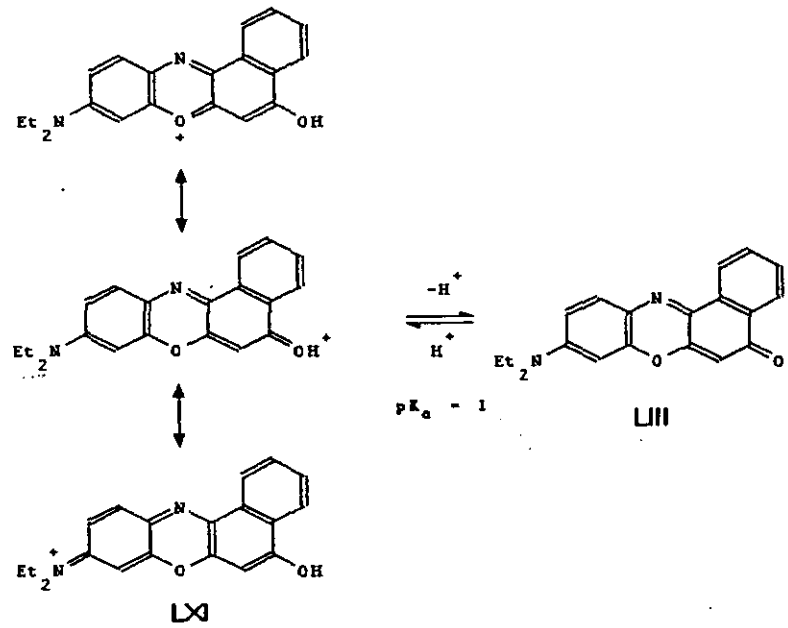


Figure 3.22: Nile Red [LIII] under acidic conditions forms the proptonated form LXI.

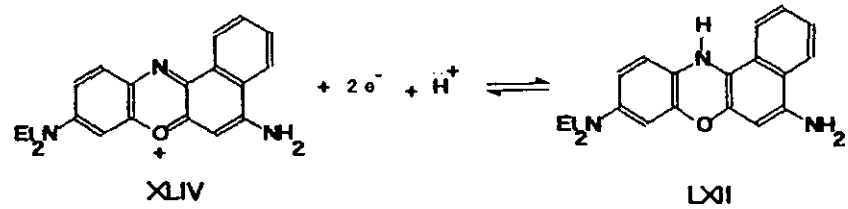


Figure 3.23: Reduction of Nile Blue A [XLIV] to the colourless compound LXII.

agents (such as 2% potassium persulphate and 2% sulphuric acid). The rate of oxidation by air was speeded up upon shaking vigorously the flask, which forced more oxygen to dissolve in the solution. The rate of oxidation was influenced by pH, temperature and by the amount irradiation. At low pH, the re-oxidation of the leuco-form did not occur or was very slow. At high pH (in the presence of sodium hydroxide), the rate of re-oxidation was increased. The absence of light slowed down the oxidation. When a solution of Methylene Blue [XLVII] and ferrous sulphate was irradiated, the leuco form of the dye was formed; the reaction was reversible and in the dark the ferric salt regenerated Methylene Blue [XLVII].

The phenoxazines and thiazines showed an approximately 30 nm Stokes' shift. The azines were bathochromically shifted according to the substituents in the 3,7-position in the azine ring as shown by the following homologous series of thiazines with their absorbances in methanol:-

Table 3.25: The influence of substituents in the 3,7-position in the azine ring on the absorbance and fluorescence wavelengths in methanol for a homologous series of xanthenes.

Substituent		Dye	Abs. (nm)	Fl. (nm
hydroxyl	[OH]	Methylene Violet [LVIII]	580	610
1° amine	[NH_2]	Azur A [LIV]	628	653
2° amine	[NH-alkyl]	Azur B [LV]	638	665
3° amine	[N(alkyl)$_2$]	Methylene Blue [XLVII]	653	680

The azines can be bathochromically shifted by exchanging the heteroatom para to the nitrogen in the azine ring from N-alkyl from oxygen to sulphur as shown in Figure 3.16. The broader absorbance bands of Cresyl Violet [XLVIII], Nile Blue A [XLIX] and Oxazine 750 [LII] were caused by the greater steric interference between the amino group and the hydrogen adjacent to it on the benzene ring in the 1,2-position.

The phenoxazines (Table 3.24) all exhibited bathochromic shifts with the increased polarity of the solvent. This was caused by the Franck-Condon excited state having a higher dipole moment than the ground state. A widening of the Stokes' shift at the longer wavelengths (higher polarity) was also observed. Nile Red [LIII] (Figure 3.20) showed both the greatest solvatochromic shift and Stoke's

shift of the dyes tested. Nile Red [LIII] has been used as a polarity probe in super-critical fluid chromatography (Deye and Berger, 1988). The addition of water to solutions of Nile Red dissolved in water miscible solvents (i.e. acetone, methanol, ethanol and DMSO) caused a bathochromic in absorption.

Methylene Blue underwent sequential alkaline hydrolytic demethylation in aqueous solution (figure 3.24) to produce what is termed Polychrome Methylene Blue, a commonly used biological stain (Dean et al., 1976). The products included Azur A [XXX], Azur B [XXXI], Azur C [XXXII], Bernsthen Methylene Violet [XXXIII] etc. This process was accelerated by the addition hydrogen peroxide, potassium dichromate, silver oxide or some other oxidising agent. Methylene Violet Bernsthen is produced commercially in up to 40% yield by treating a dilute ammoniacal solution of Methylene Blue with potassium dichromate. After the ammonia is driven off by heating with an alkali carbonate, the resulting product is recrystalised from 1,2-dichloroethane. Methylene Blue was decomposed at temperatures above 85 °C.

Toluidine Blue was in the form of the lake with tungsten (II) chloride. The free dye was isolated by precipitating a solution with barium chloride or 5% sodium hydroxide saturated with salt. The dye remaining in solution was isolated and recrystalised from methanol.

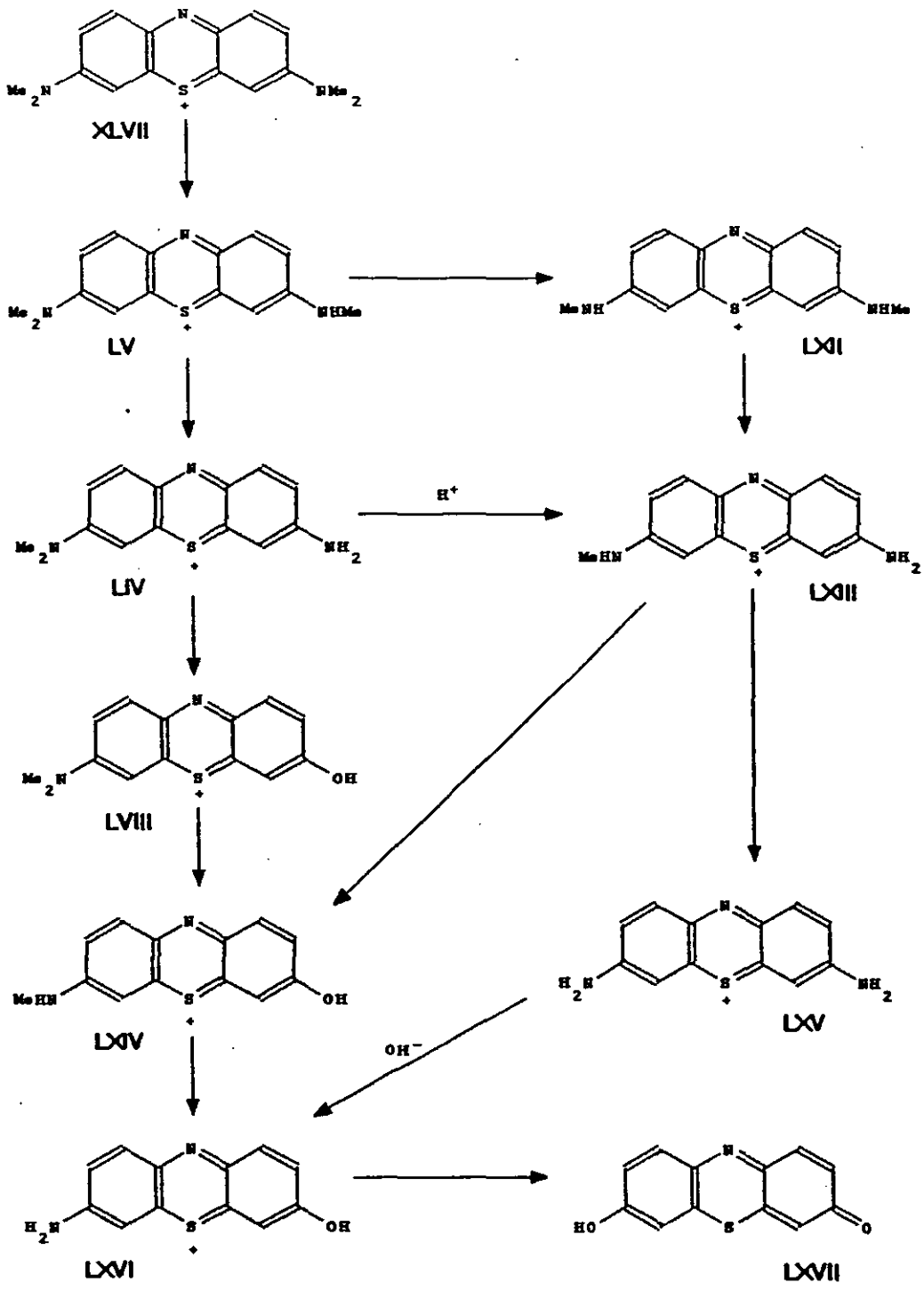


Figure 3.2.4: Sequential alkaline hydrolytic demethylation of Methylene Blue [XLVII] in aqueous solution.

XLVII	Methylene Blue		
LV	Azur B	LXI	symmetrical dimethyl Thionin
LIV	Azur A	LXIII	Azur C
LVIII	Methylene Violet		
LXIV	Methyl Thionolin	LXV	Thionin
LXVI	Thionolin	LXVII	Thionol

3.6 Other near infrared fluorophores

3.6.1 Indigoid

Indigo (Figure 3.25 [LXVIII]) is one of the oldest known natural dyes. Mummy cloths, 5000 years old, have been found dyed with indigo [LXVIII]. The chromophore is a simple crosswise arrangement of two electron donors and two electron acceptors at an ethylene double bond. In all technically important each donor and acceptor (C=O) pair is bonded to benzene or naphthalene rings. The substituents in the benzene ring have no affect on the absorbance maximum. Indigo and its derivatives cover a narrow absorbance range of 570-645nm.

In addition to NH (indigo [LXVIII]), N-alkyl [LXIX], sulphur (thioindigo [LXX]), selenium (seleno-indigo [LXXI]) and oxygen (oxi-indigo [LXXII]) can act as electron donors. The unusually high melting point (300-390 °C) and the poor solubility of indigo can be explained in terms of its crystalline structure; the X-ray diagram (von Eller, 1955) show that in the solid state indigo forms a hydrogen-bonded polymer in which each indigo molecule is linked to four surrounding molecules.

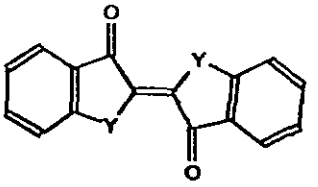
3.6.2 Natural compounds

Chlorophylls and plant proteins (Figure 3.26) are porphyrins related to porphine [LXXXI] and they fluoresce in the 700 nanometre region in water. These have been suggested as labels for immunoassay by Kronick and Grossman, 1983. The best known porphyrin are the biologically important Chlorophyll a [LXXIII] and Chlorophyll b [LXXIV] (the green pigment required by plants for photosynthesis), and haemin (the red colouring matter essential for oxygen transport in the blood).

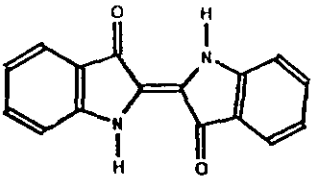
3.6.3 Quinonoid

The extended quinonoids (Figure 3.27) are naturally occurring compounds and have been synthesised for for use as dyes (Bien et al., 1985). Amino- and hydroxyl groups and their substituted forms, NHR, NR_2, NHCOR, OR are frequently present and act as auxochromes. The solubility of these dyes even in organic solvents is low and they are insoluble in water except for the sulphonic acid derivatives.

Violanthrone (e.g. LXXV and LXXVI) and isoviolanthrone dyes are derived from symmetrical or asymmetrical condensations of two molecules of benzanthrone. Violanthrone and its derivatives are fluorescent in the near infrared (Rauhut et al., 1975). The indanthrones (e.g. LXXVII and LXXVIII) are the most important of the



LXVIII to LXXI



LXVIII

Figure 3.25: Indigo type dyes with absorbance wavelengths in chloroform. (Zollinger, 1990)

			Abs. (nm)
LXVIII	Y=NH	indigo (CI-73000)	610
LXIX	Y=N-Me	N,N'-dimethylindigo	650
LXX	Y=S	thioindigo (CI Vat Red 41)	543
LXXI	Y=Se	selenoindigo	567
LXXII	Y=O	oxi-indigo	413

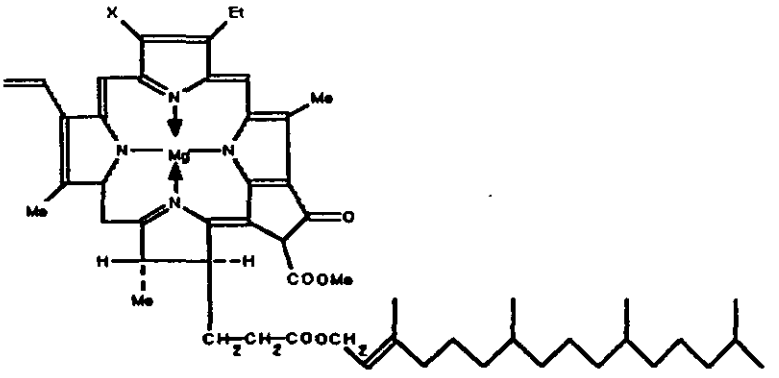
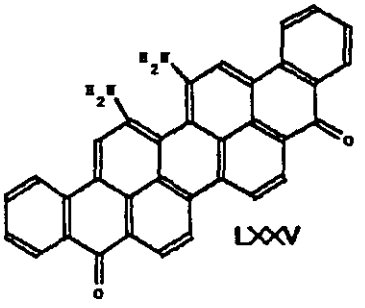
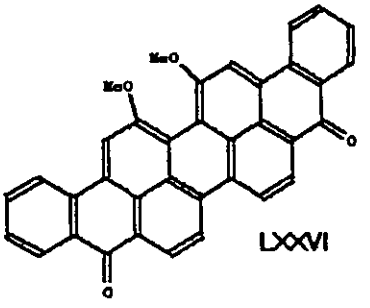


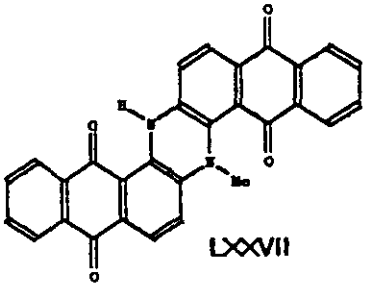
Figure 3.26: Chlorophyll a [x=Me, LXXIII] and Chlorophyll b [x=CHO, LXXIV] excite at 600 nm and emit at 700 nm in water (Kronick and Grossman, 1983).



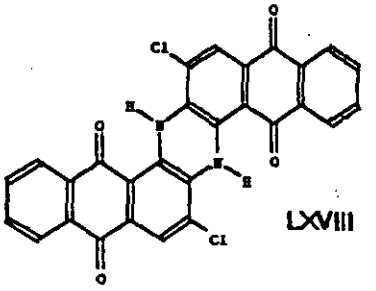
LXXV



LXXVI



LXXVII



LXXVIII

Figure 3.27: Violanthrones and indanthrones: absorbance wavelngths in toluene (Okawara et al., 1988).

		Abs. (nm)
LXXV	16,17-diamino violanthrone	640/700
LXXVI	CI-59825, CI Vat Green 1, 16,17-dimethoxyviolanthrone	636
LXXVII	6-methylindanthrene, CI-70000	720
LXXVIII	CI Vat Blue 6, CI-69825, 7,16-dichloroindanthrene	643/684

extended quinonoid dyes with blue colour in the un-substituted form which is shifted to the green on substitution.

3.6.4 Very large aromatic hydrocarbons

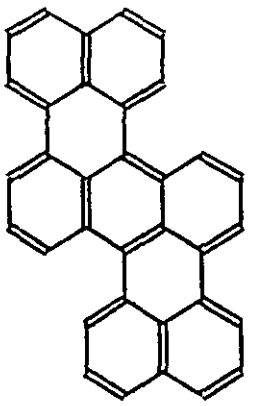
Rauhut et al. (1975) showed that some very large hydrocarbons such as LXXXIX and LXXX in Figure 3.28 that occur in soot and heavy oils fluoresce in the 700 to 820 nanometre region.

3.6.5 Phthalocyanines

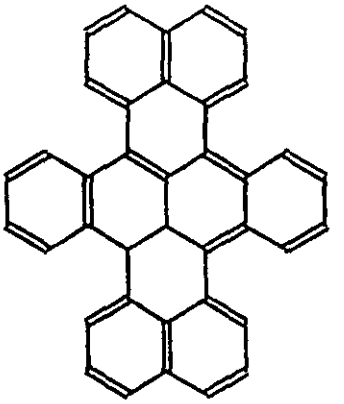
Phthalocyanine (Figure 3.29) has the same chromophoric system as porphine [LXXXI] which is a planar, cyclic 16-centre ring system consisting of four bridged pyrrole rings. Metal free phthalocyanine [LXXXII] was first prepared in 1907 by von Braun and Tscherniak, while iron phthalocyanine [LXXXIV] was first prepared in 1927 by accident during the preparation of phthalimide from phthalic anhydride and ammonia in an iron reaction vessel. The iron phthalocyanine [LXXXVI] was isolated and identified by Linstead in 1934. Since then, phthalocyanines have become important dyes and pigments. The complexes formed between the transition metals, especially copper, and phthalocyanines are chemically very stable to light and heat. The chemistry of the phthalocyanines was reviewed by Moser and Thomas in 1963 and 1983, Lesnoff and Lever in 1989. The colour chemistry of phthalocyanines were reviewed by Booth in 1971 and Gordon and Gregory in 1983. The phosphorescence and fluorescence of the phthalocyanines was described by Vincent et al. (1971).

The copper phthalocyanines [e.g. LXXXIV] are by far the most important. Metal-free phthalocyanines absorb at 686 nm in chloronaphthalene and 772 nm in the solid state. Metal-complex generally absorb at much shorter wavelengths (Figure 3.29 LXXXIII to LXXXVIII), but some, such as Lead Phthalocyanine [LXXXVII], absorb at longer wavelengths than metal-free phthalocyanine [LXXXII]. The addition of a phenyl ring to the phthalocyanines to give the naphthacyanines (Figure 3.29 LXXXVIII to XC) produces a bathochromic shift of 67 nm.

Most phthalocyanines dissolve in strongly coordinating solvents, for example pyridine, in concentrated sulphuric acid, and in highly aromatic solvents, such as α-chloronaphthalene and dichlorobenzene. Also most phthalocyanines are soluble in dimethylamine (DMA), dimethyl sulphoxide (DMSO), dimethyl formamide (DMF) and toluene. Solubility varies greatly with coordinating central metal ion. Ruthenium, Lithium and Magnesium Phthalocyanine [LXXXVI] are readily soluble in acetone,



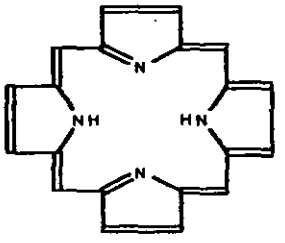
LXXIX



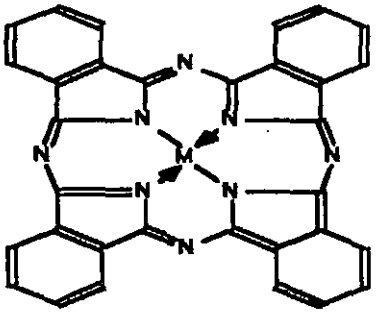
LXXX

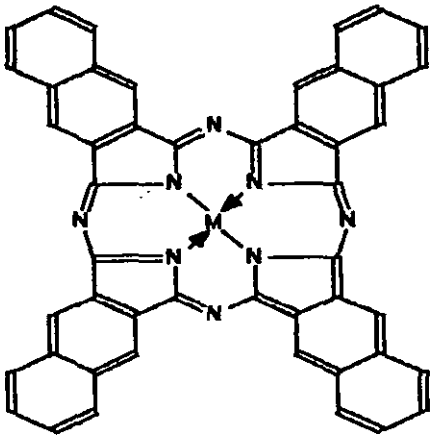
Figure 3.28: Large conjugated hydrocarbons: excitation and emission wavelengths in benzene (Rauhut et al., 1975).

		Ex (nm)	Em (nm)
LXXIX	tetrabenzo[de, hi, op, st]pentacene	628	690
LXXX	7, 8, 15, 16-dibenzoterrylene	750	810



		Abs. (nm)
LXXXI	Porphine	529/619





			Abs. (nm)
LXXXII	M=H_2	Phthalocyanine	686
LXXXIII	M=Co	Cobolt Phthalocyanine	657
LXXXIV	M=Cu	Copper Phthalocyanine	658
LXXXV	M=Fe	Iron Phthalocyanine	676
LXXXVI	M=Mg	Magnesium Phthalocyanine	666
LXXXVII	M=Pb	Lead Phthalocyanine	698
LXXXVIII	M=Zn	Zinc Phthalocyanine	661

			Abs. (nm)
LXXXVIII	M=H	Naphthalocyanine	765
LXXXIX	M=Al(F)	Aluminium Naphthalocyanine	717
XC	M=Mg	Magnesium Naphthalocyanine	720

Figure 3.29: Porphine, phthalocyanines and naphthacyanines with absorbance wavelengths in chloronaphthalene (Matsuoka, 1990).

acetonitrile and methylene chloride, solvents that most other phthalocyanines do not dissolve in at all. The solubility in organic solvents may be improved by the introduction of branched chain long-alkyl groups which prevent the aggregation by the steric interactions between the bulky and long-chain alkyl substituents. Wheeler et al. (1984) synthesised Silicon Naphthacyanine whose solubility can be controlled by the alkylsilyl substituents. Iron (III), aluminium (III) and cobalt phthalocyanine also have ligands outside the phthalocyanine π-conjugated plane so these cannot be aggregated to each other because of the steric hindrance and thus become soluble in high concentrations in organic solvents. The introduction of sulphonic acid groups increase their solubility in water.

Phthalocyanines are widely used in electrophotography (xerography), in photochemical hole burning laser disk memory, in laser printer systems and as catalysts (Matsuoka, 1990). Metal phthalocyanines and naphthacyanines have been investigated by Chan et al. (1987a,b) and Bresseur et al. (1987) as photosensitisers in photo-destructive therapy of tumours.

3.7 Dye aggregation

The formation of an aggregate by dye molecules implies the direct operation of molecular binding forces and the development of a series of equilibria in solution.

$$\begin{aligned} 2\,D_1 &\rightleftharpoons D_2 \\ D_1 + D_2 &\rightleftharpoons D_3 \\ D_1 + D_3 &\rightleftharpoons D_4 \\ \text{or } 2\,D_2 &\rightleftharpoons D_4 \text{ etc.} \end{aligned} \qquad (3.1)$$

Where D_1, D_2, D_3 and D_4 are the monomer, dimer, trimer and tetramer respectively.

The formation of dye aggregates (D_2, D_3, D_4 etc.) is shown by the appearance of another absorbance band either lower or higher than that of the monomer (D_1). The fluorescence of the monomer is quenched and the fluorescence, if there is any, from the excimer (see Section 1.4.1) appears either at higher or lower wavelength.

Aggregation can be modified by the presence of other solutes which lessen the dipolar and hydrophobic interactions leading to molecular associations. This includes surfactants (SURFace ACTive AgeNTS) or polar solvents (such as methanol, ethanol and acetonitrile). Surfactants can be subdivided into three groups according to whether they are anionic, cationic or non-ionic.

Dyes have been used extensively for the ion-pairing of surfactants and have been reviewed by Llenado et al. (1981, 1983) and more recently by Walters and King (1991). The quantification of surfactants by ion pairing then to dyes was first demonstrated by Longwell and Manence (1955), the Methylene Blue-anionic surfactant complex was separated into chloroform for the subsequent measurement of absorbance. This was modified by Abbot in 1962 to determine the concentration of anionic surfactants in drinking water. Flow injection analysis of anionic surfactants using Methylene Blue [XLVII] has been carried out by Motomizu et al. (1988), de Valle et al. (1988) and Kawase and Yamanaka (1979) to name but a few. Other cationic dyes that have been used include two other thiazines; Azur A [LIV] (van Steveninck et al., 1966), Toluidine Blue [LVII] (McGuire et al., 1962) and triphenylmethanes; Rosaniline (Cropton and Joy, 1963), Methyl Yellow (Fabre and Kamenka, 1976.) Anionic dyes have been used to determine the concentration of cationic surfactant, for example, the anionic monoazo dye Orange II (Kawase and Yamanaka, 1979; Kanesato et al., 1987).

Cyanines have been used to determine ionic surfactants, such as surfactants (Imasaka et al., 1984; Robertson et al., 1990). The use of near infrared fluorescence can reduce sample preparation time because of less interference in this spectral region as shown by the use Rhodamine 800 [XI] by Hindocha et al. (1993). The labelling of an anionic drug by a cationic dye by ionic bonding allows the drug to be extracted from the aqueous phase to an organic phase and hence reduce the interferances.

3.7.1 Method

The following two experiments were used to demonstrate the formation of aggregates. In the first experiment, a drop of Nile Red [LIII] dissolved in methanol from a pasteur pipette was added to 20 ml of water. This solution was immediately passed through a 0.8 micrometer Acrodix syringe filter (Gelman). Every two minutes the precipitant was passed through a 0.8 micrometer syringe filter. Each time approximately 1 ml was retained each time for comparison. The colour of the solution was recorded and the amount of dye left on the filter was noted.

The second experiment monitored the formation of aggregates of Nile Red [LIII] using fluorescence. A drop from a pasteur pipette of a stock solution of Nile Red [LIII] in methanol was added to distilled water and the fluorescence spectra was scanned automatically every minute for twenty minutes on the Shimadzu RF-5001PC Spectrofluorimeter. Excitation was at 550 nm and the slits were set at 10 nm. Figure 3.31 shows the fluorescence spectra collected at two minute interval up to ten minutes.

A third experiment showed the influence of surfactants on dyes. An aqueous solution of dye was monitored by fluorimetry and spectrophotometry. Added to this was increasing amounts of surfactants. The surfactants used were the non-ionic surfactants, Brij 35 (polyoxyethylene lauryl ether), Tween 20 (Polysorbate 20: polyoxyethylene sorbitan mono laurate), Tween 80 (Polysorbate 80: polyoxyethylene sorbitan mono oleate) and Triton X-100 (polyoxyethylene tert-octylphenol); the anionic surfactant, sodium lauryl sulphate (sodium dodecyl sulphate: SDS) and the cationic surfactants, tetradodecylammonium bromide and cetyltrimethylammonium bromide (CTAB). The concentration of the dye was kept approximately constant because the amount of surfactant added was less than 1% of the total volume of solvent. Either a fluorescence spectrum on a Perkin Elmer MPF-44B Spectrofluorimeter or absorption spectrum on a Kontron UVIKON 810 Spectrophotometer was scanned after each addition of surfactant. Figures 3.30, and 3.31 to 3.33 are examples of the absorbance spectra produced.

A fourth experiment showed the influence of polar solvents on aqueous solutions of dyes. To an aqueous solutions of dye was added varied amounts of polar solvent (either acetonitrile or methanol). The concentration of the dye was kept constant. Absorption spectra were taken after each addition, such as those shown in Figures 3.35.

3.7.2 Results

Table 3.26: The absorption and emission wavelengths of monomer and dimer of xanthenes (fluoresceins and rhodamines), cyanines and azines (phenoxazines, phenoxazones and thiazines) in water. The emission of the dimer of the thiazines and some of the other dyes were not determined. (ND = not determined)

	monomer		dimer	
	Abs. (nm)	Em (nm)	Abs. (nm)	Em (nm)
Fluoresceins				
Fluorescein [IV]	492	521	420	ND
Rose Bengal [VII]	549	590	510/545	none
Rhodamines				
Rhodamine 6G [VIII]	524	550	500	none
Rhodamine B [IX]	552	572	510	none
Rhodamine 800 [XI]	685	700	605	ND
Cyanines				
DTTC [XXIII]	750	770	650	none
IR125 [XXV]	788	810	690	825
Phenoxazines				
Cresyl Violet [XLVIII]	590	628	570	ND
Nile Blue A [XLIX]	638	667	590	685
Oxazine 4 [L]	612	635	565	ND
Oxazine 750 [LII]	674	692	610	705
Phenoxazones				
Nile Red [LIII]	575	665[1]	520	605[1]
Thiazines				
Azur B [LV]	644	670	600	ND
Methylene Blue [XLVII]	663	682	570	ND
Methylene Green [LVI]	655	678	618	ND
Toluidine Blue [LVII]	632	674	595	ND

[1] value from the determination of Nile Red [LIII] in 100% aqueous solvent on the Perkin Elmer MPF-44B Spectrofluorimeter. In less than 5% methanol the wavelength on the Shimadzu RF-5001PC Spectrofluorimeter was 637 nm and was similar to Nile Red in 100% methanol.

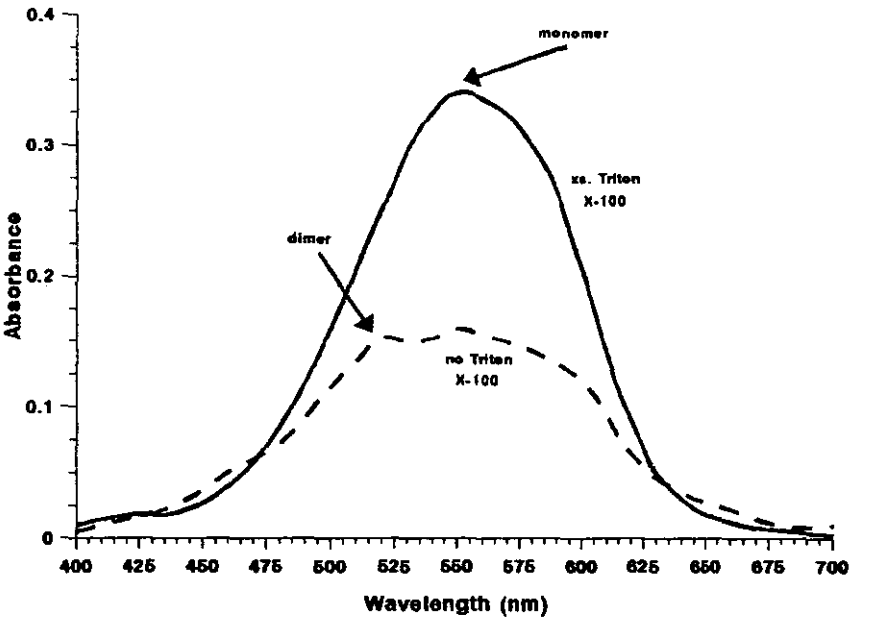
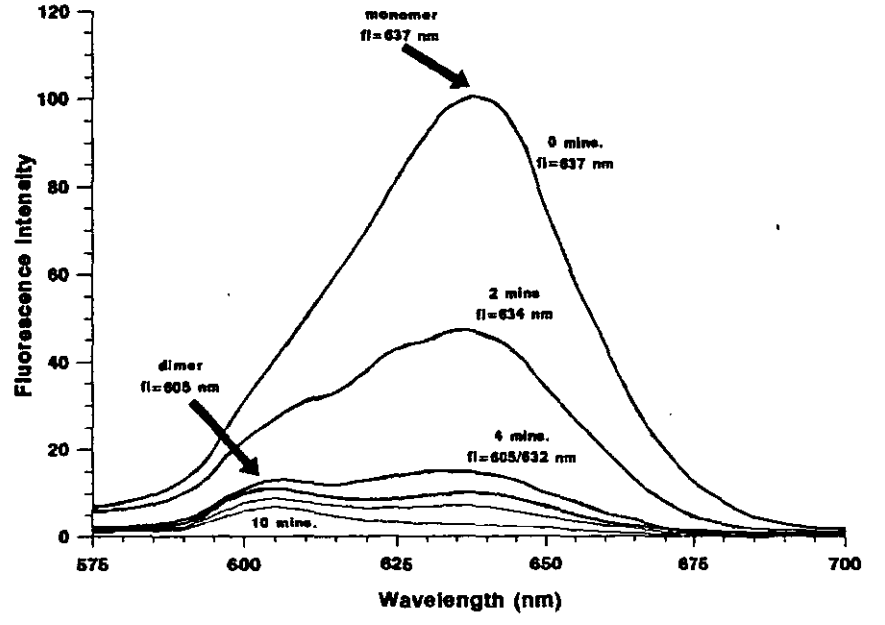


Figure 3.30: The influence of Triton X-100, a non-ionic surfactant, on the absorbance spectra of Nile Red [LIII] in water.



3.31: Repeat fluorescence spectra every 2 minutes of Nile Red [LIII] added in distilled water using the Shimadzu RF-5001PC Spectrofluorimeter. Excitation at 550 nm with 10 nm slits. Dimer formation was shown by the hysochromic shift in fluorescence from 637 nm to 605 nm.

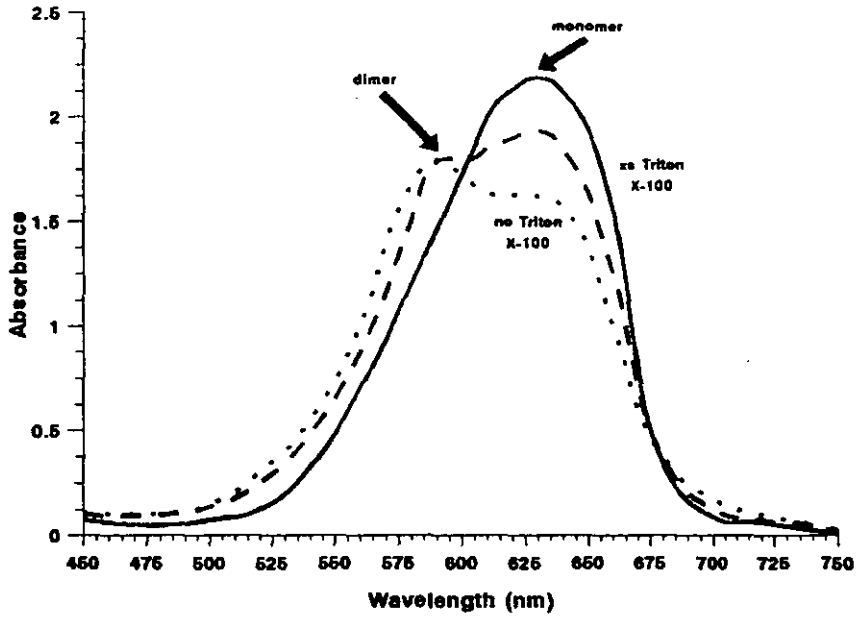


Figure 3.32: The influence of Triton X-100, a non-ionic surfactant, on the absorbance spectra of Nile Blue A [XLIX] in distilled water.

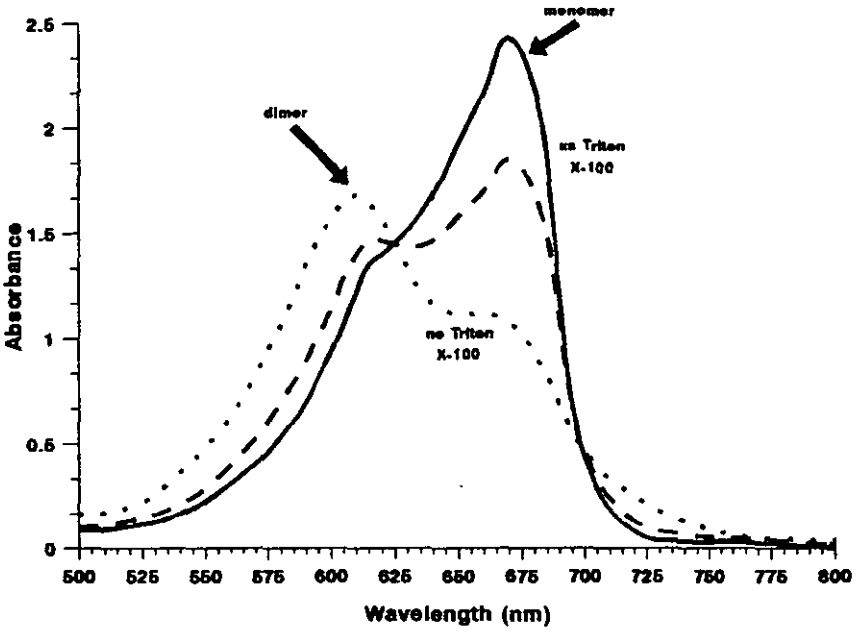


Figure 3.33: The influence of Triton X-100, a non-ionic surfactant, on the absorbance spectra of Oxazine 750 [LII] in distilled water.

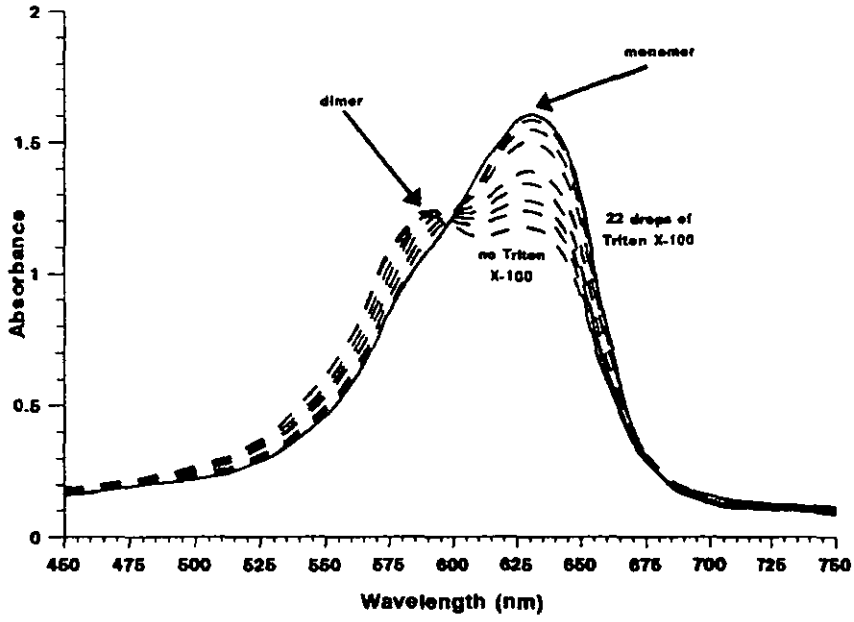


Figure 3.34: The influence of adding increasing amounts of Triton X-100 (1, 2, 4, 6, 10, 14, 18, and 22 drops) to the same concentration of Toluidine Blue [LVII] in distilled water.

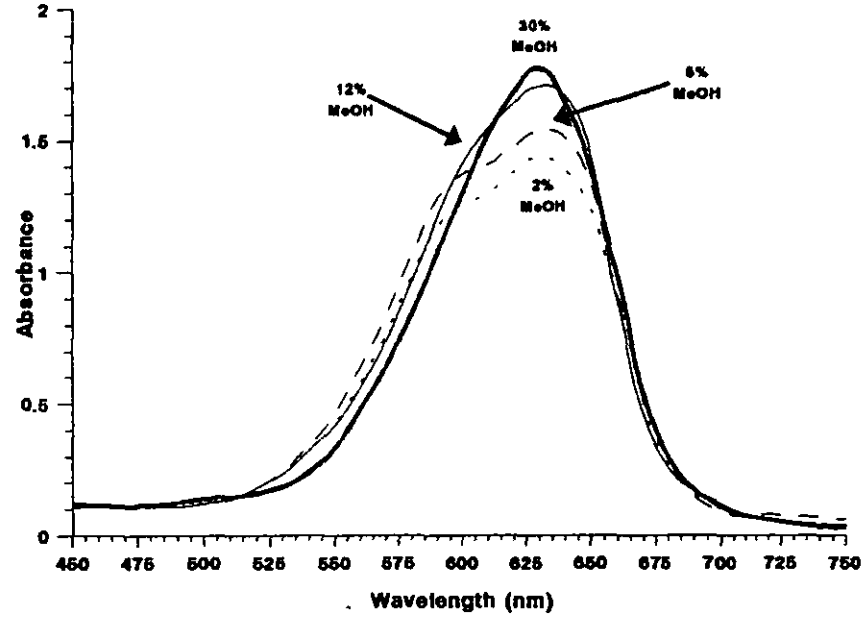


Figure 3.35: The influence of the percentage methanol on the absorbance spectra of Nile Blue A [XLIX] in distilled water.

3.7.2 Discussion

All the dyes tested formed aggregates in water to varying degrees. Most of the dyes showed the usual lower wavelength absorption band of the aggregate. This form of aggregation is termed J aggregates. The fluorescence at the monomer wavelength was always attenuated and some cases completely quenched.

Xanthenes formed aggregates in water. The absorbance maxima of the dimers of the xanthenes (Table 3.24) were always at lower wavelength than that of the monomer. Dimerisation of Rose Bengal [VII], Rhodamine 6G [VIII] and Rhodamine B [IX] prevented fluorescence. Aggregation of some xanthenes, in particular the fluoresceins, was reviewed by Valdes-Aguilera and Neckers in 1989.

Cyanines formed reversible aggregates. The dimers of DTTC [XXIII] and IR125 [XXV] showed absorption at lower wavelength. In order for the IR125 to form aggegates, higher concentrations of IR125 [XXV] were used and this was probably due to its longer alkyl chain on the N-alkyl substituent that inhibits aggregation.

Nile Red [LIII] was insoluble in water. In experiment one, the precipitant colour was reduced from the intense red at time zero to colourless after ten minutes. It was impossible to record absorption spectra due to severe light scattering caused by the aggregation of Nile Red. The colourless solution still contained enough Nile Red [LIII] to give intense fluorescence. The absorption spectrum, as shown in Figure 3.30, was measured using a 4 centimetre path length cuvette because of the necessity to use a low concentration of Nile Red [LIII] in order to eliminate scattering. Figure 3.31 shows the dynamic decay in fluorescence of Nile Red [LIII] in water by the formation of aggregates. The severe reduction in fluorescence of the Nile Red [LIII] dissolved in methanol added to water was caused by the sequential replacement of the methanol solvent shell around the dye molecule by water molecules. The dye molecules have greater affinity to each other than the water molecules and so form dimers. This explains the fluorescence decay of Nile Red [LIII] in water. The decay in fluorescence was accelerated by using increasing concentrations of Nile Red [LIII]. There was no reduction in absorbance or fluorescence when more than 20% methanol or surfactants were added to the aqueous solution, which indicated the aggregation of Nile Red was inhibited by these additives.

The degree of aggregation between two or more dye molecules depended on the structure of the dye, the solvent, and the temperature. The more hydrophobic the dye, the greater the tendency of the dye to form aggregates. Therefore, dyes

that had sulphonic or carboxylic acid groups were less likely to form aggregates due to their increased solubility. Dyes aggregated more strongly in water than in any organic solvents and there was no evidence of the dyes tested aggregating in polar solvents (i.e. methanol, ethanol, acetonitrile etc.). Branch chain long-alkyl groups were used in the phthalocyanines (see Section 3.6.5) to prevent the aggregation by the steric interactions between the bulky and long-chain alkyl substituents. A rise in temperature caused the equilibrium to shift from the dimer to the monomer. The rate of dissolution and solubility were very dependent upon the crystalline size and form. Recrystalised Nile Blue A chloride [XLIX] (99.9% pure) with needle like crystals was more soluble than the un-recrystalised dye (less than 95% pure). The counter ion and the purity of the dye also influenced the solubility and hence the degree of aggregation, e.g. the perchlorates of the carbocyanines were more soluble than the iodides in water.

Primarily cationic dyes had a tendency of depositing a mono-layer of dye on solid surfaces, especially glass. This layer once formed does not increase with further exposure. This phenomenon, known as plating, can be very troublesome when using these dyes. The leuco-form of azines, Nile Blue [XLIX] and Nile Red [LIII] were the worst offenders. The interaction with glass seems to be related to the negative charge of the silanol groups of glass and the positive charge of the dye coupled with the degree of aromaticity of these dyes. This was discovered to be a serious problem after measuring the absorbance of an aqueous solution of Nile Red [LIII] and the silica cuvette was rinsed out with water. To this seemingly clean cuvette was added methanol which was consequently coloured red, characteristic of Nile Red. Since this discovery the acid washing and then detergent washing of glassware became routine as described in Section 2.3.1.

There were three techniques used to overcome the problem of plating: glassware was cleaned very carefully after each analysis, a solvent system or an additive was used to eliminate plating (e.g. surfactants, polar solvents), or the glassware was pre-plated. Pre-plating of the glassware was not employed because leaching of the dye from the glass surface could not be guaranteed. The pH, ionic strength and the solvent greatly influenced the amount of dye adsorbed. The plating of Nile Blue A [XLIX] on glass was almost eliminated by the addition of surfactants such as Triton X100 (a non-ionic surfactant) as this inhibited aggregation and hence its dissolution. Insoluble cationic dyes caused the greatest problem of plating.

Surfactants were shown to both disaggregate and inhibit the formation of dye

aggregates (Figures 3.32 to 3.34). The non-ionic surfactants (e.g. Triton X-100) and anionic surfactants (sodium lauryl sulphate) were more efficient in dissociating the aggregates than the cationic surfactants (e.g. cetyltrimethylammonium bromide). This was due to the dyes being predominately cationic and the low solubility of the cationic surfactants in water.

The addition of the surfactant (i.e. Triton X-100) produced an increase in the absorption and fluorescence intensity of the azines because the aggregates formed in water were dissociated to the mono-molecular form. This equilibrium was dependent on the ratio of dye to surfactant as shown in Figure 3.34, the monomer/aggregate equilibrium was shifted to the left with the increasing concentrations of Triton X-100. On dilution the aggregates in solution dissociated. The variable ratios of the longer to shorter absorbance band were indicative of the presence of the formation of aggregates.

The ability of dyes to form complexes varied widely. Dyes form adducts (complexes) with the surfactant that were more soluble in water than the dye monomer and so did not aggregate. The equilibrium present in solution with the dye and surfactant is shown below.

$$\text{surfactant molecules} \rightleftharpoons \text{surfactant micelles}$$

$$\text{surfactant molecules + dye} \rightleftharpoons \text{dye-surfactant complex} \qquad (1.2)$$

Datyner and Delaney (1971) showed chain branching in the alkyl chain in the surfactant inhibited the dye surfactant complex, whilst lengthening the ethylene oxide chain of the surfactant increased it. An increase in temperature reduced the complex formation (Craven and Datyner, 1967).

Micelles are conglomerates of surfactants in solution. Micelles usually form in water and the individual surfactant molecules or ions aggregate to minimise interfacial energy. The hydrophilic "heads" of the surfactant face the aqueous solution, while the hydrophobic "tails project inwards. The micelle formation starts at a narrow and characteristic surfactant concentration, termed the critical micelle concentration (CMC) and the number of monomers that aggregate is given by the aggregation number, as shown in Table 3.27.

The optimum concentration of surfactant molecules (critical micelle concentration) increases with the length of the polyethanoxy side chain so that the first equilibrium is forced to the left and the second to the right, that is increasing the surfactant-dye complex concentration. Figure 3.34 shows graphically the there is

linearity between the concentration of surfactant when the dye concentration was kept constant. This relationship as already stated has been used in the quantification of surfactants.

Table 3.27: The molecular weight (Mol.Wt.), critical micelle concentrations (CMC) and the aggregation number of surfactants (Dawson et al., 1989; von Wandruszka, 1992; Fasman, 1985).

Surfactants	Mol.Wt.	CMC (g/l)	CMC (mM)	Aggregation Number
Non-ionic surfactants				
Brij 35		0.058	0.06	40
Tween 20		0.14		
Tween 80		0.13		
Triton X-100	625	0.16	0.25	140
Cationic surfactants				
CTAB	364.5	0.33	0.92	60
Anionic surfactants				
SDS	288.4	2.3	8.2	62

It is generally accepted that micelles enhance the fluorescence of dyes associated with them. However, the results of such measurements could easily be misinterpreted because surfactants also increased, as already stated, the solubility of hydrophobic fluorophores. The resulting increase in concentration (for instance, drawing material previously absorbed onto glass surfaces) could be mistaken for a more fundamental effect. When this increased dissolution is factored out, the main cause for the enhancement could be attributed to disaggregating dimers and so increasing the monomer concentration, and the micelle forming a protective shell around this monomer excluding quenchers.

Polar solutes such as acetonitrile and methanol were shown to greatly assist dissolution and disaggregation of dyes. The dye industry has used dimethylformamide (DMF), pyridine and ethanol (Venkataraman, 1977). Figure 3.35 shows the characteristic narrowing of the absorbance band with the subsequent hyperchromic shift with increasing concentrations of methanol. The monomer absorbance maximum remained unchanged even when the solvent content increased

to 30% methanol. According to Rattee and Breuer (1974), urea was used to assist dye dissolution and was shown to be more effective to mix the dry dye powder with urea and then paste with water before dilution than to add urea to an unsatisfactory dye solution. The interaction with urea was very complex.

3.8 Photostability

The photostability of dyes in aerated solvents illuminated by a broad band light source (i.e. daylight, tungsten lamp and a xenon arc lamp) is an important limit on the usefulness of dyes. A dye that is destroyed within a week would not be of any use as a fluorophore for further investigation as a label or a probe. Few papers have been published on dye photostability to broad band light. The first problem is what is a proper measurement of dye stability? Dye stability depends on dye concentration, temperature, composition of the environment, excitation source power and wavelength distribution. The destruction of the chromophore is caused by the absorption of light and the subsequent reactions when it is in the excited state. It should be stated that the definition of an organic dye is based on the neurological process of colour, i.e. compounds absorbing light in the spectral region between 400 and 750 nanometres. From the chemical point of view, dyes have all the properties of related colourless compounds.

Absorption of visible light and the subsequent radiative transitions to the ground state (fluorescence) and after intersystem crossing to a triplet state (phosphorescence) have been discussed in Section 1.1. Dyes in excited electronic states have occupied and unoccupied molecular orbitals which are different from those in the ground state. Therefore, the chemical reactivity of the two states differ. The energy level of the excited singlet state is always higher than that of the corresponding triplet state and so has a higher reactivity. However, the triplet state is longer lived and so has a better chance to react. For bimolecular reactions, the availability of the reagent to the excited state is the rate determining step.

The form of photochemical reaction depends on the dye structure, solvent, reagents, additives and wavelength of irradiation. The mechanisms for reaction which start from interactions between an excited dye molecule (or ion) and the ground state (D-D), or between an excited state and an oxidizing or reducing agent (Norish Type 1; D-O and Norish Type 2; D-R, respectively). Figure 3.30 (Koizumi et al., 1978) summarises primary processes in the photo-redox reactions of photosensitizer dyes. For simplification, only the reactions of the triplet (^{3}Sens*) are included. The chemical reactions of singlets are less important because as already stated the singlet excited state is so short lived the number of reactions is a small proportion of the whole. In addition to thermal deactivation (k_d) and,

phosphorescence, the triplet state may react with the ground state dye to produce a half oxidized dye radical and a half reduced dye radical (D-D). The reaction mechanism depends on whether the solution is aerated or de-aerated. Type I reactions produce primarily dye radicals and type II do not produce dye radicals. The latter is important in producing singlet oxygen. The light fading of dyes in air is usually an oxidation process. For many dyes the rate of fading is highest in oxygen, lower in nitric oxide and carbon dioxide, and practically zero in nitrogen or hydrogen, although changes due to reduction sometimes occur in hydrogen. Also peroxides may be formed on the exposure to light.

Fluorescence of the parent molecule is reduced either temporally by energy transfer to solvent molecules and intramolecular relaxation states or permanently by the destruction of parent molecule. The photoproducts may themselves be fluorescent and may have higher fluorescence quantum yields even at the same wavelength as the parent molecule.

The photostability of dyes is empirical as one dye that in unstable in one environment may be very stable in another. An example of this is Astra Cyanine B (a cyanine dye) that is very unstable on cotton in the presence of moist oxygen and very stable on polyacrylonitrile (Orlon) in the presence of nitrogen (Schwen and Schmidt, 1959).

3.8.1 Method

1. *Exposure on bench* - A dilute solution of the dye (about 10^{-5} molar) made either in methanol, water or acetonitrile was prepared. Ten millilitres was left exposed on the bench to daylight, another ten millilitres was kept in the dark at room temperature. The final ten millilitres was kept in the refrigerator (about 4 °C). The change in colour and the absorbance spectra was recorded at various time intervals for a couple of weeks. The fluorescence was measured when the original solution was diluted by one hundred fold.

2. *Illumination by a daylight simulation bulb* (100 W tungsten light bulb with a blue filter) with spectral characteristics similar to daylight (figure 3.2) was used to illuminate dilute dye solutions in methanol at about ten centimetres. The concentration was such that the initial concentration had an absorptivity of about two. The absorbance was periodically taken.

3. *Illumination by xenon arc lamp* - A dilute solution in methanol (approximately 0.1 micromolar) of the dye was illuminated by zero order light with a glass slide to eliminate ultra-violet light below 340 nm and the fluorescence intensity over time at constant temperature was measured.

3.8.2 Results

Table 3.27: Photodegradation of some xanthenes (fluoresceins and rhodamines), polymethines (cyanines and merocyanines), and azines (phenoxazines, phenoxazones and thiazines) when illuminated by a 100 watt Daylight Simulation Bulb or a 150 watt xenon arc lamp. The solvent used was methanol and the absorbance maxima. The photochemical absorption decay constant (α) was compared with that of Fluorescein (α^*). The half life ($t_{½}$) in hours was estimated by equation .

	Abs. in MeOH nm	Daylight Simulation Bulb α^*/α	$t_{½}$ hr.:min.	Xenon Arc Lamp β^*/β	$t_{½}$ hr.:min.
Fluoresceins					
Fluorescein [IV]	491	1.0	417:	1.0	9:
Rhodamines					
Rhodamine B [IX]	545	3.4	1500:	ND	ND
Rhodamine 800 [XI]	674	2.3	950:	ND	ND
Carbocyanines					
DODC [XXI]	579	0.41	167:	ND	ND
DOTC [XXII]	695	0.01	4:20	0.46	4:15
DTTC [XXIII]	755	0.007	2:55	1.2	11:30
HITC [XXIV]	739	0.41	167:30	ND	ND
IR125 [XXV]	770	0.26	109:	3.7	85:00
Merocyanines					
Merocyanine 540 [XXVIII]	540	0.61	256:30	ND	ND
Phenoxazines					
Nile Blue A [XLIX]	627	12.5	5290:	ND	ND
Oxazine 750 [LII]	662	7.7	3330:	1.4	132:
Phenoxazones					
Nile Red [LIII]	550	20.0	9000:	ND	ND
Thiazines					
Toluidine Blue [LVII]	629	4.8	2000	ND	ND

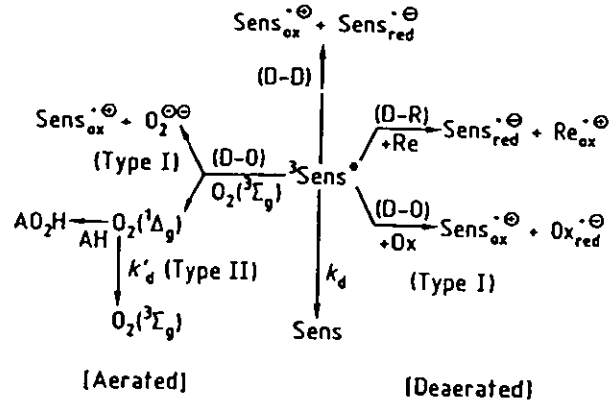


Figure 3.36: The primary processes in photo-redox reactions of dyes (Koizumi et al., 1978). Where Re is the reducing agent, Ox is oxidising agent, and Sens is the dye. The reaction mechanism D-D, D-O and D-R for the interactions between the excited dye and the ground state dye, oxidizing agent and the reducing agent respecively.

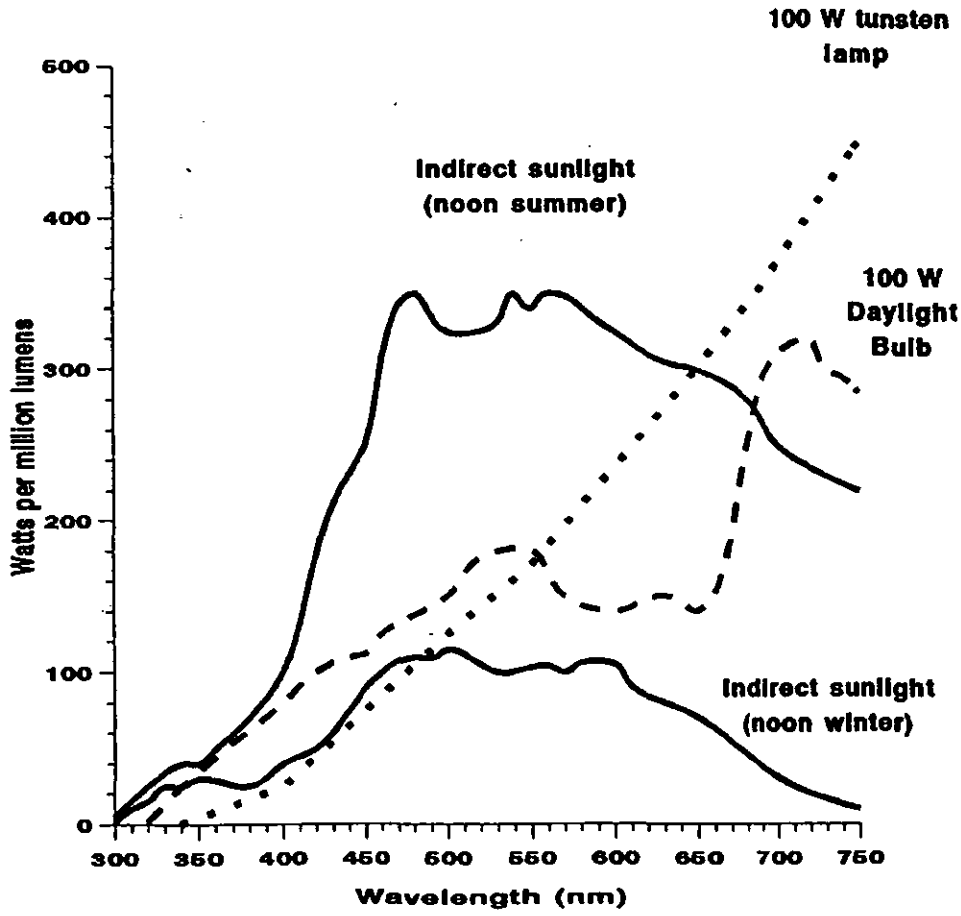


Figure 3.37: Irradiance of the 100 W Daylight Simulation tunsten light bulb compared to a conventional 100 W tungsten light bulb and sunlight.

3.8.3 Discussion

The absorption spectrum before irradiation showed the S_0-S_1 absorption band in the red and the overlapping weaker absorption band to S_2 and the higher excited states in the blue and ultraviolet. After irradiation, the S_0-S_1 band became narrower (reduced parent dye concentration) and a new absorption band of a photoproduct on the blue side of the S_0-S_1 band was formed. The decay in absorption and fluorescence in all the dyes measured approximated first order. The assumption was that none of the photoproducts produced absorbed or fluoresced at the same wavelength as the parent dye.

The photochemical absorbance decay of dyes in methanol was determined as follows:

$$A = A_o.e^{-\alpha t} \qquad (3.3)$$

$$\ln A = \ln A_o - \alpha t \qquad (3.4)$$

The photodecomposition decay constant (absorption) [α] was compared with that of Fluorescein (α^*) to derive the photostability ratio (absorbance) [α^*/α]. The half life ($t_{½}$) in hours was estimated by the following equation.

$$t_{½} = \frac{\ln 2}{\alpha} \qquad (3.5)$$

The photodecomposition fluorescence decay of dyes in methanol was determined as follows:

$$F = F_o.e^{-\beta t} \qquad (3.6)$$

$$\ln F = \ln F_o - \beta t \qquad (3.7)$$

The photodecomposition decay constant (fluorescence) [β] was compared with that of Fluorescein (β^*) to derive the photostability ratio (fluorescence) [β^*/β]. The half life of the dye was estimated by the following equation.

$$t_{½} = \frac{\ln 2}{\beta} \qquad (3.8)$$

The photostability of Fluorescein [IV] was about three times less than that for the two Rhodamines tested as can be seen from the table. This was also shown by Viriot and Andre (1989) when they used a xenon arc lamp as the light source. Rhodamine B [IX] showed a reduction in the absorbance (destruction of the

chromophore), and a hypsochromic shift of the absorbance maxima as caused by N-dealkylation of the dye solution. This was also observed by Evans, 1973.

The polymethines were chemically unstable and far less photochemically stable than any of the other dye groups tested. Solutions of these dyes were repeatedly discoloured after a few days. In the case of DTTC [XXI], it changed from blue to grey-green and then colourless within five hours when exposed on the bench. This photobleaching was observed for all polymethines and after a week of illumination on the bench top by natural light, all the polymethines were brown to colourless solutions with the corresponding severe reduction in absorbance and fluorescence. The preponderance of polymethines showed lower wavelength absorbance bands of photo-by-products. Photodecomposition of the polymethines was greater in methanol and water than acetonitrile. Miyazoe and Maeda (1970) showed that acetone was a better solvent for storing polymethines than ethylene glycol, dimethyl sulphoxide and methanol. The destruction of the polymethines was halted or slowed when stored kept in the dark and practically halted if stored in refrigerator (approximately 4°C). Miyazoe and Maeda (1970) showed that polymethines in solution could be preserved for about a month in a refrigerator and much less than a week in the dark. They also stated that polymethines as a powder can be preserved for a few years.

The polymethines with shorter conjugation bridge were more photo-stable than those with longer conjugation bridge. This would be improved by rigidising the conjugation bridge. The end-groups also influence the photostability. The indotricarbocyanines (HITC [XXIV] and IR125 [XXV]) were more stable than oxatricarbocyanine (DOTC [XXII]) and thiatricarbocyanine (DTTC [XXIII]). This relationship was commented upon by Matsuoka in 1990.

The photostability of the azines tested was excellent and this was supported by Basting et al.'s statement in 1976 that Nile Red [LIII] was more photochemically stable than Rhodamine 6G [VIII]. At high concentrations (one micromolar or more) the reduction in absorbance, in some cases, could be attributed to plating. In dilute solution they showed no significant degradation. The Colour Index (1971) reported that Methylene Green [LVI] was the most fast basic green dye known. Methylene Blue [XLVII], which was not tested, would be far less photo-stable as it has been used to produce singlet oxygen for photochemical reactions.

3.9 Discussion

The most promising of the dye groups investigated are the phenoxazines and thiazines due to their longer wavelength absorbance/fluorescence than the xanthenes combined with superior photostability over the xanthenes and polymethines. The main problem with the phenoxazines and thiazines was their tendency to plate solid surfaces. This could be alleviated by a combination of the use of disaggregating agents (such as surfactants or polar solvents) and the addition water solubilising groups to the dye (such as sulphonic acid groups.)

The only xanthene tested that had a long enough wavelength to show any promise was Rhodamine 800. It was considered that it was not possible to derivatise this dye without destroying the conjugation. The reason for the long wavelength absorption is the cyano group.

The tricarbocyanines had the longest wavelength absorbance/fluorescence but the worst chemical and photochemical stability. This suggested that the tricarbocyanines as a fluorophore for a covalent label was difficult to produce by derivatisation. This was indeed the case, see chapter 4. The Stokes' shift of the polymethines were smaller than the xanthenes and azines with the exception of the asymmetrical carbocyanines, i.e. Styryl 7 and Styryl 11.

The phthalocyanines have according to the literature the best chemical and photostability of all dyes but were not investigated. These are very promising as fluorophores for covalently labelling but suffer from their very low stability in water and very small Stokes' shift of only 5 to 10 nm.

3.9.1 Design of a Near Infrared Dye

The colour-structure relationship is the most important factor for the design of infrared absorbing dyes. Chromogenic theory was first rationalised in 1876. Most of the commercial dye chromophores were developed from the resonance theory developed by Bury in 1935. This gives only a qualitative evaluation of the chromophore. The great advance was the Pariser-Parr-Pople molecular orbital (PPP MO) in 1953 (Pariser and Parr, 1953; Pople, 1953), which gave a method of predicting the absorbance maximum of a dye chromophore. The early applications of the PPP MO method for dye chromophore have been summarised by Griffiths (1976).

A dye may be considered as being composed of an electron donor group connected by a conjugation bridge to an electron acceptor group. The absorbance

maximum of dyes may generally be extended by:

1. Lengthening of the conjugation bridge. For example, the absorbance of the carbocyanines is extended by approximately 100 nm for each extra methine group added (see figure 3.1).
2. Increasing the strength of the electron donors and/or acceptors. The strength of the electron donors increase of going down figure 3.11.
3. Substitution of the conjugation bridge by a heteroatom. The changing of a carbon to a nitrogen produces short chain near infrared dyes.
4. Anionic dyes which contain hydroxyl substituents absorb at shorter wavelength than cationic dyes with amino groups (See Table 3.7 and 3.25).
5. Substitution para to the heteroatom causes a bathochromic shift. The homologous series of azines in Figure 3.15 are examples of this. The bathochromicity increases on passing from phenazines (nitrogen) to phenoxazines (oxygen) to thiazines (sulphur).

All these features can be identified in the dye groups that were investigated. The other properties that are desirable, such as solubility in water, reduced or no plating of solid surfaces and stability should be taken into consideration when looking for a suitable fluorophore. Water solubility can be achieved by adding sulphonic or carboxylic groups. These solubilising groups should be added before the final formation of the dye. Neutral or anionic dyes have much reduced or no plating characteristics.

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Chapter 4

4.1 Covalent Labels

Covalent fluorescent labels have two components, a fluorophore (D in Figure 4.1) and a method of linking to a substrate (Q), which may be a protein, peptide, amino acid or other biochemically important compound. There are two strategies that have been followed, either using the technology of the textile reactive dyes or biological cross linking agents as reviewed by Davidson and Hilderbach in 1990. The former was pursued because this was the more flexible and less expensive alternative.

Reactive dyes were first developed for the textile industry to provide wash fast dyes for cloth and were introduced in 1956 (Venkataraman, 1972). Covalent bonds are formed with the hydroxyl groups of cellulose fibres, with amino, hydroxyl and thiol groups of protein fibres and the amino groups of polyamides.

There are various problems with reactive dyes. Hydrolysis of the electrophilic group (X in Figure 4.1) of the reactive group (RG) is in competition with the formation of the covalent bond between the dye and the substrate. The hydrolysed dye cannot react with the substrate. The high resistance to hydrolysis of the reactive dye means that a higher proportion of the reactive dye can covalently bond with the substrate. The covalent link (Q in Figure 4.1) from the dye to the substrate should be resistant to alkaline or acid hydrolysis. Hence, for a useful reactive dye, the rate of hydrolysis of the dye-substrate bond must be very small to allow a reasonable shelf-life for the conjugate.

The labelling or conjugation reaction should be in aqueous media, fast and quantitative so that denaturing of the protein is unlikely to occur. The conjugation reaction should be specific for the functional groups in the protein so non-specific binding is reduced or eliminated. The excess reagent should be easily separated from the conjugate by gel chromatography or dialysis. The reactive dye should have a high molar absorptivity at the wavelength of the excitation and a high fluorescence quantum efficiency when bound to the protein. The reactive dye should have a large Stokes' shift so there is as small as possible overlap between the excitation and emission bands. The reactive dye and the subsequent conjugate should be stable to heat and light. The conjugate's covalent bond to the protein should be stable to hydrolysis.

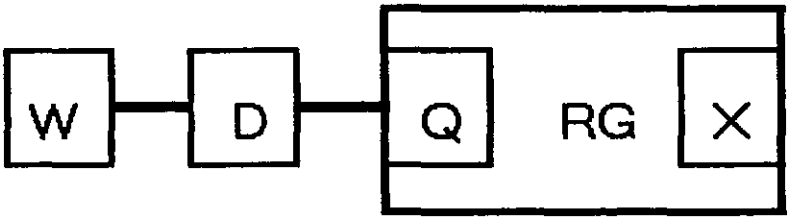


Figure 4.1: Characteristic features of a reactive dye shown schematically.

Water-solubilising groups (W) are very important for reactive dyes used to covalently bind to cellulose and proteins. One to four sulphonic acid groups are usually necessary. The pH of the aqueous dyeing solution is important for the wet fastness. The maximum stability for Remazol Reactive Dyes (-SO2-CH2-CH2-OSO3H) to hydrolysis is at pH=5 compared with pH=6 to 7 for all other dyes. On the acid and alkaline side of this maximum the rate of dye-fibre bond hydrolysis increases by a factor of 10 per pH unit [Zollinger 1987].

The chromogenic part of the dye (D) for the VNIR may be any of those discussed in chapter 3.

The Bridge link (Q) between the chromophore [D] and the reactive group influences:
1. The reactivity of the reactive system. The dissociation of the imino bridge may reduce the reactivity of the reactive groups by several orders of magnitude.
2. The selectivity or degree of fixation.
3. The stability of the reactive dyeing. It is possible to split not only the dye-fibre bond but also the link between the dye and the reactive group.

Electrophilic reactive groups (RG).

Nucleophilic leaving group (X) should form a stable ion or molecule after dissociation, for example, the halogens (F-, Cl- and Br- ions), and the sulphuric acid ester of β-hydroxyethylsulphones that forms a sulphate ion.

Also it is very important that the fluorophore is not carcinogenic or has serious toxic effect upon the user of the label as already stated in Section 3.1.1. Covalent labels are by definition mutagenic, in that they have the potential to react with DNA.

Table 4.1: Requirements of a fluorescent label.

High rlative fluorescence	(molar absorptivity x fluorescent quantum efficiency is greater than 10000
Spectral or temperal resolution	long Stokes' shift and/or long fluorescent lifetime
Hydrophilic	good solubility in reagents, especially water. Low affinity to serum proteins or solid-phase surfaces.
Negative net charge	low non-specific binding to surfaces
Photostability	long-term stability of reagents and the avoiding of bleaching of the fluorophore.
Simple coupling	retained immunoreactivity of labeled antibodies
Small size	no steric hinderance.

Fluorescence in the near infrared region requires extensive conjugation in the molecule, which in turn creates some disadvantages and so they may not fully satisfy the requirement for fluorescent labels as given in Table 4.1. The labels are necessarily large, often with molecular weights of more than a thousand in the case of the phthalocyanines (Section 3.6.5), hence hydrophobic molecules. The use of water solubilising groups is important (W in Figure 4.1). The large molecular size is not disadvantageous if large bio-molecules (i.e. proteins) are labelled but labelling smaller molecules become more difficult from the problem of steric hindrance. The extensive conjugation in the label molecule increases instability, which can result in photobleaching or shorten the shelf life of the label.

4.2 Indocyanine Green sulphonyl chloride

IR125 (Figure 4.2: XXV) is a symmetrical carbocyanine dye with a sulphonic acid group at the end of the butyl aliphatic chains attached to the indole rings. IR125 [XXV] absorbs at 750 nm and fluoresces at 770 nm in methanol. The sulphonic acid groups could be derivatised to sulphonyl chloride groups to produce a covalent label for amine groups. The methods investigated needed by necessity to be very mild because of the high probability of destroying the chromophore. The first method tried was using oxalyl chloride. This method was abandoned after three attempts because of the problems of extracting the product from the reaction mixture. The method finally used was phosphorous oxychloride. Because this is inorganic which makes the separation much simpler of this from the product.

4.2.1 Method

IR125 [XXV] (0.5 g) was treated with 2 ml phosphorous oxychloride in a 50 ml round bottom flask. After standing for approximately twelve hours, the darkened solution was poured onto ice to remove the excess phosphorus oxychloride and the other water soluble contaminants, such as sodium chloride. After about fifteen minutes the product was extracted into 150 ml. of chloroform, washed four times with 50 ml of water, and then dried over anhydrous sodium sulphate. The chloroform soluble-solution material was dried by evaporation, re-dissolved in a 100 ml of chloroform, and precipitated with an excess of n-hexane. The product was filtered, vacuum dried, and stored in ampoules in the freezer.

IR125 [XXV] and the product [XCI] were characterised by FT-IR (Figure 4.3 and 4.4), absorbance, fluorescence (Table 4.3: Figure 4.5 and 4.6), TLC and mass spectrometry. Proton NMR of the product was not possible because of the very low solubility of the product in the usual deuterated NMR solvents. The proton NMR only showed up the solvent impurities of the product. Absorbance and fluorescence spectra of both IR125 [XXV] (Figure 4.5) and Indocyanine Green sulphonyl chloride [XCI] (Figure 4.6) were measured in anhydrous acetonitrile to reduce the hydrolysis of the sulphonyl chloride groups.

The thin layer chromatography TLC of Indocyanine Green sulphonyl chloride [XCI] and IR125 [XXV] was performed on silica gel sheets developed in chloroform : methanol : acetic acid (14 : 5 : 1)

The results of Oxygen Flask Combustion and CHN analysis carried out on both the starting material and the product by MEDAC are shown in Table 4.2.

4.2.1.1 Protein Conjugation

Indocyanine green sulphonyl chloride [XCl] formed a covalent linkage with amino groups on proteins when they were mixed. The conjugation was carried out at about 4 °C, in order to slow down the rapid hydrolysis of sulphonyl chloride groups. Indocyanine Green sulphonyl chloride [XCl] was dissolved in anhydrous acetonitrile and an aliquot of the solution added to the protein solution.

0.5 mg of Indocyanine Green sulphonyl chloride [XCl] was conjugated with 1 mg/ml bovine IgG in pH 8.8 Tris saline buffer for at least 4 hours below 4 °C and in the dark light.

10 ml of 1 mg/ml human transferine in pH 8.8 phosphate buffer saline was incubated with 1 mg of Indocyanine Green sulphonyl chloride for at least 12 hours at 4 °C and protected from light.

The desalting and the removal of unbound Indocyanine Green sulphonyl chloride [XCl] from the protein was carried out by gel filtration on Sephadex G-25 (PD-10 columns, Pharmacia). Concentration and solvent exchange was then carried out using a Centricon 30 microconcentrator.

4.2.2 Results

Table 4.2: Percentage composition sulphur, nitrogen and chlorine by oxygen flask and ion chromatography of IR125 [XXV] and Indocyanine Green sulphonyl chloride [XCI]. The theoretical values were calculated.

	Theoretical							Experimental		
	H	C	N	O	Na	S	Cl	S	N	Cl
IR125[1] [XXV]										
$C_{43}H_{47}N_2NaO_6S_2$	6.1	66.6	3.6	12.4	3.0	8.3	-	9.2	5.0	-
Indocyanine Green sulphonyl chloride[2] [XCI]										
$C_{43}H_{46}N_2NaO_5S_2Cl$	5.8	65.1	3.5	10.1	2.9	8.1	4.5	9.1	4.6	5.0

[1] MEDAC found by CHN analysis C=58.83%: H=6.29%: N=3.16%.

[2] MEDAC found by CHN analysis C=45.06%: H=4.97%: N=2.37%.

Table 4.3: Emission and excitation maxima of IR125 [XXV] and Indocyanine Green sulphonyl chloride [XCI] in anhydrous acetonitrile and the conjugated indocyanine green with various proteins in varying buffers.

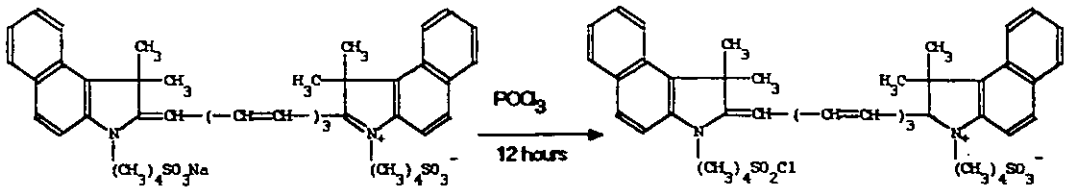
	Ex (nm)	Em (nm)
IR125 [XXV]	783[1]	815[1]
Indocyanine Green sulphonyl chloride [XCI]	778[1]	808[1]
Indocyanine Green-IgG in pH 8.8 buffer	794[2]	808[2]
Indocyanine Green-IgG in pH 7.0 buffer[3]	none	none
Indocyanine Green-transferine in pH 7.0 buffer	790[4]	810[4]

[1] Anhydrous acetonitrile:

[2] pH 8.8 Tris saline buffer

[3] pH 7.0 phosphate buffer after concentration using the microconcentrator.

[4] pH 7.0 phosphate buffer



XXV XCl

Figure 4.2: Reaction scheme for the synthesis of Indocyanine Green sulphonyl chloride [CVI] by the reaction of phosphorous oxychloride with IR125 [XXV] over 12 hours.

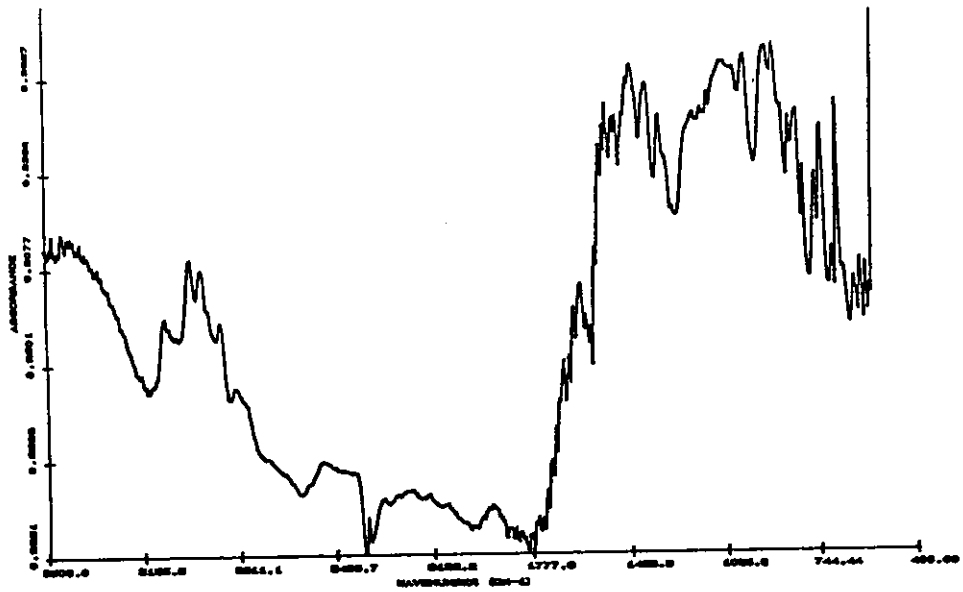


Figure 4.3: Drift FT-IR absorbance spectra of IR125 [XXV] on the Nicolet DX. Peaks at the following wavenumbers: 3086 (m), 2976 (m), 2921 (m), 2866 (m), 2811 (w), 1522 (vs), 1495 (vs), 1459 (vs), 1405 (vs), 1352 (vs), 1298 (s), 1197 (vs), 1010 (vs), 941 (vs), 900 (vs), 835 (s), 798 (s), 760 (s), 733 (s), 695 (m) and 664 (vs).

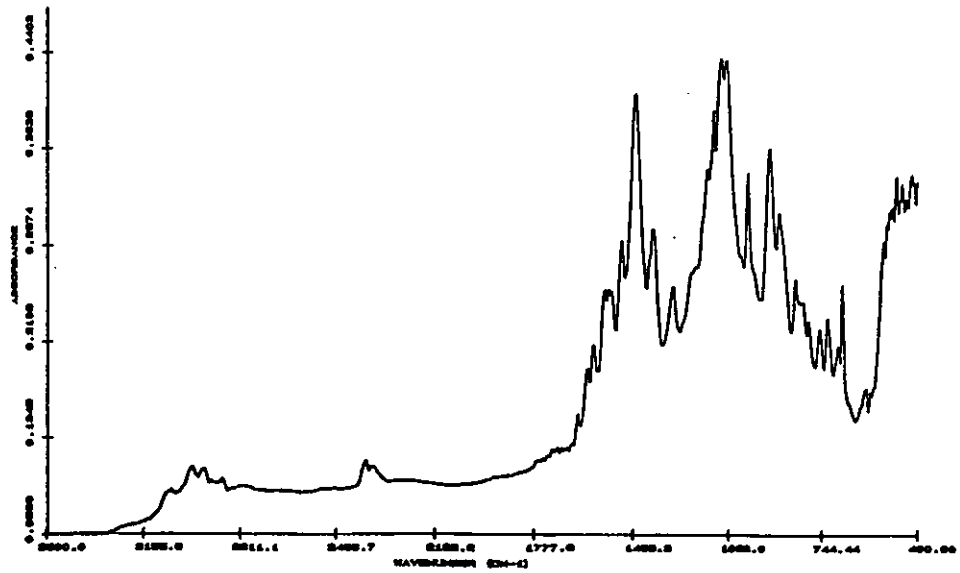


Figure 4.4: Drift FT-IR absorbance spectra of Indocyanine Green sulphonyl chloride [XCl] on the Nicolet DX. Peaks at the following wavenumbers: 3086 (w), 3003 (w), 2950 (w), 2880 (w), 2370 (w), 2315 (w) 1625 (s), 1400 (vs), 1357 (s), 1277 (m), 1123 (vs), 1085 (vs), 1070 (vs), 1000 (s), 920 (s), 877 (s), 830 (m), 803 (m), 787 (w), 752 (w), 717 (w), 680 (w) and 664 (m).

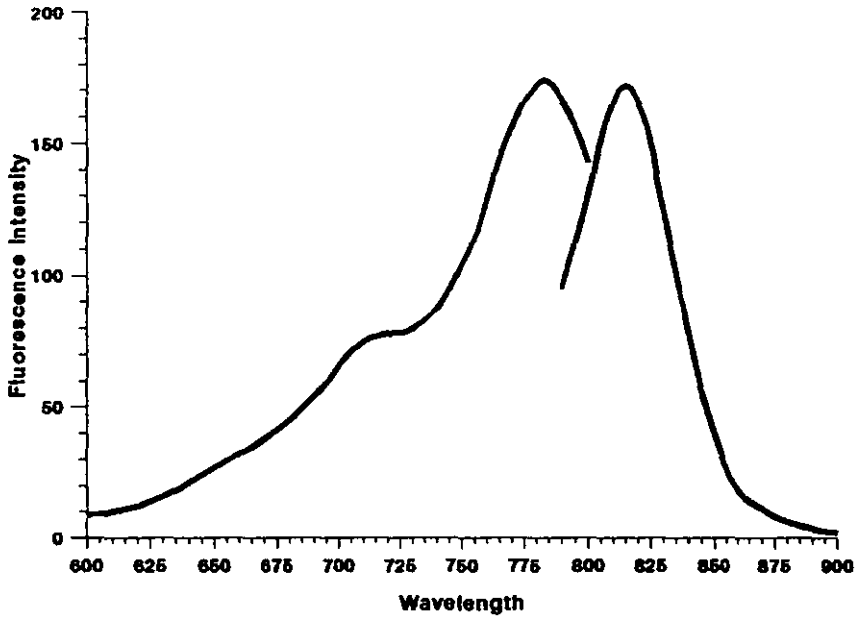


Figure 4.5: Excitation and emission spectra of a 0.1 micomolar solution of IR125 [XXV] anhydrous acetonitrile. (Ex 783 nm, Em 815 nm).

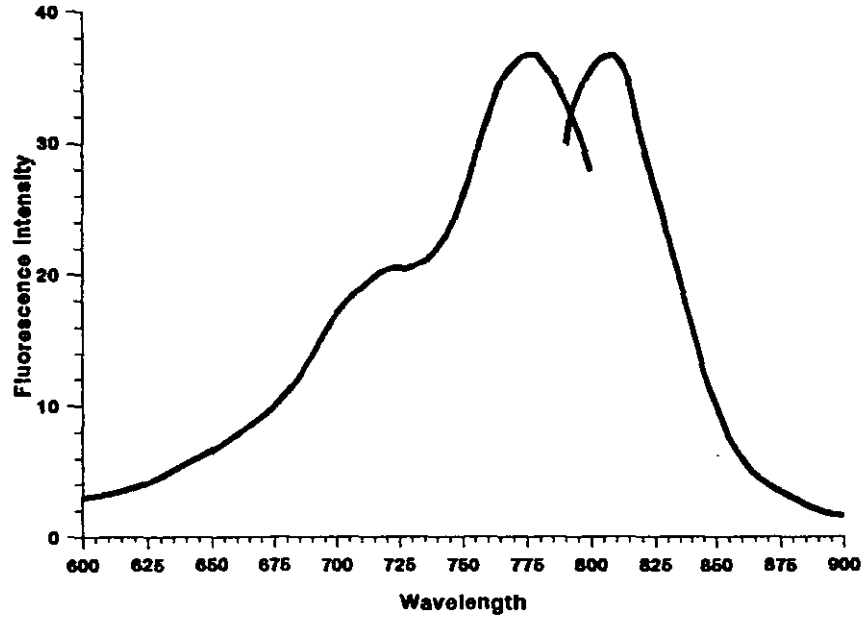


Figure 4.6: Excitation and emission spectra of a 0.1 micomolar solution of Indocyanine Green sulphonyl chloride [XCI] in anhydrous acetonitrile. (Ex 778 nm, Em 808 nm).

4.2.3 Discussion

TLC of a freshly prepared solution of product gave a small light green spot at R_f 0.35 (IR125 [XXV]) and a dark green spot at R_f 0.90 (Indocyanine Green sulphonyl chloride [XCI]). The product formed IR125 [XXV] upon standing in solution for about an hour as shown by the increased intensity of the spot at R_f 0.35.

Drift FT-IR (Figure 4.3 and 4.4) showed the absence of the characteristic O-H stretches at 3500 cm^{-1} in IR125 [XXV] and the appearance of aromatic C-Cl stretches at 800-600 cm^{-1}. The strong absorbances at 1410-1375 cm^{-1} and 1170-1205 cm^{-1} indicated the presence of sulphonyl chloride group.

Electron impact mass spectrometry gave no molecular ion. Indocyanine Green sulphonyl chloride [XCI] lost chloride and the conjugation bridge groups quickly when heated to 100 °C. At 200 °C loss of sulphur dioxide from the aliphatic carbon chain. At 300°C there was the characteristic breakdown pattern of the loss of methyl radicals. There was a good correlation between the mass spectra of IR125 [XXV] and the product [XCI], which showed that the fluorophore was intact.

Anhydrous Indocyanine Green sulphonyl chloride [XCI] was stored in a dessicator in the dark. The sulphonyl chloride group was only activated in anhydrous solvent for only a couple of hours. Indocyanine Green sulphonyl chloride [XCI] was photdecomposed comletely after a day.

The excitation and emission maximum of Indocyanine Green sulphonyl chloride [XCI] was hypsochromic shifted (Table 4.3 and compare Figure 4.5 with 4.6). The fluorescence intensity was much reduced.

The fluorescence intensity of the IgG conjugate in the pH 8.8 Tris saline buffer was very small. Solvent exchange with pH 7.0 potassium phosphate increased the fluorescence intensity. The concentration of the conjugate by microconcentrators was unsuccessful because the conjugate stuck to the membrane. No fluorescence was therefore observed. The molar ratio of dye to protein was not determined because of the large absorbance band at 280 nm of the fluorophore (IR125).

4.2.4 Conclusion

Indocyanine Green sulphonyl chloride [XCI] as a reactive dye for proteins was not pursued further due the very low light stability of the product. Storage of the dye in solvents caused loss of activity and on exposure to light destruction of the chromophore.

4.3 Nile Blue allyl

The second dye chosen for derivatisation after the problems found was Nile Blue A (Figure 4.7: XLIX). Nile Blue A [XLIX] is both chemically (see Section 3.4 and 3.5) and photochemically (see Section 3.8) more stable, and has a longer Stokes' shift than IR125 [XXV]. Also, it is thirty times less expensive. The reactivity of the primary amine on Nile Blue A [XLIX] was investigated by the reaction with allyl bromide.

4.3.1 Method

O.5 g of Nile Blue A chloride, 4 g of anhydrous sodium carbonate, 1 ml of allyl bromide and 50 ml of toluene were refluxed for 6 hours. When cooled, solvent extraction with 50 ml of 10% (w/v) sodium hydroxide was performed and the aqueous layer was discarded. The organic layer was washed a couple of times with water. The organic layer was rotary evaporated to yield the Nile Blue A allyl (Figure 4.3 [XCII]).

The Nile Blue A [XLIX] and Nile Blue allyl [XCII] were characterised by FT-IR (Figure 4.8 and 4.9), Oxygen Flask Combustion (Table 4.4), absorbance (Table 4.5), fluorescence (Table 4.5, Figures 4.11 and 4.12), and TLC (Table 4.7). Proton-NMR of Nile Blue A [XLIX] and Nile Blue allyl [XCII] was not possible because of their very low solubility in the various NMR solvents tried and severe plating of the NMR tubes. TLC was carried out using two solvent systems 9:1 acetonitrile:water, and 6:8:1 chloroform:2-butanone:formic acid and the results are show in Table 4.7.

4.3.2 Results

Table 4.4: Percentage composition sulphur, nitrogen and chlorine by oxygen flask and ion chromatography of Nile Blue A [XLIX] and Nile Blue allyl [XCII] with the values corrected for 95% purity of the starting material. The theoretical values were calculated.

	Theoretical					Experimental		Mol. Wt.
	H	C	N	O	Cl	N	Cl	
Nile Blue A chloride								
$C_{20}H_{20}N_3OCl$	5.7	67.9	11.9	4.5	10.0	12.1	9.8	353.85
Nile Blue allyl								
$C_{23}H_{24}N_3OCl$	6.1	70.1	10.7	4.1	9.0	11.0	9.1	393.92

Table 4.5: The absorbance and fluorescence maxima of Nile Blue A chloride [XLIX] and Nile Blue allyl [XCII].

	Abs. (nm)	Em. (nm)	Solvent
Nile Blue A chloride [XLIX]	622	658	methanol
	619	650	chloroform
Nile Blue allyl [XCII]	627	662	methanol
	625	658	chloroform

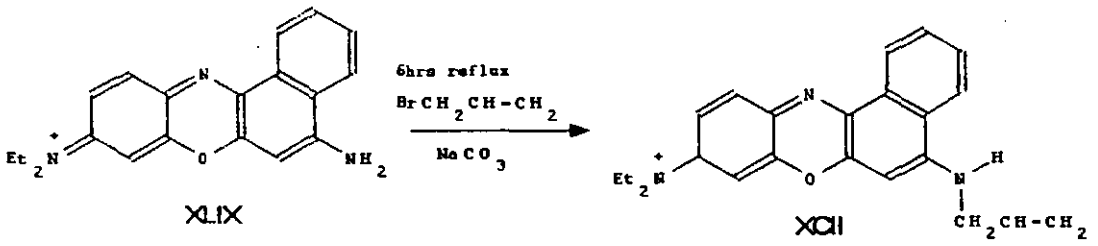


Figure 4.7: The synthesis of Nile Blue allyl [XCII] by the refluxing Nile Blue A [XLIX] with allyl bromide for six hours in the presence of sodium carbonate.

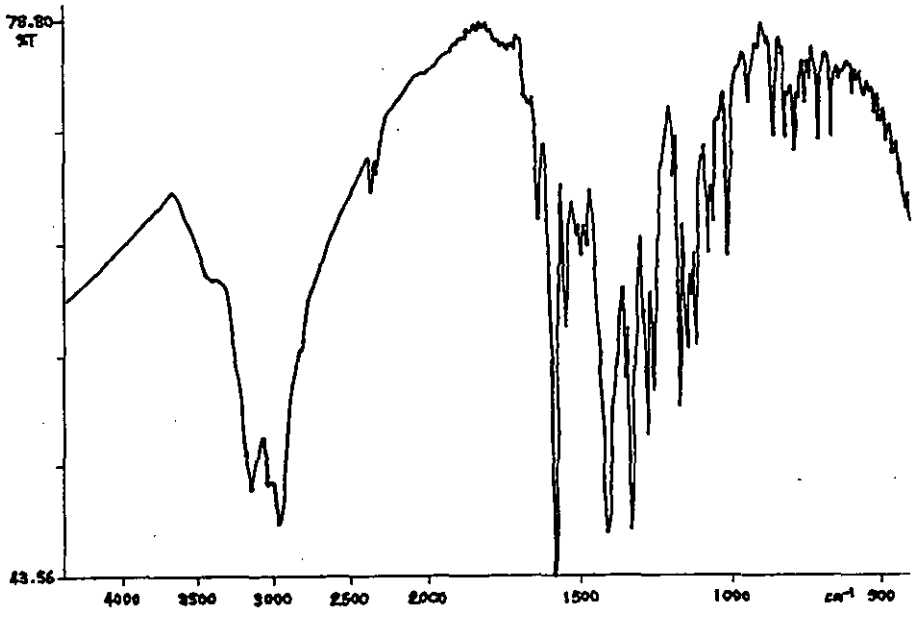


Figure 4.8: FT-IR transmission spectrum of Nile Blue allyl [XCII] on the Perkin Elmer 1600 Series. Peaks at the following wavenumbers: 3131 (s), 2968 (s), 1640 (w), 1585 (vs), 1549 (m), 1499 (w), 1411 (s), 1350 (m), 1332 (s), 1279 (s), 1257 (m), 1192 (w), 1170 (m), 1145 (m), 1116 (m), 1074 (w), 1061 (w), 1011 (w), 943 (w), 816 (w), 789 (w), 751 (w), and 706 (w).

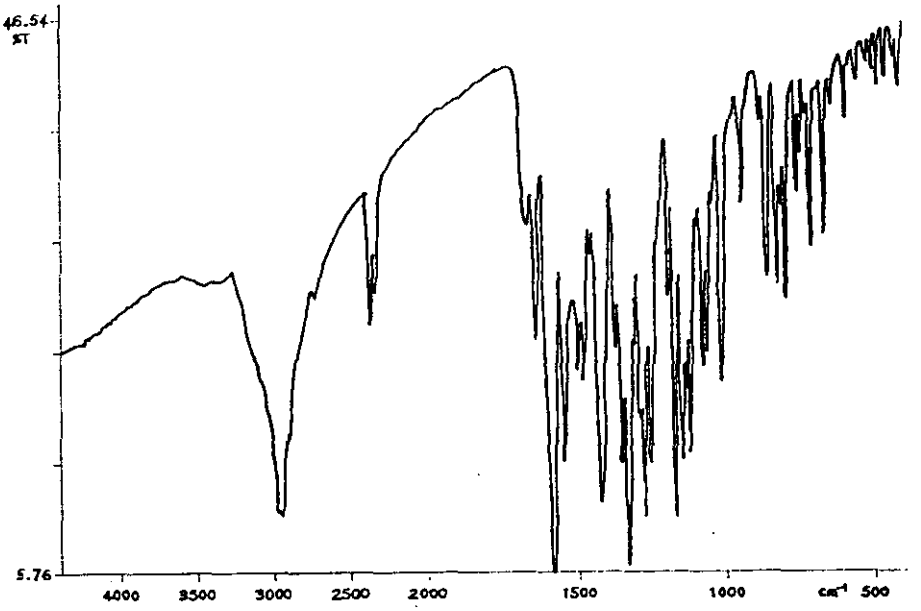


Figure 4.9: FT-IR transmission spectra of Nile Blue A [XLIX] on the Perkin Elmer 1600 Series. Peaks at the following wavenumbers: 2949 (vs), 2361 (m), 2343 (m), 1676 (w), 1641 (m), 1586 (vs), 1550 (s), 1500 (m), 1483 (m), 1424 (s), 1374 (m), 1351 (s), 1333 (vs), 1280 (s), 1258 (s), 1192 (m), 1171 (s), 1145 (s), 1128 (m), 1116 (s), 1074 (m), 1061 (m), 1011 (m), 943 (w), 858 (m), 817 (m), 790 (m), 751 (w), 740 (w), and 706 (m).

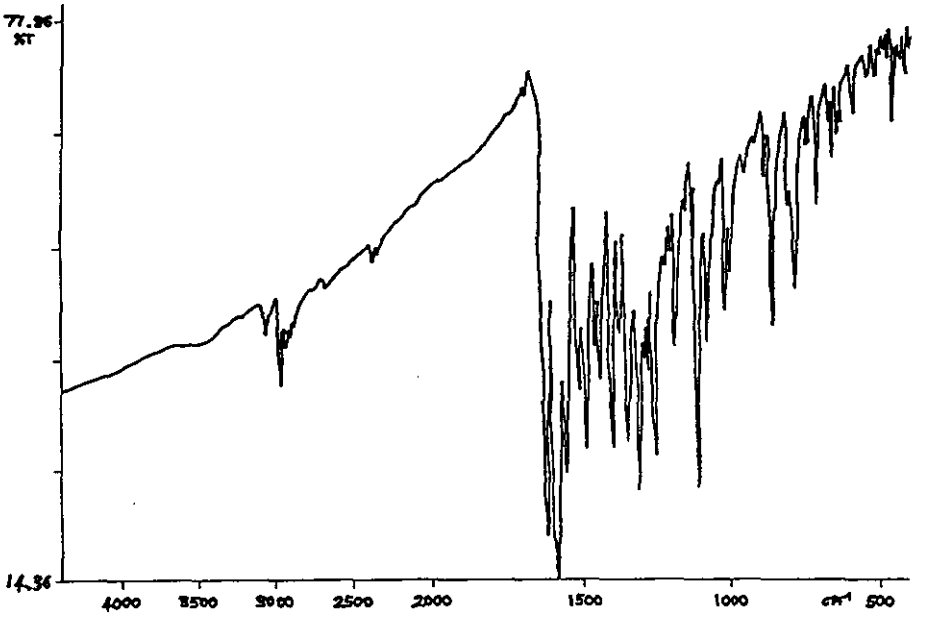


Figure 4.10: FT-IR transmission spectra of Nile Red [LIII] on the Perkin Elmer 1600 Series. Peaks at the following wavenumbers: 3057 (w), 2965 (w), 2925 (w), 1622 (vs), 1584 (vs), 1558 (s), 1514 (m), 1493 (s), 1460 (m), 1445 (m), 1406 (s), 1378 (m), 1349 (s), 1310 (s), 1290 (m), 1276 (m), 1255 (s), 1201 (w), 1182 (m), 1182 (m), 1129 (w), 1112 (s), 1078 (m), 1015 (m), 1000 (w), 880 (w), 850 (m), 776 (m), 746 (w), and 708 (w).

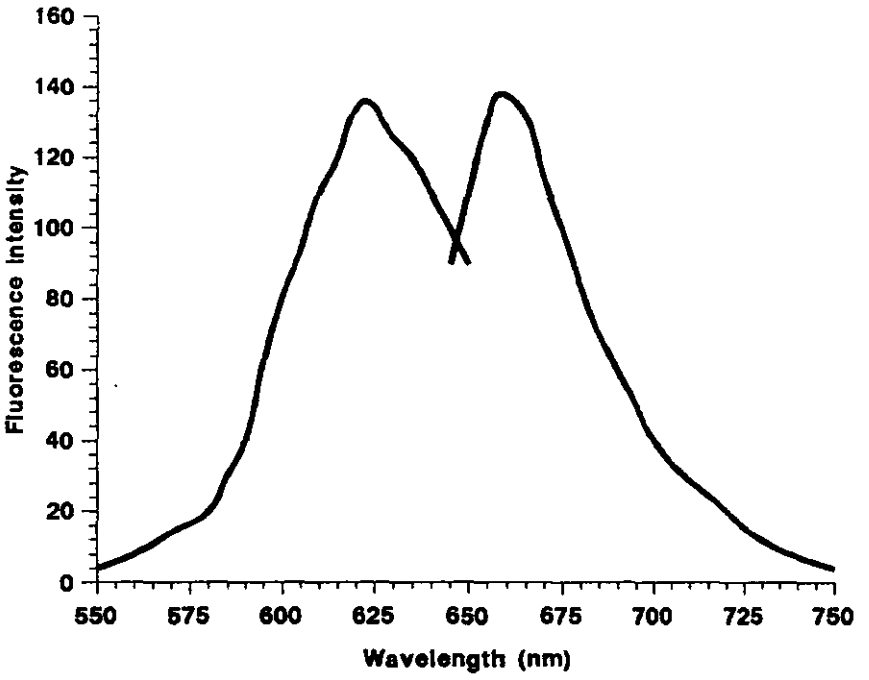


Figure 4.11: Excitation and emission spectra of a 50 nanomolar solution of Nile Blue A chloride [XLIX] in methanol. (Ex. 622 nm, Em. 658 nm)

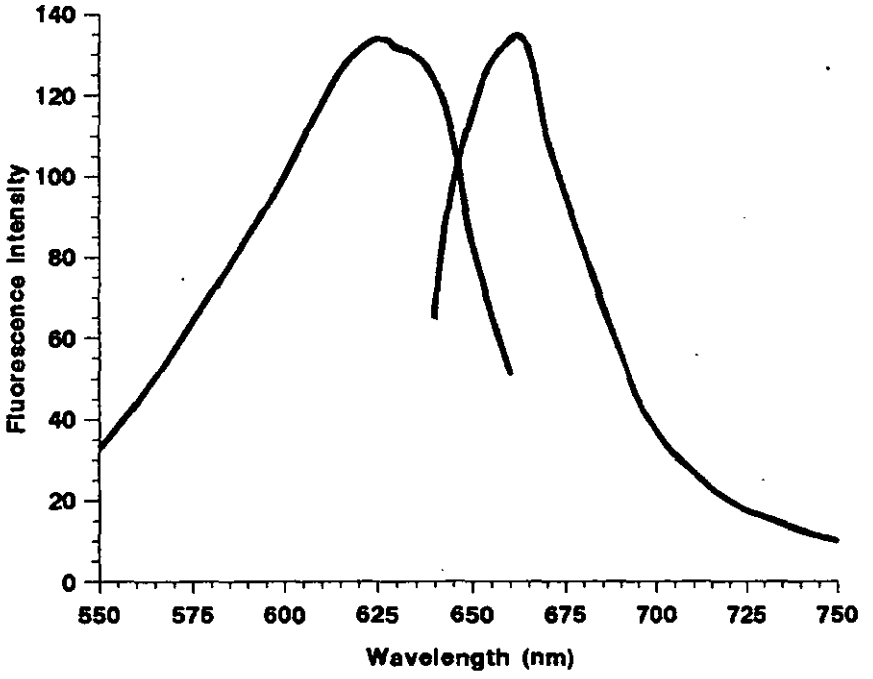


Figure 4.12: Excitation and emission spectra of a 50 nanomolar solution of Nile Blue allyl [CXII] in methanol. (Ex. 627 nm, Em. 662 nm)

4.3.3 Discussion

The product [XCII] was identified by FT-IR by additional absorption at 943 and 1011 cm^{-1} caused by C-H deformations and 3131 cm^{-1} C-H stretching of the ethylene group. TLC of the product [XCII] showed the absence of contamination from Nile Blue A [XLIX], the starting material and only a small contamination from Nile Red [LIII]. The latter was identified by its R_f, red colour and FT-IR (Figure 4.10).

Nile Blue allyl [XCII] showed a slight bathochromic shift as would be expected by transforming the primary amine of Nile Blue A [XLIX] into a secondary amine and this is consistent with the trend shown in Table 3.25 of Section 3.5.2.

Nile Blue allyl [XCI] was successfully synthesised, so the primary amine of Nile Blue A [XLIX] could be used to add a reactive group in order to produce a label as described in the next section.

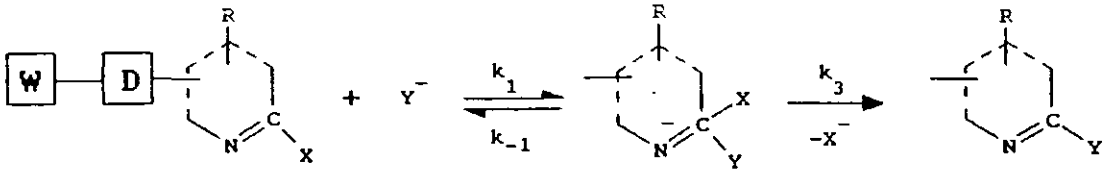
4.4 Nile Blue SS

After the successful synthesis of Nile Blue allyl [XCII], the reactive group of dichloro-s-triazine (Figure 4.15 [CIV]) was selected. Dichloro-s-triazines [CIV] belong to the most widely used reactive group of the dye industry.

Monochloro-s-triazine (Figure 4.14 [XCVIII]) and dichloro-s-triazine (Figure 4.15 [CIV]) reactive dyes were the first reactive dyes for cellulose fibres. They were commercially introduced in 1957 and 1956 respectively by the inventor companies (Imperial Chemical Industries (ICI) and CIBA). After the patent rights expired, numerous other dyestuff manufacturers started to produce these dyes. This reactive group can be subdivided into mono-functional and bi-functional sub-groups. Procion Supra (Figure 4.15 [CIX]) has two mono-functional reactive groups and so may be classed as either mono- or bi-functional.

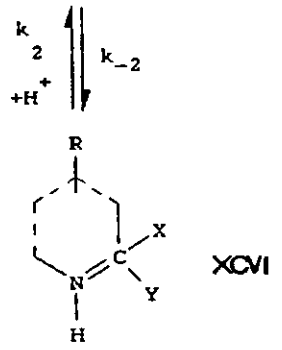
The principle synthesis of these reactive dyes is first to prepare the chromophore which has at least one primary or secondary amine group. Then this is reacted with, for example, in the case of dichloro-s-triazine reactive dyes [CIV] cyanuric chloride (2,4,6-trichloro-s-triazine [CX]). The monochloro-s-triazine dyes [XCVII to CII] are prepared from the dichloro-s-triazine dyes [CIV] by reacting them with a primary, secondary aliphatic or aromatic amine (or other nucleophilic agents). Dyes with other N-hetercyclic reactive groups can be similarly prepared.

The reactivity of N-heterocyclic reactive groups (Figure 4.13 [XCIII]) is increased by higher temperature. The addition reaction (k_1 in Figure 4.13) is the rate determining step. The rate of reaction is increased by making the electrophilic carbon-atom more positive.



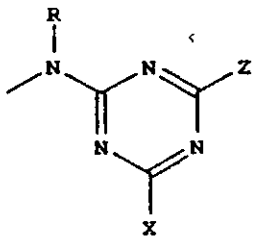
XCIII XCIV XCV

k1: the base catalysed addition of the substrate's nucleophilic functional group to the reactive group's electrophilic carbon.
k3: the elimination of the nucleophilic leaving group, if this intermediate accumulates rapidly, this reactions can be base catalysed.



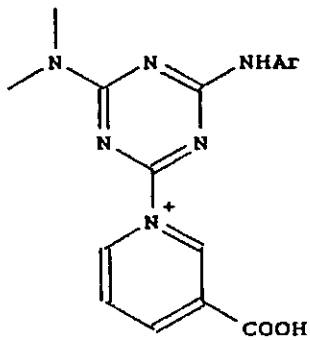
XCVI

Figure 4.13: Reactive pathway for reactive groups that react by the nucleopilic bimolecular (heteroaromatic) substitution mechanism. W is the water-solubilzing group, F is the fluorophore, X is the nucleophilic leacing group and Y is the group on the substrate (i.e. protein).



XCVII to CI

	monohalogeno-S-triazine	X	Z
XCVII	Cibacron and Procion H	Cl	NHR, NHAr, NR'R", NRAr
XCVIII	Cibacron F	F	NR'R", NRAr
XCIX	Cibacron Pront	Cl	OR
C	Procion SP	Cl	SO3H
CI	Sumifix Supra	Cl	NH-Ph-m-SO2-Et-OSO3H
CII	Cibacron C	F	NH-alkene-SO2-CH=CH2



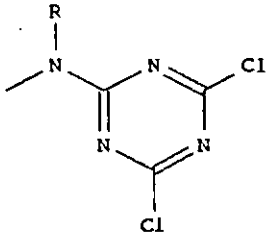
mono-(m'-carboxypyrimidinium)-s-triazines
CIII Kayacelon

Figure 4.14: Mono-functional reactive groups with one halogen which may react with one site on the substrate.

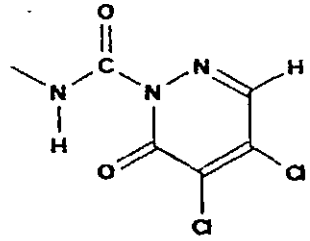
Table 4.6: Reactivity of N-heterocyclic reactive groups decrease on going down the table.

Reactive group	Commercial Name	Structure
dichloro-s-triazine	Procion MX	CIV
2,3-dichloroquinoxaline	Levafix E	CV
monofluoro-s-triazine	Cibacron F	XCIX
monochloro-s-triazine	Cibacron Pront	C
monochloro-s-triazine	Cibacron & Procion H	XCVII
2,4,5-trichloropyrimidine	Drimarene X & Z	CVII

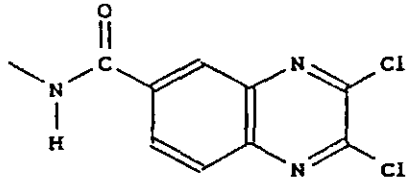
Table 4.6 shows that the reactivity of reactive groups decreases on going down the table. Dichloro-s-triazines [CIV] are more reactive than 2,4,5-trichloropyrimidines [CVII] because the former has more electron-attracting cyclic nitrogen atoms than the latter. Dichloro-s-triazines [CIV] are more reactive than monochloro-s-triazines [XCVII to CII] because the former has the one more electron-attracting substituent, chlorine. The rate of hydrolysis of the reactive dye is also increased by both of these factors as well as a reduction in the strength of the covalent between the dye-substrate with increasing reactivity of the dye.



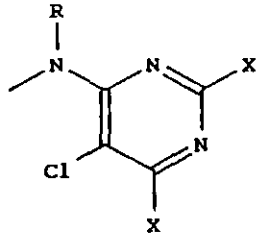
dichloro-s-triazine.
CIV Procion MX



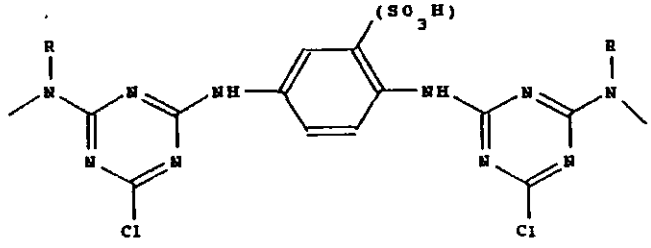
dichloropyridazone
CV Primazine P



2,3-dichloroquinoxaline
CVI Levafix E, Cavalite dyes



	2,4,5-trihalogenopyrimidine	X
CVII	Drimarene X and Z, Reactones	Cl
CVIII	Verofix, Levafix P-A, Drimalan, Drimarene R & K	F



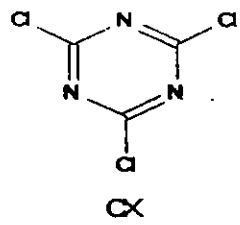
CIX Procion Supra

Figure 4.15: Bi-functional reactive groups with two halogens are present which may react with two reactive sites of the substrate.

Strongly electron-attracting leaving groups, such as quaternary ammonium groups of pyridine, increase the reactivity of the dichloro-s-triazine [CIV] or monochloro-s-triazine [XCVII to CII] reactive dyes because pyridine is a better nucleophilic leaving group. The quarternisation of the nitrogen atom increases the electrophilic character of the carbon atom and hence its susceptibility to nucleophilic attack (Banks, 1944: Banks and Controlis, 1946) and pyridine also regulates the pH of the reaction mixture by neutralising the chloride ions. In the latter case this inverse relationship between reactivity and stability can be avoided because the activating effect of the leaving group is lost during the fixation reaction. In this way, highly-reactive dyes produce very stable reactive bonds.

Dyeing with dichloro-s-triazine (Figure 4.17 [CXI]) dyes produces a mixture of CXII, CXIV and CXV. Under the mild conditions of sodium hydrogen carbonate almost entirely the mono-substituted product [CXII] is formed. CXII has a larger electron deficiency at 4-position caused by the chlorine substituent than the 2-position bonded to the substrate, so that the main alkaline hydrolysis reaction is to CXV. The di-substituted product [CXIV] is formed by the further reaction of the mono-substituted [CXII]. The hydrolysed product [CXV], formed by the hydrolysis of CXII and CXIII, exists almost exclusively in the keto form except at very high pH values. The deactivation of the keto form is caused by the destruction of the conjugation of the heterocyclic ring. This means that the dichloro-s-triazine dye-substrate bonds are more stable than those formed by the monochloro-s-triazine dyes [XCVII to CII], which cannot deactivate in this manner without bond cleavage with the substrate or the substituent in the 2-position. Under severe conditions, significant attack occurs at the 4-position causing dye-substrate rupture at the same time as hydrolysis. The mono-substituted [CXII] and di-substituted [CXIV] are permanently activated through the heterocyclic ring, the reactivity being influenced by the inductive effects of the substituents. CXIV is more stable than CXII to alkaline hydrolysis since two dye-substrate bonds need to be broken simultaneously.

Acid catalysed hydrolysis proceeds through the protonation of the nitrogen or oxygen bridging group between the reactive system and the substrate followed by nucleophilic attack by water. Only the nucleophilic substitution of amine groups has been discussed but other nucleophiles can be used, including sulphide ions and hydroxyl ions of cellulose.



CX

Figure 4.16: Cyanuric chloride [CX], also known as 2,4,6-trichloro-1,3,5-triazineand trichloro-s-triazine, is soluble in most organic solvents and is insoluble in cold water. In water above 10C, it undergoes rapid hydrolysis (Smolin and Rapoport, 1967)

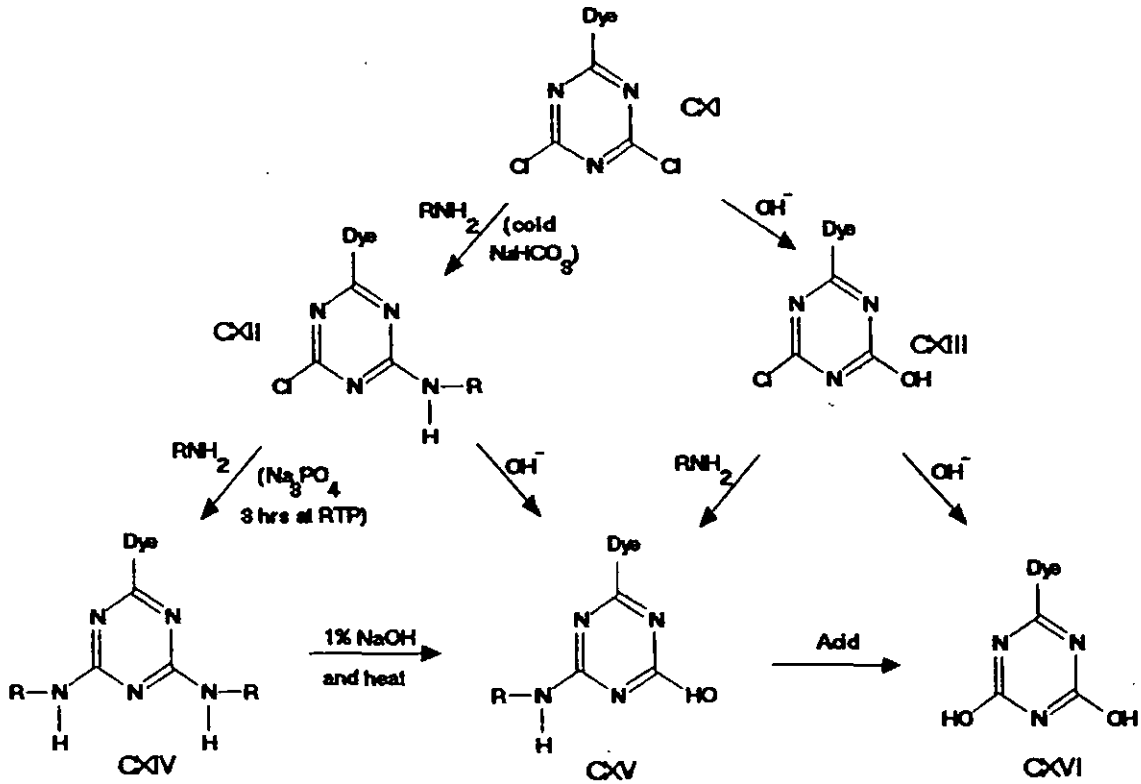


Figure 4.17: Possible reactions of a dichloro-s-triazine dye [CIV] in alkaline medium with RNH2 being a protein or amino acid. The mono-substituted product [CXII] is the main product under mild conditions with the hydrolysed form [CXV] produced slowly if the conjugate is stored in aqueous media.

4.4.1 Method

Nile Blue A anhydro base [LX] was prepared from Nile Blue A chloride [XLIX]. Nile Blue A chloride (1 g) was dissolved in 10 ml of acetonitrile and 75 ml of 2 molar sodium hydroxide was added. This was stirred for 10 minutes and then ultrasonicated for a further 10 minutes. The red brown product was filter and dried. The yield was 82%.

320 mg of Nile Blue A anhydro base was dissolved in 80 ml of 50% (v/v) acetonitrile/water and added drop wise over 30 minutes to 360 mg cyanuric chloride [CX] dispersed in 50 g of ice and 25 ml of water with 0.5 ml of 5 molar hydrochloric acid. The mixture was stirred over two hours. Then 0.5 g of sodium carbonate was added to neutralise the excess acid and stirring continued for a further 20 minutes maintaining the temperature below 4 °C. Sufficient sodium chloride was added to give a concentration of 200 g/l and the mixture was stirred for a further 30 minutes with the temperature between 0-4 °C. The product was filtered and washed with water and then hexane to remove Nile Red. The product was dried in an oven at 100 °C. The yield was 96%.

The starting material and the product was characterised by FT-IR (Figures 4.9, 4.10, 4.19 to 4.21), Oxygen Flask Combustion (Table 4.7), CHN analysis (Table 4.7), absorbance, fluorescence (Table 4.10: Figures 4.11 and 4.22), TLC (Table 4.8) and mass spectrometry. As previously stated proton-NMR of these dyes was not possible because of their very low solubility in the various NMR solvents tried and severe plating of the NMR tubes.

4.4.1.1 Protein Conjugation

An aliquot from the 10 millimolar Nile Blue SS [CXVII] in 25% (v/v) methanol / water stock solution was added to 2.5 ml of protein dissolved in buffer to give to the initial molar dye / protein ratio (Table 4.6). The concentration of the protein was between 1 and 2 mg/ml. The conjugation was carried out at room temperature for over 12 hours with constant stirring. The percentage content of the conjugation mixture was less than 1% v/v methanol. The conjugations carried out are summarised in Table 4.10.

Desalting and the removal of unbound Nile Blue SS [CXVII] was carried out by gel filtration on Sephadex G-25 (PD-10 columns, Pharmacia) and followed by exhaustive dialysis with pH 7.2 PBS.

Two conjugations were carried out with Nile Blue A [XLIX] (the starting material) and Nile Red [LIII] (the major contaminant of Nile Blue A) respectively.

These form non-covalent interactions with proteins. A ten fold molar excess of dye to human apo-transferin in pH 9.2 sodium carbonate-sodium bicarbonate buffer saline were prepared and the mixtures were stirred for 12 hours. The reaction mixtures were then passed down a PD-10 column and dialysised with pH 7.2 PBS. After each stage the fluorescence was measured.

Covalent bonding of Nile Blue SS [XCVII] was tested by dialysing 2 ml of the 10:1 Nile Blue SS-human apo-transferin conjugate with 8 molar urea in pH 7.2 PBS for three days.

4.4.2 Results

Table 4.7: Percentage composition sulphur, nitrogen and chlorine by oxygen flask of Nile Blue A [XLIX], Nile Blue anhydro base [LX], Nile Red [LIII], and Nile Blue SS.

	Theoretical					Experimental		Mol. Wt.
	H	C	N	O	Cl	N	Cl	
Nile Blue A chloride [XLIX]								
$C_{20}H_{20}N_3OCl$	5.7	67.9	11.9	4.5	10.0	12.1	9.8	353.85
Nile Blue A anhydro base [LX]								
$C_{20}H_{19}N_3O$	6.0	75.7	13.2	5.0	-	12.5	-	317.39
Nile Blue SS[1] [CXVII]								
$C_{23}H_{19}N_6OCl_3$	3.8	55.1	16.8	3.2	21.2	17.0	22.0	501.80
Cyanuric chloride [CX]								
$C_3N_3Cl_3$	-	19.5	22.8	-	57.7	21.2	58.1	184.85

[1] MEDAC found by CHN analysis C=51.38%: H=5.20%: N=15.6%: Cl=13.73%. MEDAC stated that sample lost weight before analysis so the loss of one of the chlorine atoms from the dichloro-s-triazine group occurred upon their drying procedure.

Table 4.8: Rf values of Nile Blue A [XLIX], Nile Red [LIII], Nile Blue allyl [XCII] and Nile Blue SS.

	9:1 MeCN:water				6:8:1 $CHCl_3$:2-butanone:HCOOH			
	Rf_1	Rf_2	Rf_3	Rf_4	Rf_1	Rf_2	Rf_3	Rf_4
Nile Blue		0.51		0.91[1]		0.52		0.95[1]
Nile Red			0.91					0.95
Nile Blue allyl			0.56	0.91[1]			0.60	0.95[1]
Nile Blue SS	0.20			0.91[1]	0.31	0.53		0.95[1]

[1] Nile Red was formed by the heating of Nile Blue A [XLIX] and was a minor contaminant of all Nile Blue A derivatives.

[2] Nile Blue A showed as very small contaminant of Nile Blue A.

Table 4.9: Summary of proteins conjugated with Nile Blue SS at room temperature for 12 hours. The initial molar ratio was the amount of Nile Blue SS added to protein at conjugation. Nile Blue SS:protein molar ratio in pH 7.2 PBS carried out by spectrophotometry after desalting and dialysis.

Initial molar ratio	protein	Conjugation buffer	protein conc. (mg/ml)	dye/protein molar ratio (pH 7.2 PBS)
	apo-transferin	pH 9.2[1]	2.0	ND
1:1	apo-transferin	pH 9.2 saline[2]	2.0	1.1:1
2:1	apo-transferin	pH 9.2 saline[2]	2.0	1.5:1
3:1	apo-transferin	pH 9.2 saline[2]	2.0	2.9:1
5:1	apo-transferin	pH 9.2 saline[2]	2.0	3.5:1
10:1	apo-transferin	pH 9.2 saline[2]	2.0	ND
1:1	human IgG	pH 10.3[3]	1.0	ND
3:1	human IgG	pH 10.3[3]	1.0	ND
10:1	human IgG	pH 10.3[3]	1.0	ND
15:1	human IgG	pH 10.3[3]	1.0	ND
2:1	human IgA	pH 9.2 saline[2]	1.0	ND
2:1	bovine insulin	pH 9.2 saline[2]	1.0	1.6:1

[1] pH 9.2 sodium carbonate-sodium bicarbonate buffer.
[2] pH 9.2 sodium carbonate-sodium bicarbonate buffer saline.
[3] pH 10.3 sodium carbonate-sodium bicarbonate buffer.

Table 4.10: The excitation and emission maxima of Nile Blue A, Nile Blue SS and Nile Blue SS protein conjugates after gel chromatography and exhaustive dyalysis in various solvents. (ND means not determined).

	Ex. (nm)	Em. (nm)	Solvent
Nile Blue A chloride [XCIX]	622	658	methanol
	625	660	10 methanol/water
Nile Blue SS [CXVII]	630	662	methanol
	633	668	10% methanol/water
Nile Blue SS-human apo-transferin	640	665	pH 7.4 PBS
	642	672	10% MeOH/pH 7.2 PBS
	634	ND	50 % methanol
	646	671	8 molar urea[1]
Nile Blue SS-IgG	640	670	10% MeOH/pH 7.2 PBS
Nile Blue SS-IgA	645	662	pH 7.4 PBS
Nile Red-human apo-transferin	none	none	pH 7.2 PBS
Nile Blue A-human apo-transferin	none	none	pH 7.2 PBS

[1] After dialysis for 3 days in 8 molar urea in pH 7.2 PBS.

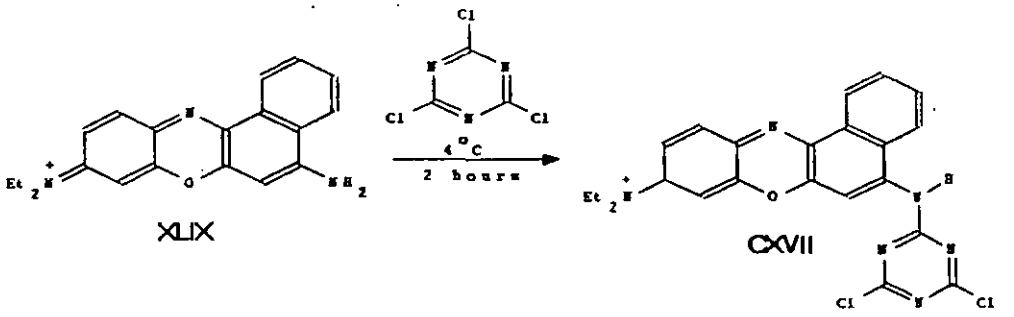


Figure 4.18: Nile Blue SS [CXVII] (the dichloro-*s*-triazine derivative of Nile Blue A [XLIX]) was synthesised by the dopwise addition of Nile Blue A [XLIX] in 50% acetonitrile to cyanuric chloride dispersed on ice with continuous stirring over two hours. A few millilitres of hydrochloric acid were added to stop the reaction. The product was isolated by salting out and washed with hexane dried.

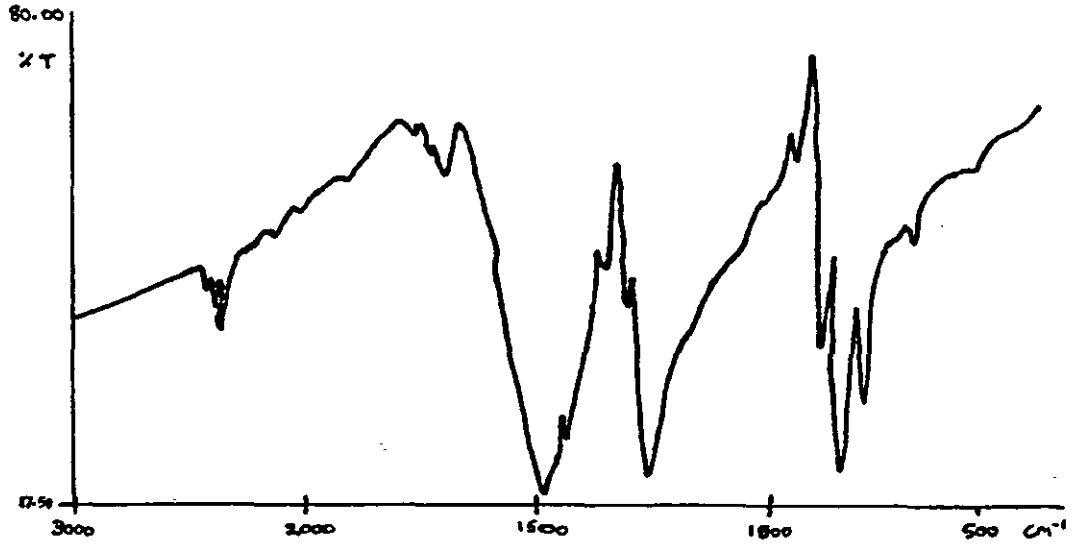


Figure 4.19: FT-IR transmission spectrum of cyanuric chloride [CX] on the Perkin Elmer 1600. Peaks at the following wavekengths: 2361 (m), 1708 (w), 1497 (vs), 1318 (m), 1270 (vs), 880 (m), 850 (vs), and 792 (s).

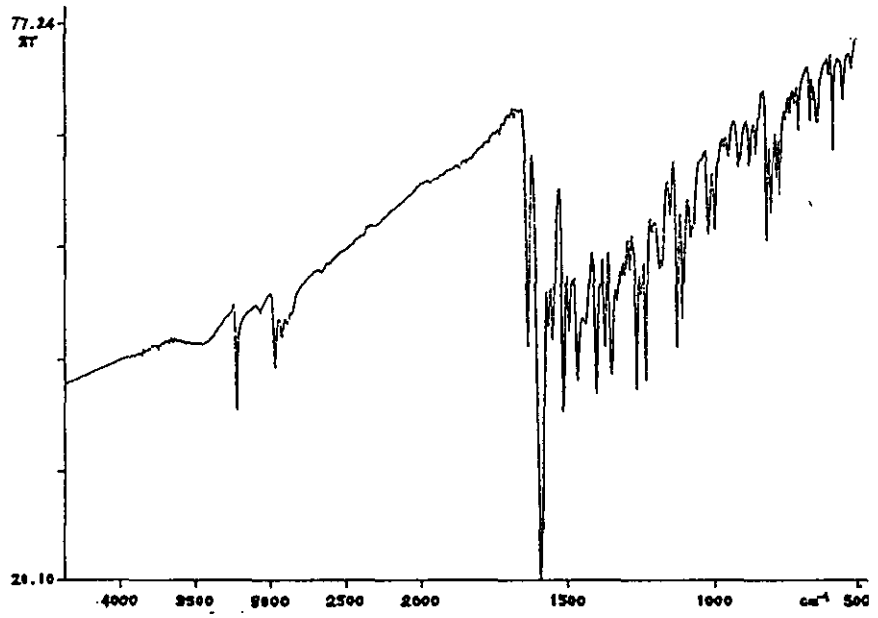


Figure 4.20: FT-IR transmission spectrum of Nile Blue A anhydro base [IX] on the Perkin Elmer 1600. Peaks at the following wavenumbers: 3226 (m), 2967 (m), 1636 (m), 1592 (vs), 1566 (m), 1551 (m), 1514 (s), 1493 (m), 1466 (m), 1406 (s), 1374 (m), 1352 (m), 1291 (w), 1267 (s), 1234 (s), 1187 (w), 1148 (w), 1124 (m), 1105 (m), 1078 (w), 1017 (w), 996 (w), 915 (w), 879 (w), 858 (w), 817 (m), 801 (w), 780 (w), 768 (w), and 699 (w).

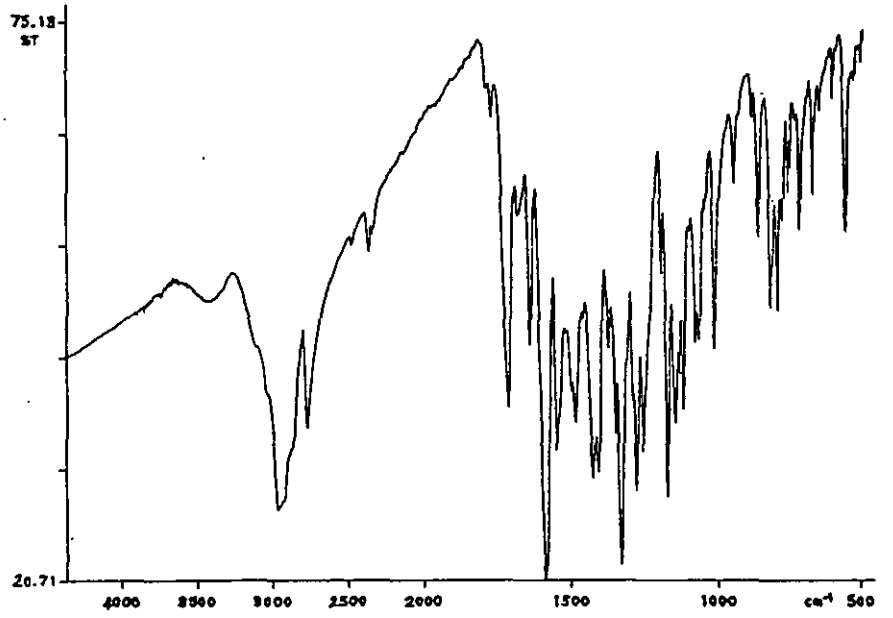


Figure 4.21: FT-IR transmission spectrum of Nile Blue SS [CXVII] on the Perkin Elmer 1600. Peaks at the following wavenumbers: 3443 (w), 2967 (s), 2776 (m), 2359 (w), 1771 (w), 1716 (s), 1681 (w), 1640 (m), 1587 (vs), 1550 (s), 1485 (m), 1426 (s), 1407 (s), 1374 (m), 1351 (m), 1333 (vs), 1280 (s), 1257 (s), 1192 (m), 1171 (s), 1145 (m), 1116 (m), 1074 (m), 1062 (m), 1011 (m), 943 (w), 881 (w), 858 (m), 816 (m), 789 (m), 770 (m), 750 (m), 740 (m), and 706 (m).

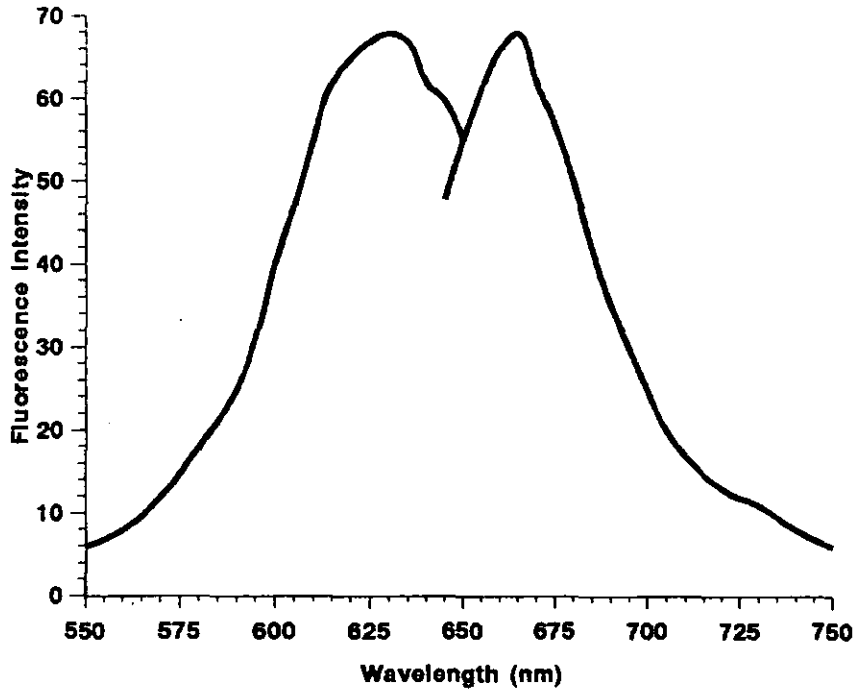


Figure 4.23: Excitation and emission spectra of a 0.1 micromolar solution of Nile Blue SS [CXVII] in 10% methanol:water. (Ex 633 nm, Em 665 nm).

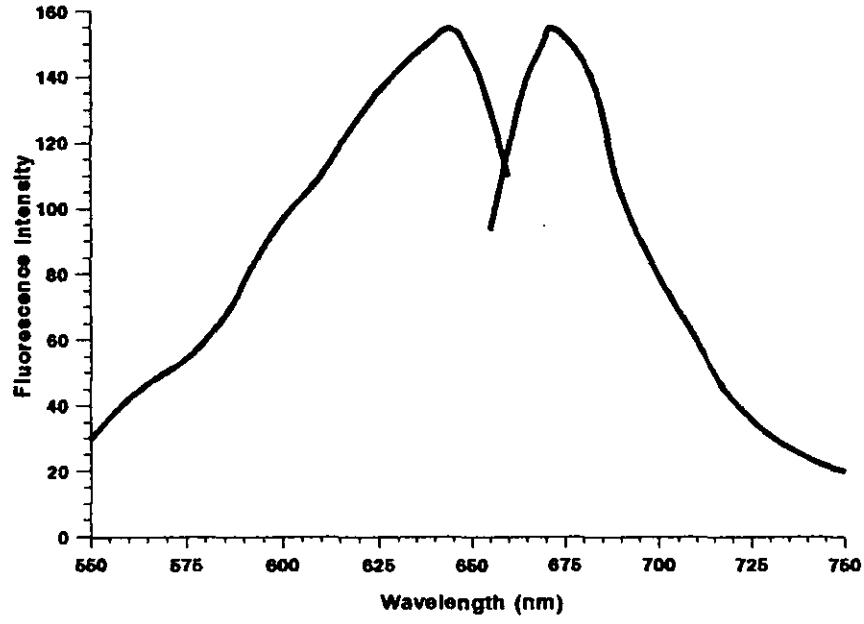


Figure 4.24: Excitation and emission spectra of Nile Blue SS-human apo-transferin in 10% methanol:pH 7.2 PBS. (Ex 640 nm, Em 670 nm).

4.4.3 Discussion

Nile Blue SS [CXVII] was identified by the presence of strong absorbance at 2967 cm^{-1}, 1761 cm^{-1} (see Figures 4.9 and 4.21).

TLC of the product [CXVII] showed the presence of Nile Red [LIII] as a minor contaminant, which was identified by FT-IR (Figure 4.8). All the starting material had reacted.

Nile Blue SS [CXVII] showed a slight bathochromic shift as would be expected by transforming the primary amine of Nile Blue A [XLIX] into a secondary amine and this is consistent with the trend shown in Table 3.25 of Section 3.5.2.

Nile Blue SS was successfully synthesised on three occasions. Each batch showed the same characteristics.

The molar dye-protein ratio (Table 4.8) was not determined for the Nile Blue SS-human IgG conjugates because of the low absorbance values. Also, human IgG precipitated out of the buffer in the absence of salt, so subsequent conjugations were with 0.1 molar sodium chloride added. The low conjugation of Nile Blue SS to human IgG was due to the use of too high a pH conjugation buffer that promoted hydrolysis of the dichloro-s-triazine group rather than covalent reaction. The conjugation with bovine insulin showed that conjugation of Nile Blue SS [CXVII] with small proteins occurred.

Nile Red [LIII] and Nile Blue A [XLIX] were approximately 90% removed by gel chromatography and completely removed by the subsequent dialysis. Therefore, the fluorescence shown by the Nile Blue SS conjugates was from the covalently bonded dye and not from non-covalent interactions.

Nile Blue SS-human apo-transferin conjugate dialysed with 8 molar urea still showed significant fluorescence, which was equivalent to the fluorescence from before dialysis if the concentration effect was taken into account. This is the strongest evidence that Nile Blue SS [CXVII] formed covalent bonds with proteins because any unbound dye would have been dialysed away.

4.4.4 Conclusion

Nile Blue SS-human apo-transferin conjugates were then used as discussed in Section 5.3 to develop an immunoassay.

4.5 References

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Chapter 5

5.1 Fluorescent probes and immunoassay

Proteins are composed of amino acids covalently linked by amide bonds between the α-carboxylic group of one amino acid and the α-amino group. Some proteins are made up of two or more chains which are linked by disulphide bonds. There are twenty naturally occurring amino acids. Amino acids can be divided into the classes as listed in Table 5.1. Various non-covalent forces such as electrostatic, hydrogen bonding, and Van der Waals forces influence the shape of the protein. Most proteins have a molecular weight between 10000 and 100000 daltons, although smaller and larger proteins also occur. *Polypeptides* are classed as having molecular weight under 5000 daltons.

Table 5.1: Naturally occurring amino acids.

Group	Amino acid
Allphatic side-chains	glycine (Gly), alanine (Ala), valine (Val), leucine (Leu) and isoleucine (Ile)
Aliphatic side-chains with secondary amine.	prolin (Pro)
Hydroxyl side-chains	serine (Ser) and threonine (Thr)
Aromatic side-chains	phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Try)
Positively charged (basic) side-chains (at neutral pH)	lysine (Lys), arginine (Arg) and histidine (His)
Negatively charged (acid) side-chains (at neutral pH)	glutamic acid (Glu) and aspartic acid (Asp)
Sulphur containing	methionine (Met) and cystein (Cys)
Special amino acids	hydroxyproline (Hypro), carboxyglutamate, phosphoserine

The architecture of proteins may be described in terms of five levels.

Primary structure is the sequence of amino acids.

Secondary structure refers to the steric relationship of amino acids that are next to each other in a linear sequence and the regular hydrogen bonding required to form the α-helix structure.

Tertiary structure refers to the steric relationship of amino acids that are far apart in the chain.

Quaternary structure refers to the spatial interaction of different polypeptide chains in a protein molecule.

The *conformation* refers to the overall three dimensional structure of a protein and is specified by the amino acid sequence that enables a given optimal occurrences of covalent (disulphide bonds) and non-covalent bonding between the participating amino acids.

The electric charge on a protein and the way in which this is produced by its amino acid residues is of primary importance for the dye-protein interaction. When a protein is considered as a globular particle, the overall charge on a protein depends on the sum of the charges on the amino acid residues and the pH of the surrounding medium. A protein's isoelectric point (IEP) is the pH where there is no net charge on the protein. A pH below this isoelectric point, the protein molecule carries a net positive charge, and a pH above the isoelectric point, it will have a net negative charge. The notions of negatively charged ("acidic") and positively charged ("basic") proteins are relative. Increasing pH results in acidic proteins behaving like basic proteins and decreasing pH results in basic proteins behaving like acidic proteins. Proteins are thus said to be amphoteric (Greek "ampho", meaning both) in character in this respect.

Almost all proteins occurring in animal and plant tissues are amphoteric, although some are at such extremes of the scale that they are virtually always basic or acidic. Under conditions of neutral pH, the majority of proteins have a net positive charge so they bind negatively charged (acid) dyes, e.g. Eosin, Rose Bengal [VII], Fluorescein [IV] etc. On the other hand, the nucleic acids in, for

example, the cell nucleus carry a negative charge at neutral pH, so that these acid macromolecules will bind basic (positively charged) dye molecules.

The occurrence of differing electrical charges on a protein molecule are brought about by ionization phenomena which are in fact properties of the amino acid residues of the protein concerned. It is necessary therefore to briefly to some properties of these basic structural units, leaving again the detailed description of these compounds to specialised textbooks on biochemistry.

The classic subdivision of proteins is into *simple proteins* which yield only amino acids in hydrolysis (Table 5.1) and *conjugated proteins* (Table 5.2) in which the protein moiety is united covalently to one or more molecules of another nature (carbohydrate, lipid).

Table 5.2: Simple proteins.

IEP	class	notes
11-12	protamines	high proportion of arginine and lysine residues.
10-11	histones	complexed with DNA
5-7	globulins	e.g. serum globulins. Sparingly soluble in water but easily soluble in dilute salt solutions.
4-6.5	scleroproteins	e.g. collagen, elasten and keratin. Found in animal tissues, insoluble in water at neutral pH
4-5	albumins	e.g. egg albumin and serum albumin. Soluble in water.

Table 5.3: Complex proteins.

class	notes
glycoproteins	e.g. membrane glycoproteins have one or more sugar residues attached to the serine, threonine, or asparagine side-chain of the protein.
lipoproteins	protein complex with fatty acids that occurs in many animal cells.
nucleoproteins	nucleic acids are non-covalently bonded to proteins.
chromoproteins	e.g. haemoglobin, haemocyanin, flavoproteins and the pigments of the sensory cells of the retina. These pigmented proteins absorb in the visible region and are readily soluble in water or dilute saline.
phosphoproteins	orthophosphoric acid linked to serine and threonine by ester linkage occur in milk and egg yolk.

5.2 Fluorescent probes

Proteins form complexes predominately with anionic or neutral small molecules. The fluorescence character of the small molecules and that of the protein may be altered upon formation of the complex. If neither the small molecule nor the protein has suitable fluorescence characteristics, the interactions between the small molecule and the macromolecular can still be investigated by binding non-covalently to the macromolecule a fluorescent label. These non-covalently bound fluorescent labels have also been used to study dye-binding to macromolecules and as probes for some feature of macromolecular structure (Weber, 1992). The intermolecular forces responsible for these interactions can be ionic, van der Waals, hydrophobic or charge transfer.

There are only a few examples of the applications of non-covalently bound near infrared fluorogenic labels. Competitive binding of IR125 [XXV] to human and bovine serum albumin was studied by Kamisaka and co-workers in 1974. The earliest analytical applications of this were by Sauda et al. in 1986, who reported picomolar detection limits for the laser diode fluorimetry of proteins from human serum labelled with IR125 [XXV], after gel chromatography (Sauda et al., 1986; Imasaka et al., 1989). More recently, four different carbocyanines (DTDC, DTTC [XXIII], DODC [XXI] and DOTC [XXII]) have been evaluated to determine their binding to serum proteins (Wilberforce and Patonay, 1990).

In 1984, Taylor and co-workers traced in vivo viable cells by the fluorescence of carbocyanine dyes and hence calculated cellular life spans. In vivo cellular tracking of labelled blood cells could be utilized for diagnosing tumours and other diseases. The application of visible fluorogenic labels is severely limited for biomolecular determinations because of strong spectral interferences.

Carbocyanines have been used as molecular probes in membranes (Smith, 1990). Low interference of long wavelength labels is important when the technique is coupled with microscopy (Oseroff et al., 1986). Menzel et al. (1986) used carbocyanines and merocyanines to study electrical damage in insulators by attaching fluorogenic labels to damaged surfaces.

5.2.1 Method

The near infrared dyes, Nile Red [LIII] and DTTC [XXIII], were investigated as probes in conjunction with two proteins; bovine serum albumin (BSA) and bovine α_1-acid glycoprotein.

An aliquot of dye from a methanol stock solution was added to aqueous pH 7.2 PBS buffer solution containing 1.0 micromolar of bovine α_1-acid glycoprotein or about 2.0 micromolar of bovine serum albumin. The fluorescence spectrum was recorded immediately on the addition of the dye and then at regular time intervals. The concentration of dye in the final solution was 0.3 micromolar. The protein was never exposed to more than 1% v/v methanol.

5.2.2 Results

Table 5.4: A summary of the results of dye-protein binding studies of two near infrared dyes (Nile Red [LIII] and DTTC [XXIII],) with the proteins bovine serum albumin (BSA) and α1-acid glycoprotein.

Label	Protein	Ex. (nm)	Em (nm)	Enhancement of fluorescence
Nile Red	unbound	580	663	decays exponentially with time
	bovine serum albumin	550	614	large enhancement
	α_1-acid glycoprotein	550	655	small enhancement
DTTC	unbound	700	780	constant over time
	bovine serum albumin	700	780	moderate enhancement
	α_1-acid glycoprotein	700	780	very small enhancement

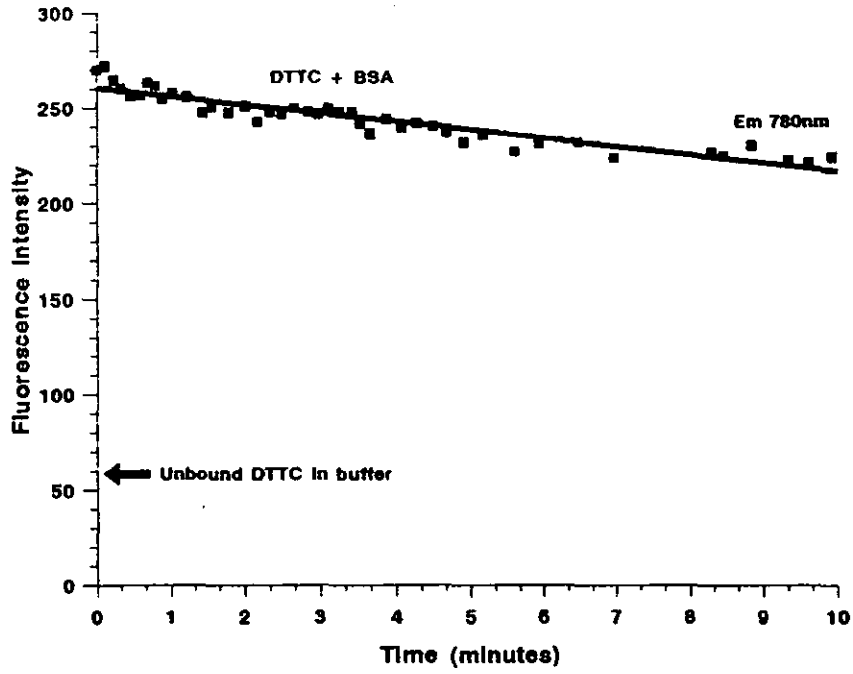

Figure 5.1: Time dependent fluorescence at 780 nm on the IMUC-7000 Photodiode Array Detector of DTTC bound to bovine serum albumin in pH 7.2 PBS. BSA and DTTC concentration were 0.3 and 1.8 μM respectively.

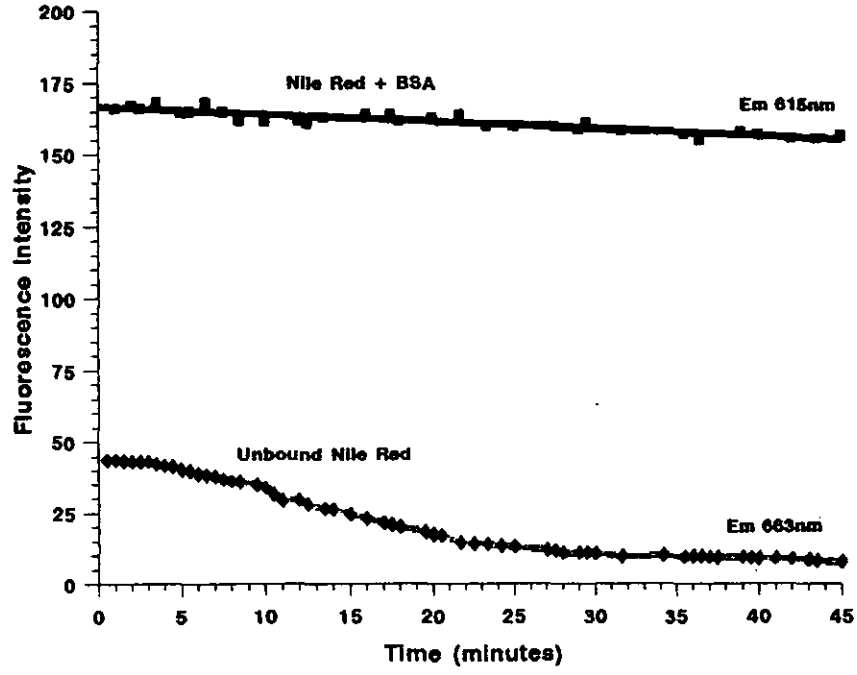

Figure 5.2: Time dependent fluorescence of Nile Red bound to BSA in pH 7.2 PBS and excitation at 546 nm on the IMUC-7000 Photodiode Array Detector. BSA and Nile Red concentration were 0.3 and 2.0 micromolar respectively.

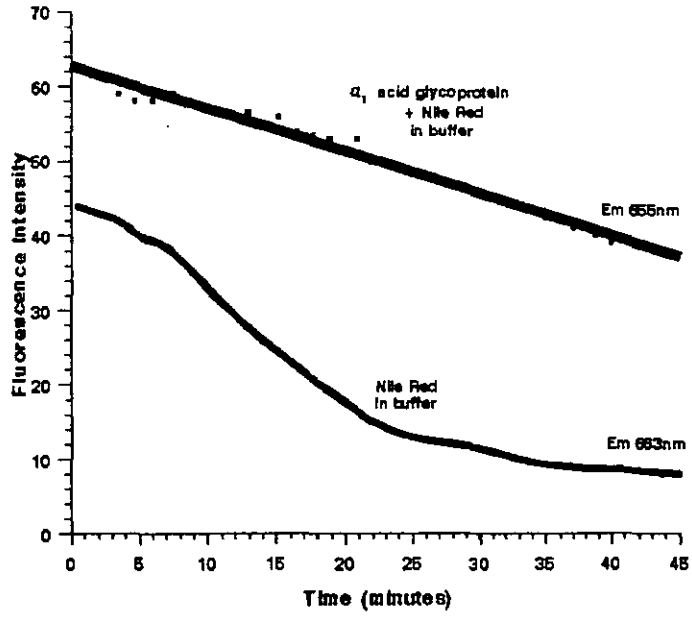

Figure 5.3: The fluorescence decay with time of 0.3 micromolar Nile Red [LIII] bound and unbound to 1.0 micromolar alpha-1-acidglycoprotein in pH 7.2 PBS buffer at 25 °C.

5.2.3 Discussion

The Nile Red-bovine serum albumin complex was very stable. The 0.2% per minute reduction in fluorescence intensity as shown in Figure 5.1 was attributed to photobleaching because in that experiment gating of the excitation beam was not used. The hypsochromic shift of Nile Red's emission wavelength from 663 nm in buffer to 615 nm when Nile Red is bound to BSA is explained by a change in environment from a hydrophilic to a hydrophobic one (Sacket and Wolff, 1987). This conclusion is supported Nile Red's [LIII] solvatochromic properties (see Figure 3.20) and was used by Deye and Berger (1990) as polarity probe for super critical fluid chromatography (SFC). Nile Red [LIII] shows good probe characteristics with bovine serum albumin because the intensity of the Nile Red bound to albumin changes slowly and the unbound dye decays quickly. The quenching of Nile Red [LIII] in aqueous buffer was due to dimerisation causing the dye to become insoluble and hence precipitate out of solution as already described in Section 3.7.

DTTC [XXIII] is a cationic dye and hence showed poor binding to BSA at pH 7.2 because at this pH the protein has a net positive charge. Albumins bind anionic and neutral dyes better under these conditions. If the pH of the buffer used was reduced below the IEP of BSA, the binding of cationic dyes would have been increased. Figure 5.2 shows that DTTC [XXIII] bound very rapidly to BSA with an initial enhancement of fluorescence of about four fold. There was no observed shift in either the excitation and emission wavelength. The fluorescence intensity then fell by about 1.7% per minute.

Nile Red [LIII] bound to α1-acid glycoprotein showed an 8 nm hypsochromic shift in emission but no appreciable gain in fluorescence. This seems to suggest that there has definitely been a change in the environment but only a small amount of Nile Red bound to the protein. In another experiment the excitation wavelength was shown not to shift with or without the α1-acid glycoprotein.

DTTC [XXIII] and α1-acid glycoprotein gave intensities that were not significantly above those observed for the aqueous buffer alone. This indicates that there was either no binding of DTTC to α1-acid glycoprotein.

From the initial results, it can be seen that the binding of the near infrared dyes (Nile Red [LIII] and DTTC [XXIII]) to proteins (such as bovine serum albumin) enhanced the fluorescence of the dye. If a substance could be found to dislodge the dye, this could be used as an assay method for example drugs.

5.3 Immunoassay

Trace analysis of organic compounds in complex matrices, such as body fluids, tissue samples, foodstuffs, and environmental samples, is one of the hardest problems encountered by analytical scientists (Sherry, 1992). The presence of similar target molecules demands a highly selective analysis. The labile nature of biological molecules and molecular interactions such as protein binding of small molecules present further problems. The high demand for such analysis, especially in medicine, environmental and food science, means that the techniques must be simple, robust, low cost and capable of automation and/or performance by relatively inexpert personnel (Miller 1979). Immunoassay is a selective and sensitive technique that exploits the immune system's ability to produce antibodies (Ab) in response to virtually any organic molecule. Immunoassay technology originated in the 1950's and is a blend of analytical and clinical chemistry.

Antibodies belong to the globular fraction of serum proteins and consist of five main types of glycoproteins: IgG, IgM, IgE, IgA and IgD. The principle human antibody is immunoglobulin G (IgG), which consists of two identical pairs of heavy and light polypeptide chains (molecular weight of 50000 and 25000 respectively) linked together by disulphide bridges. The IgG structure can be divided into three fractions: two immunologically active antigen binding sites (Fab-fragments), and into one constant non-binding (Fc) fragment. The antibody binding site of Fab consists two variable region generated by variation of the amino acid sequence, this generates perhaps 100 million chemically distinct binding specificities while the overall structure of all IgG molecules remains unchanged. The ability of the Fc part of the antibody to bind to the bacterial products Protein A and Protein G is of value in antibody purification and has been used in certain immunoassays developed by Palmer et al. (1992).

Polyclonal antibodies are produced by repeated injection into an experimental animal of antigen, or in the case of a small molecule (called a hapten) a conjugate with a polymeric carrier. The antibody molecules are recovered from the animal's blood serum. Normally, the antibodies are separated from the sera. However, due to the specificity of antigen-antibody interactions, it is often possible to use a simple dilution of this antiserum directly as an analytical reagent.

In 1975, Kohler and Milstein made the first *monoclonal antibodies* by the fusion of a spleen cell producing specific antibodies with a myeloma (cancer) cell capable of continuous growth in cell culture. Monoclonal antibodies are rapidly

gaining a dominant position in immunoassays, especially from the commercial point of view, because of their unlimited supply, molecular homogeneity, and defined, unchanged properties.

In a typical competitive binding immunoassay (Figure 5.4) a labelled antigen (Ag*) is incubated together with antibodies (Ab) to the target analyte. The label could be a radioisotope, an enzyme or a fluorophore. In some immunoassay formats, the antibody rather than the antigen is labelled. A portion of the sample, or buffer that contains a known amount of analyte, is then added to the assay tube. The labelled (Ag*) and unlabelled analyte (Ag) compete for the highly specific binding sites on the antibody (Ab). After a sufficient incubation period, the bound and the unbound antigen are separated. The amount of analyte that was bound to the antibody is quantified. For samples, the amount of bound label (AbAg*) is compared with that of a set of calibration standards. The quantity of analyte in the sample can be interpolated from a calibration curve.

Immunoassays may be divided into two distinct types: heterogeneous assays, where a separation step is necessary to separate antibody bound and unbound materials, and homogeneous assays, in which no separation step is required.

Although assays involving radio-labels remain in common use, there is a very substantial and growing trend to replace such labels with non-isotopic markers (Miller, 1990). Non-isotopic immunoassays are normally less sensitive than radioimmunoassays, but have the important advantages: they are less hazardous, the expense of disposal of radioactive material is eliminated, the ability to detect two or more analytes simultaneously is possible and there is the potential to develop a simple, robust and portable "immunosensor." The non-isotopic labels that have had widespread acceptance are enzymes, chemiluminescent groups or fluorescent groups. Table 5.5 summarises the detection limits achieved by various non-radioisotopic labelling methods.

Enzyme immunoassays have been reviewed by Kricka, 1985; Voller and Bidwell, 1985 and are favoured by clinical chemists. Many enzyme immunoassays use the enzyme linked immunosorbent assay (ELISA) approach (Engvall and Perlmann, 1971), in which the antibody (sometimes antigen) molecules are immobilized on a solid surface. The antibody-bound molecules are separated from the unbound species by decanting. The second commonly used enzyme immunoassay is the homogeneous enzyme multiplied immunoassay technique (EMIT), this was the first commercially available non-isotopic immunoassay method (Rubenstein et al., 1972).

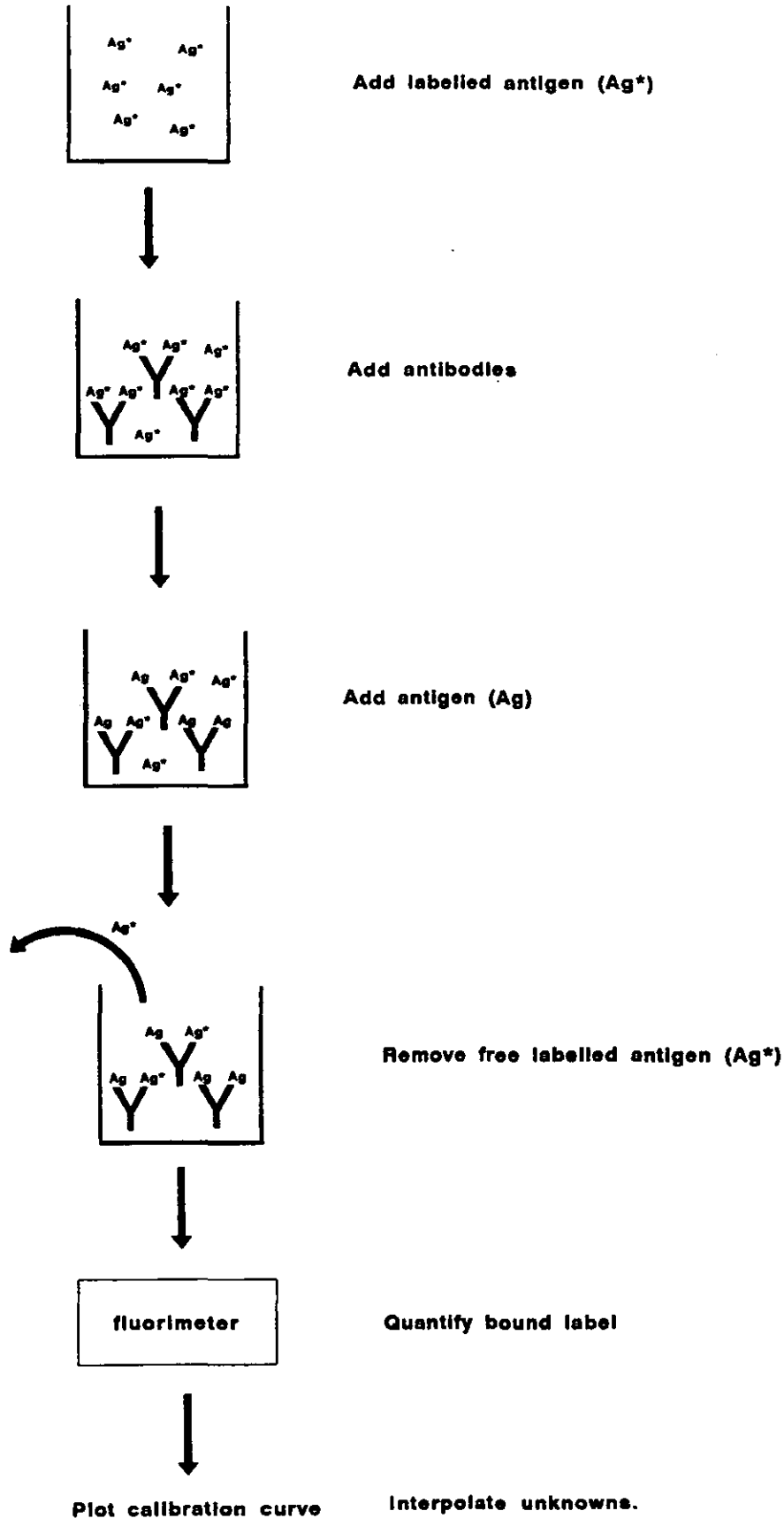

Figure 5.4: Steps in a typical competitive binding immunoassay. Y = antibody; Ag* = labelled antigen; Ag = antigen.

Table 5.5: Summary of the detection limits achieved by various non-radioisotopic labelling methods (Diamandis and Christopoulos, 1990) .

Method	mol/l
Fluorophore labels	10^{-9} to 10^{-10}
Luminol and isoluminol chemiluminescence	10^{-9} to 10^{-10}
Enzyme labels	10^{-10} to 10^{-11}
Acridinium ester chemiluminescence	10^{-11} to 10^{-12}
Europium chelate time resolved fluorescence	10^{-12} to 10^{-13}
Enzyme label with fluorogenic or chemiluminescent	10^{-12} to 10^{-13}

5.3.1 Heterogeneous immunoassays

Heterogeneous assays rely on a separation step based either on size, charge, solubility or some specific binding to Protein A etc. to distinguish antibody bound from unbound antigen. Such separations are in principle tedious, and may complicate the automation of assays, but new methods such as the use of magnetic particles to immobilise antibody molecules minimise the practical problems. The major advantage is that the separation step automatically removes sample components that would interfere with the immunoassay, hence the limit of detection is improved. The majority of immunoassays in everyday use are heterogeneous. The numerous approaches to the separation step may be split up into three major categories:-

Adsorption methods, in which one or other component of the assay is removed after incubation by adsorption on a solid or gelatinous adsorbent:

Precipitation methods, in which the antibody-containing fraction is precipitated after incubation by a non-specific chemical (such as ammonium sulphate, sodium sulphate, organic solvents and polyethylene glycol) or specific biochemical reagents (i.e. a second antibody). The double antibody precipitation utilises as the precipitant antibodies from another species (e.g. sheep, goat) reacting with the first (e.g. rabbit, guinea-pig) antibody.

Solid phase reagents, in which one component of the reaction (antigen or antibody) is bound covalently or non-covalently to a solid matrix from the start of the assay, the separation step then merely being a washing process. This is also called Immunometric immunoassay and has been extensively investigated by Miller, 1992.

5.3.2 Homogeneous immunoassays

In homogeneous immunoassay, the extent of the antigen-antibody reaction can be determined by changes in signal of the labelled molecules without physical separation of the bound and unbound forms. The sensitivity of an immunoassay procedure is dependant upon the choice of label and its limit of detection in the matrix of interest. This is especially important in homogeneous immunoassays which rely on the use of a label whose characteristics change when antibody-antigen combination occurs. This change in properties allows the antibody-antigen to be monitored directly, but the disadvantage is that all the other sample components are still present. Considerable "background" effects can arise. Passing serum samples through small pre-columns containing one or more affinity matrices provides only a partly satisfactory approach to the reduction of background and cannot be used if the analyte is itself a protein, or is a small analyte bound to a protein.

The practical limit of detection of homogeneous fluorescence immunoassays is in the nanomolar region. This is much poorer than would be expected from studies of fluorophores in pure solution, where background signals are very low. The absorption and emission bands of the commonly used fluorescent labels (Fluorescein [IV] derivatives, Fluorescamine, Rhodamines B [IX] derivatives) lie in the spectral region where interference is likely from intrinsic fluorophores such as proteins, haemin, NADH, bilirubin and albumin bound bilirubin, drugs, etc. The practical applicability of any fluorogenic labelling method depends on its molar absorptivity and fluorescence quantum yield and on the presence of interfering fluorophores. The background scattering and fluorescence of a 1% blood sera sample (see Figures 1.4 and 1.5) was reduced by at least 100 in the near infrared region as compared to the visible region. There are very few fluorophores that absorb and emit in near infrared region (600 to 1000 nanometres) so there are less likelihood of background fluorescence.

Fluorescent labels with small Stokes shift, such as Fluorescein, make them vulnerable to Rayleigh scattered light from protein containing samples. As already noted in Section 1.6, the intensity of Rayleigh scatter at right angles to the direction incident light beam is inversely proportional to the forth power of the wavelength. So Rayleigh scatter is six times as intense at 450 nm than at 700 nm. Raman scattering is simply resolved at longer wavelength because the separation between the excitation wavelength and Raman becomes greater at longer wavelength (see Table 1.3).

Fluorescence is environment dependent (see Sections 1.4 and 1.5). The binding of a fluorescent labelled molecule to an antibody frequently leads to a change in the observed fluorescence signal. Such a change means that a separation step may not be required to distinguish antibody-bound from unlabelled molecules, so a competitive binding assay can be performed homogeneously. Fluorescence polarisation assay is where the labelled species is excited using vertically polarised light, with the emission signal measured alternatively between vertically and horizontally orientated polarisers. Small molecules rotate polarised light rapidly and lose their orientation within about 5 nanoseconds, which is less than the fluorescent lifetime of the fluorophore so the emitted fluorescence is unpolarized. The relaxation times of a small molecule bound to an antibody is greatly increased and so the emitted fluorescence is polarised. This method is limited to small molecules.

In singlet-singlet energy transfer (Ullman et al., 1976) homogeneous immunoassay, an analogue of the analyte is labelled with one fluorophore (the donor)and the antibodies are labelled with another fluorophore (the acceptor). The energy transfer quenches the fluorescence of the donor fluorophore and subsequently enhance the fluorescence of the acceptor fluorophore occurs when the labelled antigen and antibody are specifically bound. The donor fluorophore should have a emission spectrum that overlaps with the the absorption (excitation) spectrum of the acceptor fluorophore. Also the acceptor should not absorb or fluoresce at the same wavelength as the donor fluorophore and there is negligible fluorescence at the acceptor emission wavelength by the donor.

5.3.3 Method

The suitability of Nile Blue SS-human apo-transferin conjugate (see Section 4.4) for use as a labelled antigen was investigated by determining the immuno-reactivity in a typical antibody immunoassay protocol as outlined in Figure 5.5.

400 microlitres of Nile Blue SS-human apo-transferin conjugate (Figure 5.5: Ag*) and 100 microlitres of a one in five dilution of anti-transferin antibody raised in goat (Figure 5.5: Ab) were incubated together for 2 hours with stirring in pH 7.2 PBS buffer. Excess second antibody (anti-goat antibody) (50 microlitres) was then added to precipitate any complex formed between the anti-transferin and Nile Blue SS labelled human apo-transferin. This was then centrifuged for 10 minutes at 5000 rpm. The supernatant was decanted into micro-cuvette and the fluorescence was determined using a Perkin Elmer (Beaconsfield, Buckinghamshire, UK) LS-50 Spectrofluorimeter. This procedure was carried out on 3:1 and 5:1 Nile Blue SS-human apo-transferin conjugates (see Section 4.4).

In order to check the cross reactivity between anti-goat antisera and the Nile Blue SS-human apo-transferin conjugate, the same procedure as described above was followed, 100 microlitres of pH 7.2 PBS buffer replaced anti-transferin antibody.

5.3.4 Results and Discussion

On the addition of the anti-goat antibody visible precipitation occurred immediately. After centrifugation, the supernatant had about one fifth of the fluorescence recorded for the Nile Blue SS-human apo-transferin before the addition of the anti-goat antibody. This suggested that the human apo-transferin was still immuno-reactive after labelling with Nile Blue SS.

There was no precipitation upon the addition of excess of anti-goat antibodies to Nile Blue SS-human apo-transferin conjugate. This showed that there was no cross reactivity between them. There was a about 5% reduction in the fluorescence of the supernatant. These results suggest that Nile Blue SS-human apo-transferin conjugate would be a suitable labelled antigen for an immunoassay.

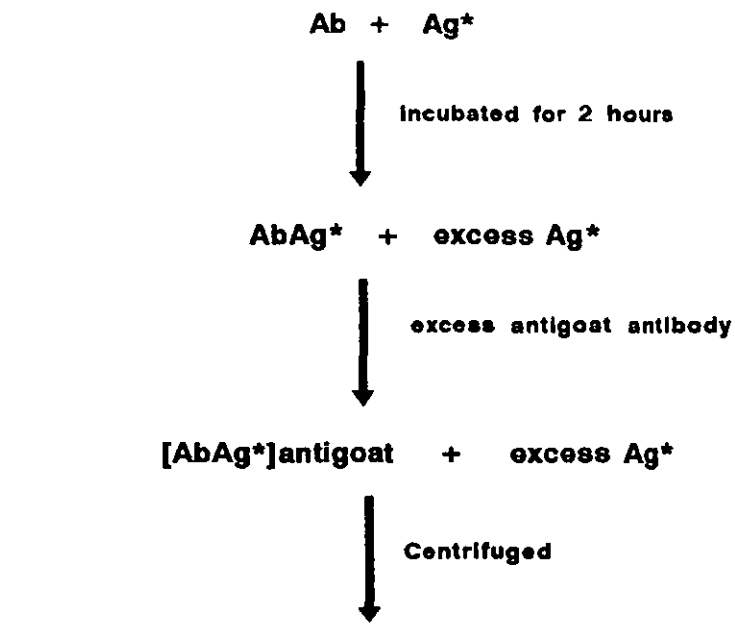

Figure 5.5: Schematic diagram of the 2nd antibody immunoassay Ab = anti-transferin antibody raised in goat, Ag* = Nile Blue SS-human apo-transferin conjugate.

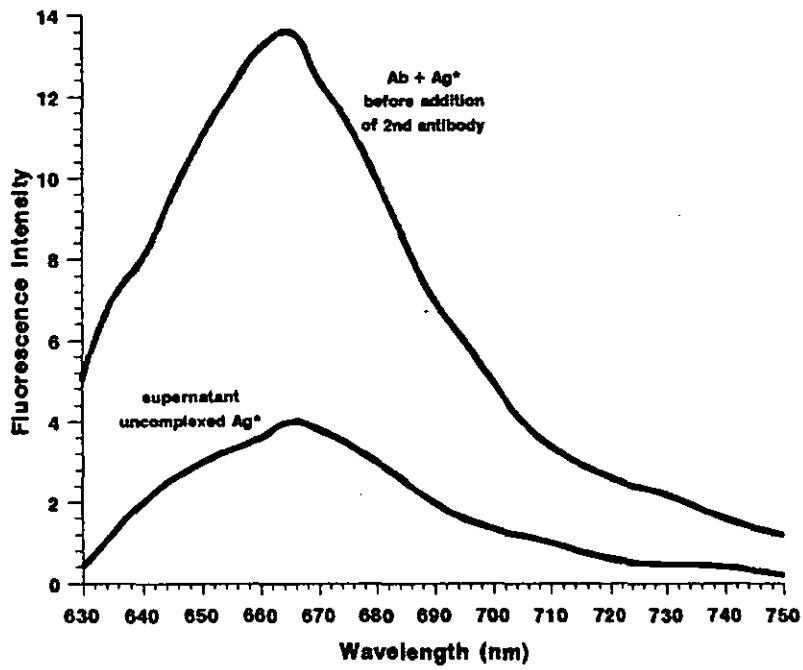

Figure 5.6: Fluorescence spectra of the Nile Blue SS labelled human apo-transferin and anti-transferin complex before and after the addition of anti-goat antibody.

5.4 Conclusion

Nile Red [LIII] shows very good probe properties especially with bovine serum albumin, this should be developed more fully. Other dyes that are further into the near infrared require investigation.

Nile Blue SS showed promise as a covalent label for immunoassay. However the problems of near infrared fluorophores has not as yet been fully investigated. Near infrared fluorophores that have long wavelength fluorescence have extensive conjugation and normally have larger molecular weight than those that fluorescence in the ultraviolet-visible region. This adds complications to their use as labels because non-specific binding to surfaces and hydrophobicity is increased with increased conjugation (Section 3.7.3) and this is especially so if the fluorophore is cationic. This is not really a problem if large molecules are labelled, however it is a problem if small molecules are labelled as was found by Palmer and Webster (1992) when Nile Blue A [XLIX] was used to label a sulphonamide. However, Palmer and Miller (1993) have shown that the small drug Theophyline can be labelled with Nile Blue A [XLIX]. This suggests that Nile Blue SS should be used to label large molecules such as proteins and another labelling technique should be employed for small molecules.

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Chapter 6

6.1 Instrumentation

The design considerations for a fluorimeter can be surmised from the following equation:

$$F = I_0(\lambda)\ \varepsilon\ b\ c\ \phi_f\ f_{ex}(\theta,\lambda)\ f_{det}(\theta,\lambda) \qquad (6.1)$$

I_0 is the incident radiation at wavelength λ, ε is the molar absorptivity ($dm^3\ mol^{-1}\ cm^{-3}$) of the solute with a molar concentration c ($mol\ dm^{-3}$) and with cuvette path length b (cm). ϕ_f is the fluorescence quantum efficiency of solute. $f_{ex}(\theta,\lambda)$ is the throughput of excitation monochromator/filter at wavelength λ and solid angle θ. $f_{det}(\theta,\lambda)$ is the detector responsivity at the specified wavelength λ and solid angle θ.

Fluorimeters built for the ultraviolet-visible region (240-600 nm) are unsatisfactory for the near infrared because of the low efficiency of both the optics and photomultipliers in this region. The exchanging of the photomultiplier tube (PMT) to a red sensitive type (for example R928 PMT) improves the sensitivity but is only a compromise.

A fluorimeter consists of a light source (usually a xenon arc lamp), excitation dispersing element, a sample area, an emission dispersing element, a photodetector and a data read-out device (see Section 1.3 and Figure 1.2).

6.2 Light Sources

Fluorimetry requires an intense and stable light source. A filter fluorimeter may use line sources (such as atomic lines or lasers) or filtered continuum light sources (see Section 1.3.1). A spectrofluorimeter requires a continuum source over a large spectral range (see Section 1.3.2).

6.2.1 Conventional Light Sources

Incandescent sources emit continuous radiation by thermal excitation of the source atoms or molecules. The spectral distribution and total radiant power is dependent on the temperature, area and emissivity of the surface. The *tungsten filament lamp* provides an inexpensive, reliable and stable light source. The life of the source is limited by the tungsten filament evaporation that causes the darkening of the glass envelope and gives large variations in output long before the lamp fails. Operational range is 330nm to the near infrared (Figure 6.1).

The *tungsten halogen lamp* has a small quartz envelope filled with a very small amount of halogen gas, normally iodine. Tungsten halide is formed from the evaporated tungsten and the halide. This decomposes on contact with the hot filament and deposits the tungsten back on the filament. A 50W tungsten halogen lamp was shown to have almost the same radiance in the near infrared as a 275W xenon arc lamp (Summerfield and Miller, 1993).

Low pressure discharge sources consist of an electric current passes through a gas, the gas is ionised and some of the absorbed energy is released as optical radiation. At low current density and low pressures, the electrons bound to the gas atoms become excited to higher energy levels and radiation is emitted as the electrons fall back to the ground state, which gives several narrow atomic spectral lines. *Low pressure mercury vapour lamps* are frequently used in filter fluorimeters. Individual mercury lines appear at 253.7nm, 313nm, 365nm, 404.7nm, 407.8nm, 435.8nm, 546.1nm, 577.0nm and 579.1nm. The lamp may be coated with a phosphor that gives a nearly continuous spectrum.

Short arc lamps, a high current discharge through a noble gas at 70 atmospheres and 3000°C gives the brightest conventional sources of optical radiation. The gap

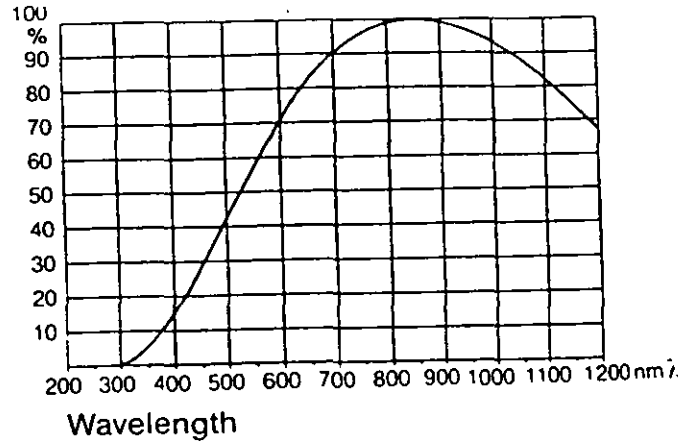

Figure 6.1: Percentage output power of a tungsten filament lamp.

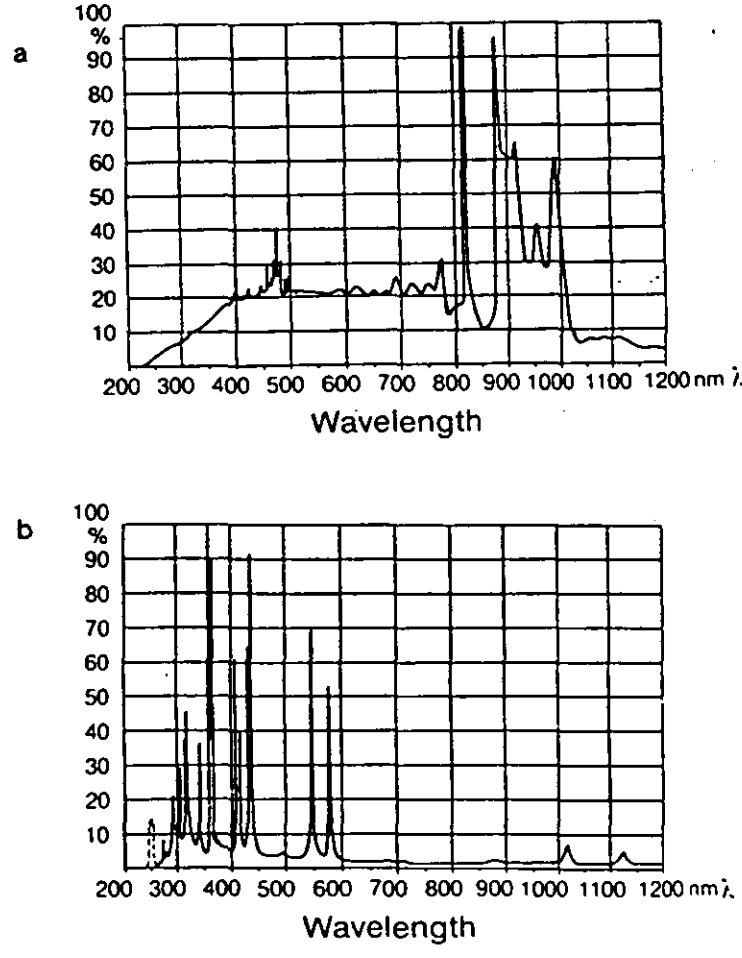

Figure 6.2: Percentage output power of (a) xenon arc lamp and (b) mercury arc lamp.

between the anode and the cathode is so small that it acts as a point source. The small pointed cathode, made of tungsten doped with materials such as thoria, allows the high temperature required for efficient electron emission. The larger tungsten anode withstands the electron bombardment and dissipates the heat produced. The quartz or silica envelope is filled with either a rare gas or a little noble gas and a measured amount of mercury. An expensive power supply is required to start and operate them. Ignition occurs when a fast high voltage pulse is applied between the electrodes causing a spark. The gas is ionised. A high direct current (DC) must be used to ensure the arc is maintained. Arc flicker determines the short-term stability. Life of the lamp is limited by the slow evaporation of tungsten causing electrode wear that deposits on the envelope interior and especially reduces the ultraviolet radiation.

Xenon arc lamps are filled with purified xenon and have the anode at the top. The continuous spectrum produced is from 190 to 750nm (Figure 6.2a) with negligible line structure. From 800 to 2500nm the continuum declines severely and has a few intense lines at specific wavelengths especially between 800 to 1000nm.

Mercury arc lamps contain an exactly measured amount of mercury and either argon or xenon as the starter gas. The anode is at the bottom to ensure the proper vaporisation of the mercury. The spectrum (Figure 6.2b) is dominated by the strong mercury lines through the visible and ultraviolet with a weak continuum to 2500nm.

6.2.2 Lasers and solid state light sources

Lasers ("Light Amplification by Stimulated Emission of Radiation") produce spectrally pure light. Piepmeier (1986) gives a detailed discussion of the use of lasers in luminescence. A laser consists of an active medium in which population inversions are caused by optical pumping (continuous wave operations) or pulsed electrical excitation. The intensity, spectral purity and both temporal and spatial coherency of laser light is much greater than an ordinary incandescent lamp. Greater coherence in ordinary light sources is done at the expense of reduced throughput. Table 6.1 shows the lasing wavelengths of commonly used lasers.

Solid state lasers have an active medium consisting of a rod (usually fifteen by one centimetres) of host material doped with a laser active substance and is placed in the pumping cavity where it is illuminated by the pumping lamp.

A *gas laser* operates by passing a low pressure electrical discharge through the gaseous active medium and so producing a very high lasing output.

Organic dye lasers allow lasers to be tuned over a limited range using an appropriate dye solution. The laser dye, optically pumped by either a gas laser, solid state laser or flash lamp, lases at a higher wavelength. The wavelength may be tuned by changing the solvent or dye.

Table 6.1: Lasing wavelengths of lasers, light emitting diodes and laser diodes.

Type	Wavelength (nm)
Solid State Lasers	
Nd-YAG frequency doubled	532.4
Ruby (aluminium oxide doped with chromium)	694.3
Nd-glass (glass doped with neodymium)	1064.8
Nd-YAG (yttrium aluminium garnet doped with neodymium)	1064.8
Gas Lasers	
Neon (ion) - pulsed operation only	235.8, 332.4
Nitrogen (N_2)	337.1
Helium-cadmium (He-Cd)	325.0, 441.6
Argon (ion) laser	457.9, 465.8, 472.7, 488.0 496.5, 501.7, 520.8
Krypton (ion) laser	468.0, 476.2, 482.5, 514.5 568.2, 647.1, 676.4
Argon-krypton (Ar-Kr) laser	457.9 to 676.4
Helium-neon (He-Ne)	632.8, 1153
Carbon dioxide (CO_2) laser	10600
Light emitting diodes	
Blue	455 to 470
Green	560 to 570
Yellow	580 to 590
Red	635 to 695
Near infrared	780 to 820
Infrared	930 to 950
Laser Diodes	
GaInAsP/GaInP diode	635 to 690
GaAlAs/GaAs	700 to 900

Light emitting diodes (LEDs) and their close relative the laser diode are semiconductor p-n junction devices. The major difference is that regardless of the degree of forward bias, LEDs will only emit incoherent light (see Figure 6.3). LEDs are low cost and have very stable outputs with deviations of about 0.001% of intensity in favourable cases. The possibility of pulsing allows simple discrimination between the fluorophore emission stray light but increases the complexity of the electronics. LEDs are currently available in wavelengths that span from the visible into the near region (See Table 6.1).

Laser diodes (LD) provide intense, polarised, coherent and monochromatic light that can be operated using conventional dry cells. Laser diodes operate mainly in the near-infrared and have only recently been manufactured to operate in the visible as low as 635 nm. When a voltage is applied to the junction between p-type and n-type semiconductors, the Fermi level on each side of the junction shifts and current flows through the junction (see Figure 6.4). The system then relaxes back through spontaneous emission. Each relaxing electron causes the emission of a single photon the same energy as the band gap. A population inversion of electrons occurs if the current is high enough. Lasing is produced if the junction is enclosed in an optical cavity that is aligned along the junction and not through it. At low operating currents, laser diodes will behave like LEDs (see Figure 6.3).

Laser diodes are usually controlled by driving current (as opposed to voltage). A laser diode will operate with just a fixed current providing the current is above the lasing threshold. The *output beam* emitted from a laser diode is both highly divergent and elliptical in cross section (see Figure 6.4). The stigmatised beam of gain guided laser diodes appears to diverge from a point located 10 to 50 micrometers inside the laser diode chip. Index guided lasers exhibit little or no astigmatism due to the beam being constrained in both directions by internal reflections. Laser diodes are sealed on the output end with a plane glass window (0.1-0.3 mm thick). The thicker this window the greater the aberrations. The output of low powered, single cavity laser diodes are polarised with plane of polarisation parallel to the laser diode junction.

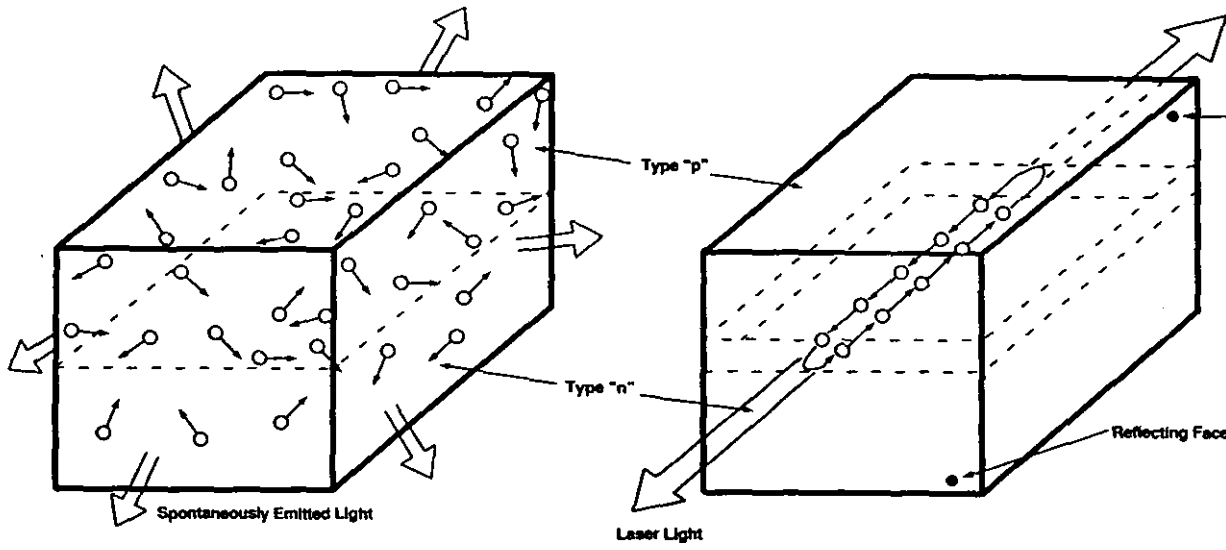

Figure 6.3: Light emitting diode (left) emits incoherent light and a laser diode (right) emits polarised coherent light above the operating current.

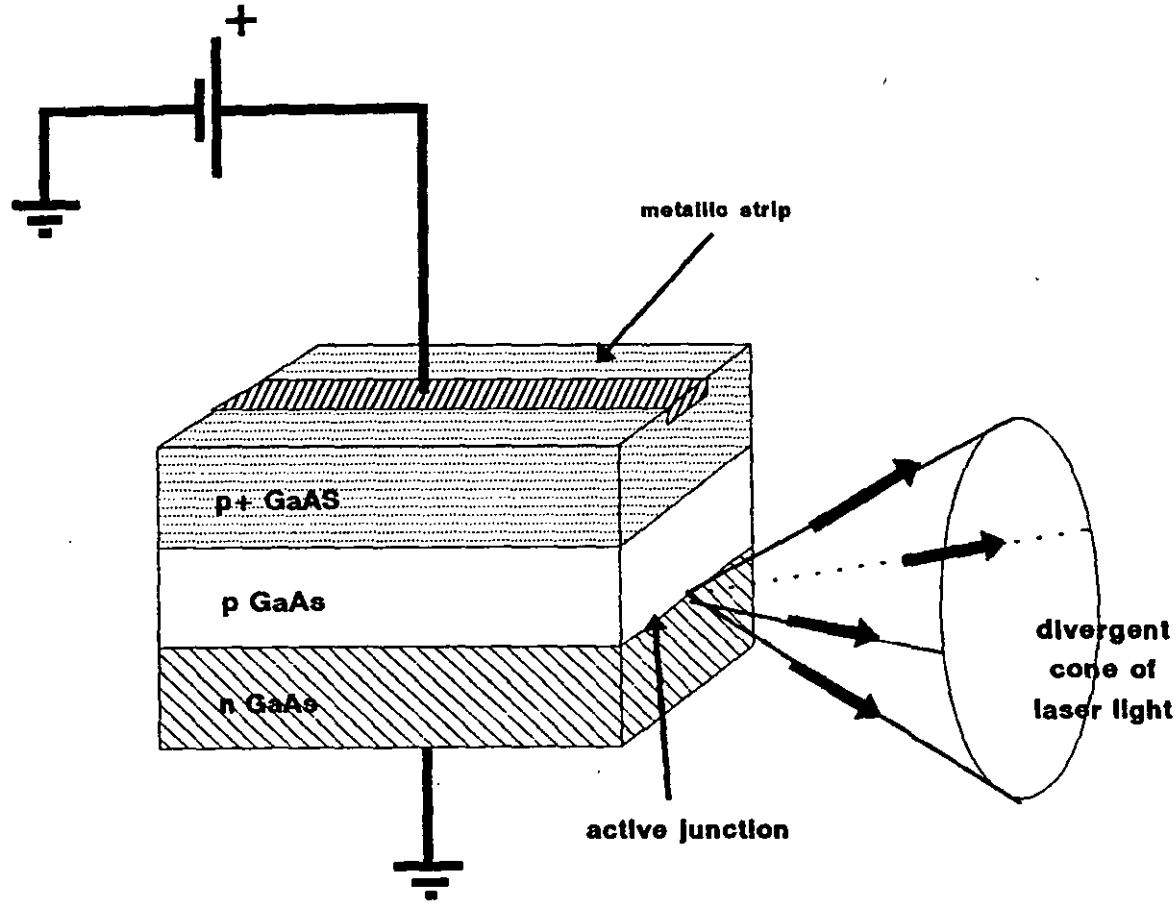

Figure 6.4: A schematic diagram of a gallium arsenide laser diode.

6.3 Dispersive Elements

The dispersive elements, which convert the polychromatic light into monochromatic light, are the heart of a fluorimeter and the deficiencies in their performance cannot be made up by higher quality parts elsewhere in the optical system. The light intensity measured at a particular wavelength is the average intensity for the sample over the wavelength range (bandwidth). A narrow bandwidth gives measured values near to the true value (i.e. wavelength selective) but allows less light to reach the photodetector, hence low sensitivity. A broader bandwidth gives a smoother spectral bands and allows more light to the photodetector so has higher sensitivity but lower selectivity.

6.3.1 Prisms

Prisms disperse polychromatic light non-linearly: the separation of ultraviolet wavelengths is much better than longer wavelengths so prisms are of very little use in the near infrared. The wavelength separation is very temperature dependent. The efficiency of prism monochromators is much better than grating monochromators.

6.3.2 Grating monochromators

Gratings have far superior wavelength separation, lower stray light and temperature sensitivity than prisms. A diffraction grating consists of a glass or silica substrate with as many as 1200 fine parallel grooves per millimetre. These may either be a replica grating produced from an original ruled by a ruling machine or holographic grating formed from the laser interference patterns recorded on the photoresist layer and finished with a reflective layer of aluminium. The groove profile is a shallow triangle, with the wide faces of each groove tilted at a known blaze angle. The groove acts as a very narrow mirror when it is illuminated. The radiation is propagated only if the groove separation in the direction of the radiation is whole number of wavelengths. Otherwise the light waves cancel each other and so no radiation is propagated.

The resolving power is improved by increasing the number of grooves. The blaze of a grating is the wavelength at which the grating has the maximum efficiency and the blaze efficiency decreases rapidly the further away from it. Grating spectrofluorimeters, like the Perkin Elmer LS50 and the Perkin Elmer MPF-44B, modified ultraviolet-visible spectrofluorimeters have their excitation and emission monochromators blazed at 300nm (optimal between 200-600nm) and

emission grating blazed at 500nm (optimal between 335 to 1000nm). Therefore, the grating efficiency in the near infrared is low. Blazing at higher wavelengths would mean higher light throughputs.

Slits regulate the wavelengths that excite the sample and ultimately pass onto the detector. The slit width is the most important factor in determining the resolution of the instrument. In a grating monochromator the bandpass for a given slit is constant throughout the spectrum and depends on the ruling of the grating. There are three types of slits:

Quantum counters are a solution of a dye in a viscous solvent, such as Rhodamine 101 in ethylene glycol and are used to correct the spectral anomalies caused by the monochromator and light source. The quantum counter Rhodamine 101 above 600 nm loses its ability to compensate for variation of output from the source. Various dyes have been proposed as replacement for the near infrared, such as benzopyrylium by Brechte (1986) and Oxazine 725 by Demas et al. (1985).

6.3.3 Filters

The throughput of filters is greater than a grating monochromator, whose throughput is dependent on slit size, the grating efficiency, input optics and so on. A filter is optically homogeneous and obeys the Bouger-Lambert Law that requires that the spectral transmittance of two of more optical filters used simultaneously must be equal to the product of the spectral transmittance of each filter.

An *Absorption filter* is a piece of tinted glass or gelatin containing organic dyes usually sandwiched between glass plates and may be used between 400-1000 nm. These may be in the form of a bandpass filters (about 25nm spectral bandwidth), a cut-off filter that absorbs light below a certain cut-off wavelength, a cut-on filter that absorbs light below a certain cut-on wavelength or a neutral density filter that has constant transmission over a wide wavelength range and decreases the light intensity of strongly fluorescing compounds.

An *Interference filter* consists of glass or silica coated with different thicknesses of materials of varying refractive indices. The wavelengths not required are either reflected or absorbed in the filter by destructive interference of the light waves.

Interference bandpass filters have a bandwidth of 10 to 17 nm. Very little light is absorbed so an interference filter is well suited to use with intense spectral sources.

Wedge filters give a continuous range of wavelengths and are produced by depositing increasing thicknesses of the material on a glass base. The sliding or rotating of a filter brings different thicknesses into the beam and allows radiation of a new wavelength to reach the sample. This type of dispersion element is not widely used because its wavelength range is limited.

Holographic laser bandpass filters have recently been introduced in this country by Glen Spectra Limited (Stanmore, Middlesex, UK) in the wavelength range of 400 to 1064 nm. These filters have a 2 nm bandpass and greater than 90% throughput.

6.4 Sample Area

Fluorimeters generally have standard rectangular cuvette holders of one centimetre pathlength. The sample holder allows reproducible cuvette location with the optical faces of cuvettes perpendicular to the light beam. Cuvettes are fabricated from four highly polished pieces of glass or silica with a path of 1 cm and 2-3 ml working volume. The transmission ranges of cuvettes is shown in Table 6.2. Cuvettes normally transmit over 80% of incident radiation over their range. Moulded polystyrene or acrylic cuvettes are a cheap and disposable alternative but offer lower accuracy than the fused cuvettes due to the lower tolerances in construction.

Table 6.2: The transmission range of cuvettes made of various materials.

	Wavelength (nm)
glass	340 - 2400
silica	220 - 2400
ultraviolet grade silica	185 - 2400
polystyrene	400 - 1100
acrylic	390 - 1100

Reflective coated cuvettes have the window opposite the incident light and that perpendicular to it coated with a reflective coating either of gold or aluminium (see Figure 6.5). The incident and emitted light is reflected from the coated windows. The effect is to increase the fluorescence and background scatter. Scatter is reduced at higher wavelength so lower limits of detection are likely in the near infrared.

Test tubes are cheap and could be used for routine measurement because only a small area of the cuvette is viewed by the detector. The variation in cuvette wall thickness and native fluorescence of the material produces large blank values that are much reduced in the near infrared.

Flow cells are devices that allow semi-automatic filling and emptying. Unless a flow cell is designed to empty efficiently, droplets may remain in the cell and associated tubing may influence the measurement of the next sample, cross contamination or carry over.

The *temperature-controlled sample holder* can control temperature in the range in the range 0-40°C. Generally fluorescence intensity increases as the temperature of the sample is reduced.

6.4.1 Method

The limit of detection (LOD) for silica, acrylic and reflective coated cuvettes were determined on the Perkin Elmer MPF-44B Spectrofluorimeter at 25 °C in HPLC grade methanol (BDH, Poole). The fluorescence intensity was measured for at least five different concentrations of dye. The LOD was defined as the intercept of the regression line with 3 standard deviations of the background (Miller and Miller, 1988).

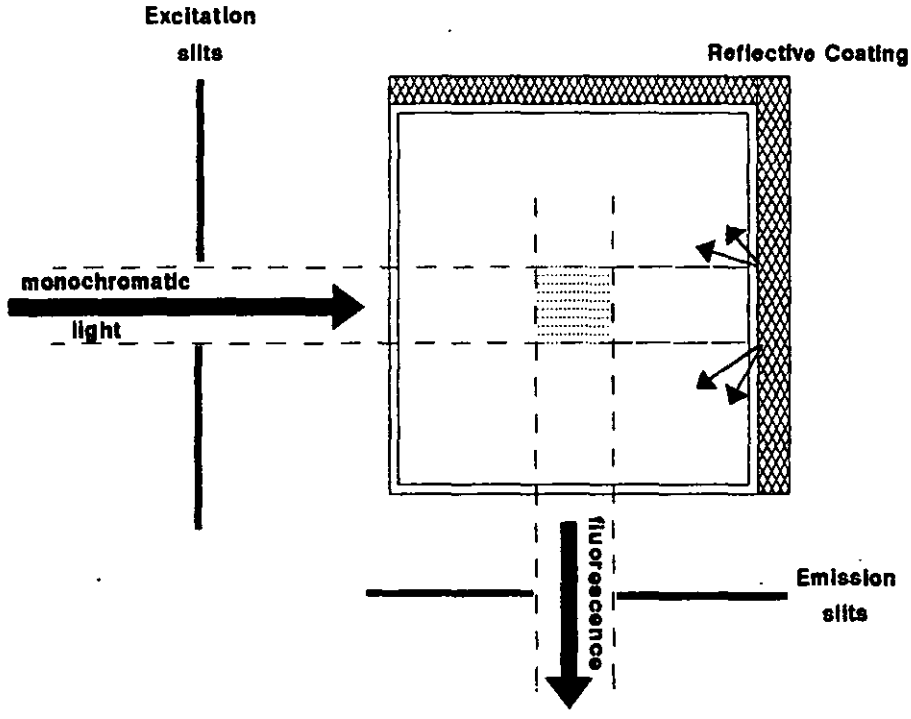

Figure 6.5: Reflective coated cuvette has two adjacent sides coated with aluminium.

6.4.3 Results

Table 6.3: Limit of detection of dyes in methanol using silica cuvettes, reflective coated cells and acrylic cuvettes. The λ_{Ex} was the excitation wavelength used and for some of the dyes was lower than the absorption wavelength maximum because of very narrow stokes shift. λ_{Em} is the emission wavelength maximum.

	λ_{Ex} (nm)	λ_{Em} (nm)	Silica cuvette (g/ml)	Reflective coated cell (g/ml)	Acrylic cuvette (g/ml)
Xanthenes					
Fluorescein	480	530	2.39x10-10	4.92x10-10	
Phenoxazones					
Nile red	565	638	3.00x10-11		
Phenoxazines					
Oxazine 4	608	633	4.00x10-11		
Nile blue A	622	660	7.00x10-11		
Carbocyanines					
DOTC iodide	665	705	1.40x10-10	3.48x10-10	3.64x10-10
HITC iodide	720	780	2.56x10-10	2.81x10-10	
DTTC iodide	730	788	5.10x10-10		
IR125	770	830	5.83x10-10	3.86x10-10	

6.4.4 Discussion

The reflective coating on the cuvettes increased the amount of light getting to the detector. Rayleigh scatter reduced by the inverse forth power of the wavelength. So there is less scatter in the near infrared region than the ultraviolet-visible region, so it was assumed that reflectively coated cuvettes would give lower limits of detection. The LOD of the reflectively coated cuvette was slightly lower than the non-reflectively coated silica cuvettes. The reflective coating significantly increased both the background scatter and dye fluorescence intensity by about three fold. The excitation wavelength for both HITC [XXIV] and DTTC [XXIII] in the reflective coated cuvettes was backed off by 10 to 15 nm in order to have comparable scattering as uncoated cuvettes. This could be useful if a light source such as a laser diode is used for increasing the signal from a fluorophore that does not exactly coincide with the excitation wavelength.

There was no significant difference between the acrylic cuvette and the silica cuvette limits of detection. The signal was noticeably attenuated by about 20% and slightly noisier baseline was obtained for the acrylic cuvette. The result seems to suggest that the routine use of acrylic cuvettes at longer wavelength of aqueous solutions is possible. This overcomes the severe problems of proteins and dyes adhering to solid surfaces which are difficult to remove, because the acrylic cuvettes are disposable.

The differences in the limit of detection between the fluorophores can also be attributed not only to the quantum efficiency of the dye but also to instrument artifacts. The xenon arc lamp continuum is considerably reduced in the near infrared region and has sharp atomic emission lines of xenon superimposed on the continuum at 820-900nm. The MPF-44B spectrofluorimeter has the excitation monochromator grating blazed at 300nm and the emission blazed at 350nm. So below and above the blazing wavelength the efficiency falls of severely. Beyond 650 nm this is significant. The red sensitive photomultiplier R928 has a sensitivity from 185 to 900 nm, with maximum sensitivity at 400nm, which decreases beyond 700nm. Obviously a different instrument would yield a change in the limit of detection.

6.5 Detectors

The purpose of a detector is to convert the radiant energy into an electrical signal that is then processed and displayed so that the operator can interpret it. Some forms of noise increase with detector area, so the active area should be no larger than is needed to capture all the light. Low light level measurements are limited by noise so the use of concentrating optics and a small detector may give better results than a large detector. The detection limit is usually determined by the output impedance, dark current, noise and the output signal level. Detectors without internal amplification are often limited by the noise of an external amplifier rather than the detector's intrinsic capabilities. Amplification carried out at the detector boosts the signal so the noise picked up between detector and read-out device is less significant. Several types of detectors are available in the near infrared.

6.5.1 Photomultiplier Tubes (PMT)

Photomultipliers are several orders of magnitude more sensitive in the ultraviolet-visible region than any other detection system. This is due to the noise free amplification of the photocurrent by the multiplier stages in the tube. Light passing through the silica envelope strikes a photocathode and emits electrons. These are attracted to a second electrode (the first dynode D_1 in Figure 6.6), which is maintained at a higher voltage than the cathode. Each electron reaching the first dynode causes two or more electrons to be emitted. These electrons fly off to hit the second dynode (D_2) that is at a higher potential. Thus, an avalanche effect is set up. There are two types of PMTs. In Side Window PMTs where the electrons follow a complicated path between dynodes before arriving at the anode and is limited to nine dynode stages: End-on PMTs permits more dynode stages, so this is more photosensitive and more expensive.

Photomultipliers are prone to failure if daylight is allowed to fall directly on them. Red sensitive PMT, such as the R928 PMT, are sensitive up to 900 nm. PMTs are very delicate and require a special power supply to keep the dynodes at the high potential required.

The S number specifies the spectral responsivity distribution of both the photocathode and the envelope (see Figure 6.7). This defines the spectral responsivity of the photoemissive detector. S-1 devices have a Ag-O-Cs

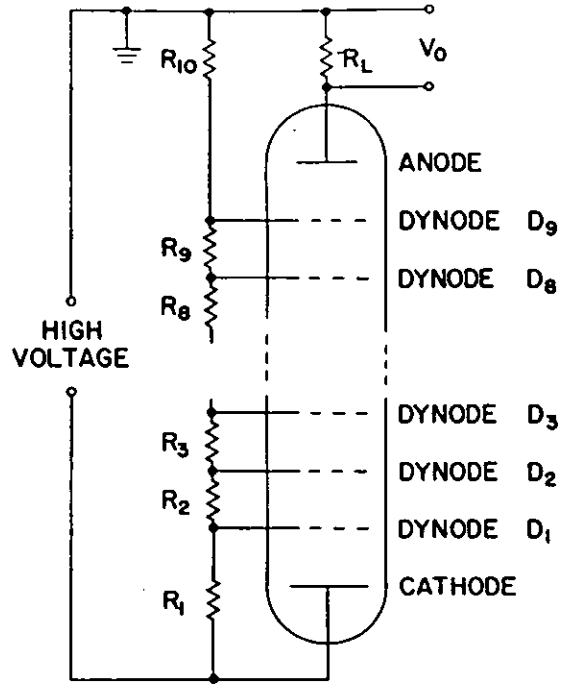

Figure 6.6: Schematic circuit diagram of a photomultiplier with resistor R_L and resistor chain for supply of dynode voltages (Budde, 1983).

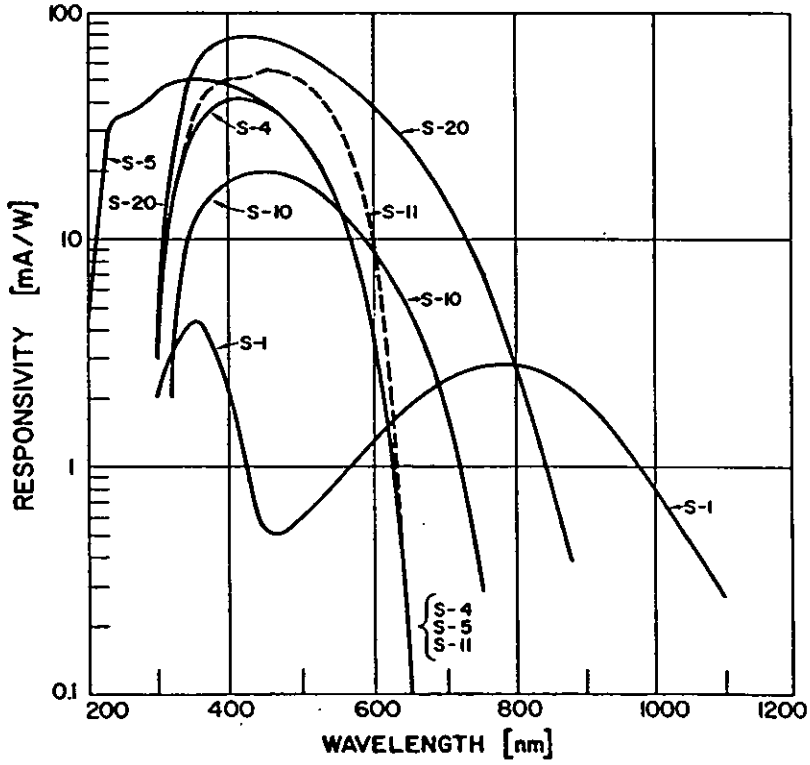

Figure 6.7: Standardised spectral responsivity functions of photoemissive cathodes (Budde, 1983).

photocathode and a lime glass envelope and have a 400-1200nm responsivity range as compared to 200 to 950nm for the other types of photoemissive detectors (S-2 to S-24). The R928 PMT is of the latter type.

6.5.2 Microchannel Plate Photomultipliers

Microchannel plate photomultipliers resemble, in their basic functions, normal photomultipliers except that the amplification is achieved in the microchannel plate rather than in a system of separate dynodes. The basic principle of secondary emission amplification (see Figure 6.8). The inner surface of a glass tube is coated with a material having high resistance and good secondary emission. Electrodes are attached to both ends for the application of a high voltage in the order of 600-1000 volts. A small current flows through the material on the inner surface of the tube generating a potential gradient. A primary electron entering from the left strikes the wall, exciting secondary electrons, which are accelerated toward the wall in the direction of the positive electrode. This secondary emission process continues until a high number of electrons per primary electron leave the tube.

6.5.3 Photodiodes

Silicon photodiodes are cheap and robust, and can be run off batteries. Their peak sensitivity is between 750-900 nm (see Figure 6.9). The characteristics of semiconductor detectors is outlined by Grayson (1984). A single photodiode can be used for single wavelength detection or producing a spectrum by sweeping the spectrum across it using a monochromator.

Unbiased (photovoltaic) detectors are designed for low noise frequency applications (below one kilohertz). No external bias (voltage) is applied and the semiconductor generates an emf during the period of illumination.

The *biased (photoconductive) detector* is designed to detect light pulses or continuous light beam high frequency modulations. An external bias is applied in the reverse direction at the p-n junction and current flows under illumination. The current generated is composed of both photo induced and reverse leakage (dark) current. The dark current is constant at certain bias and temperature. The reverse voltage accelerates the electron/hole transit times by increasing the junction field strength.

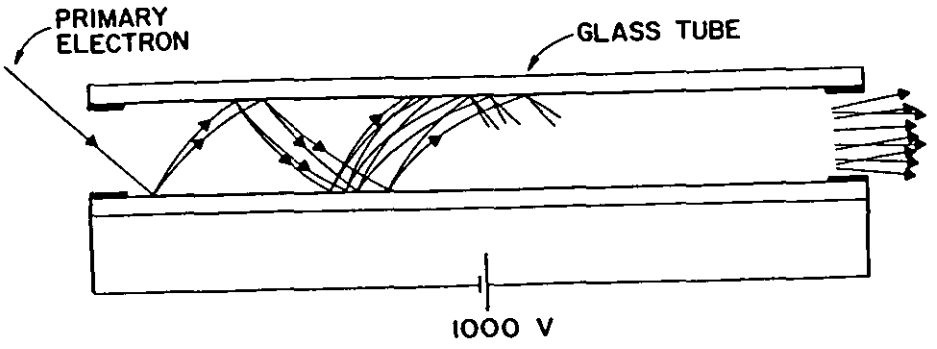

Figure 6.8: Schematic diagram of secondary electron amplification in a microchannel. The primary electron at left is assumed to come from the photocathode (Budde, 1983).

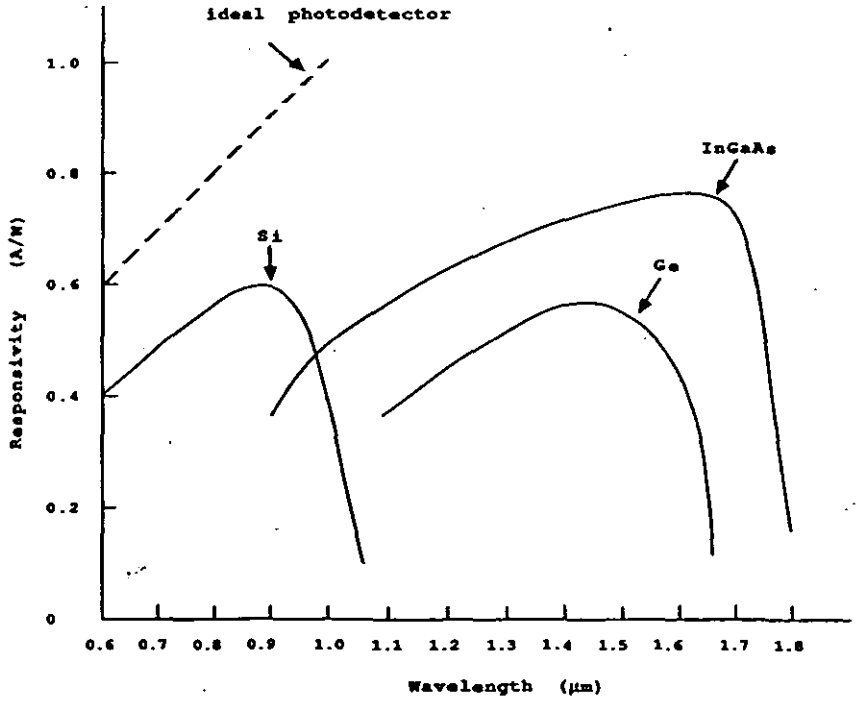

Figure 6.9: Typical current responsivity of silicon, germanium, and InGaAs p-i-n photodiodes. The responsivity of an ideal photodiode is shown as the dashed line. (Yeh, 1990)

Photodiode arrays (PDAs), plasma coupled devices (PCDs) and charge coupled devices (CCD) consist of array of silicon detectors constructed as an integrated circuit. Light causes a charge to accumulate in the series of diodes that are sequentially read and reset. The voltage is amplified and converted by an analog-digital (A/D) converter into a series of digitized readings for computerised processing. The time to scan these diodes is determined by clock pulses of an external controller and is typically five to one hundred milliseconds. A spectrum can be read quickly by spreading the light over the array using a grating or prism and monitoring the appropriate diode. Once the diode array is triggered, each element collects both the dark current and photo-induced charge. So without illumination, the array becomes saturated if the integration time is too long. This limits the maximum integration time. The dark current can be reduced by cooling.

6.5.4 Avalanche Photodiodes (APD)

An APD is a reverse-biased junction photodiode that is biased just below its breakdown voltage, which causes an avalanche effect of electrons similar to PMTs. These have greatly improved sensitivity over normal photodiodes and under certain circumstances even approach the sensitivity of PMTs (McIntyre, 1970). In the wavelength region 800-1100 nm the quantum efficiency of photomultiplier tubes is considerably lower than that of APDs, and therefore APDs have the better detectivity in this region (Budde, 1983).

Structurally, APDs are similar to non-avalanche junction detectors except a region of very low concentration doping material where the avalanche multiplication occurs. APDs are characterised by very fast response times and avalanche gain amounting to values of several hundreds. Consequently, APDs have been used for the detection of weak but fast pulses in laser ranging, optical communication and high speed switching.

Spectral responsivity function of APDs are very similar to non-avalanche junction detectors. Total responsivity, which includes gain (multiplication M), of an APD depends on the bias voltage V_b. The breakdown voltage V_{br} is dependent on temperature. The higher the temperature the lower the breakdown voltage.

The dark current of APDs comprises of surface leakage and bulk leakage, the latter being several orders of magnitude smaller. The total noise is rather small and noise is mostly from the input noise of the amplifier. Lowering of the temperature reduces the noise and the dark current.

6.5.4 Comparison of the detectors

The wavelength range encompasses a group of detectors that can cover the range rather than a single detector. Photomultipliers have the largest sensitive areas at 12000 mm^2. Silicon and germanium photodiodes are the smallest and fastest detectors with areas of approximately 0.008 mm^2 and rise times of 30 picoseconds. The linear range, response time, and capacitance decrease with size for most detectors.

The responsivity is a measure of the sensitivity of a detector and is given in amperes per watt and applies to the wavelength of maximum responsivity. The responsivity of biased detectors depend on the bias voltage. At low voltages and constant irradiation the response increases with voltage, but at high bias close to saturation the responsivity becomes independent of voltage. The responsivity for photomultipliers and microchannel plates is the anode responsivity assuming a gain specified for the suggested operating voltage.

Table 6.4: Wavelength range, size, responsivity, normalised detectivity, temperature range, bias voltage and response time. The data was compiled from Budde (1983) and manufacturers' catalogues.

	Wavelength range nm	Size mm^2	Spectral responsivity A W^{-1}	Temperature range °C	Bias voltage V	Response time ns
Photoemissive detectors:						
Photomultiplier tube						
S-1	400-1200	80-280	470-2000	-196-20	10^3-5000	3.5-15
S-2 to 24	180-950	14-12000	10^3-3×10^4	-20-50	750-6000	.5-15
Microchannel plate	300-900	120-500	100-10^4	-	600-10^3	.3
Junction semiconductor detectors:						
Silicon photodiodes						
unbiased	250-1100	.85-800	.45-0.62	-50-100	zero	9-1500
biased	250-1100	.01-800	.45-0.6	-50-100	1-200	.030-200
APD	250-1100	.007-7	2-85	-40-125	30-550	.08-2
Germanium photodiodes						
biased	400-1900	.01-80	.6-2.5	-200-55	0-20	.5-200
APD	400-1900	.007-7	2-85	-200-70	30-550	.08-2

The temperature range is either the safe operating temperature or the suggested operating temperature for a detector. S-4 photomultipliers may be operated at lower

temperature, but cooling below -20 °C reduces responsivity. S-1 photomultipliers may be operated at room temperature; however at this temperature the anode dark current is quite high and often close to the maximum current for linear and fatigue-free operation.

The smaller detectors of a given type have faster response times. An increased bias voltage reduces the response time, i.e. biased silicon photodiodes are faster than unbiased photodiodes.

A bias voltage of zero volts indicates unbiased operation. For semiconductor junction detectors, the maximum reverse bias is occasionally given by the breakdown voltage which is strongly dependant on temperature.

6.6 Multi-Channel Detection

Near infrared fluorescence of three dyes in conjunction with various light sources and a fibre optic light collection system was performed. It is shown that nanogram per millilitre detection limits can be obtained, that laser diode excitation is particularly efficient when the lasing wavelength coincides closely with the absorption wavelength of the dye, and that the detection system can be used advantageously where there are temporal changes in fluorescence.

6.6.1 Experimental

The Otsuka Electronics IMUC-7000 intensified multi-channel photo-detector (Hakuto International Ltd., Waltham Cross, UK), Figure 6.10, used a F/4.5 Czerny-Turner grating monochromator blazed at 500 nm which disperses the light over a proximity focused image intensifier coupled to a Peltier-cooled 512 element silicon photo-diode array (PDA). Spectrally dispersed light was converted to photoelectrons by the photocathode and the high voltage microchannel plate performs exponential amplification as the electrons were directed towards the exit. The amplified photoelectrons were reconverted to light by the phosphor and by means of an optical fibre coupler, an image was formed on the photodiode array. The wavelength range per scan was 280 nanometres with a 1.2 nanometre resolution. The detector was further cooled with nitrogen gas and cooling water to reduce the build-up of dark current within the diode array elements to a minimum. The fluorescence intensity recorded by the diode array detector system varied linearly up to at least 30 s: an integration time of a thousand milliseconds (one second) was used in all fluorescence intensity comparison studies and an integration time of five hundred milliseconds was adopted for limit of detection (LOD) determination. The IMUC-7000 was interfaced to an IBM-AT compatible personal computer. The shortest repeat time for this instrument was twenty one milliseconds, which comprised five milliseconds integration time, and sixteen milliseconds for the 512 element diode array to be interrogated and its output transmitted the via the IEEE 488 board to the personal computer.

Light from the source was transmitted to the side of the sample compartment via a two metre industrial stainless steel encased fibre optic (1.05 millimetre internal diameter bundle of twelve quartz fibres). The sample compartment (figure 6.11) containing a one centimetre silica cuvette had three ports for the fibre optics. The two ports at right-angles to each other were used for fluorescence. A

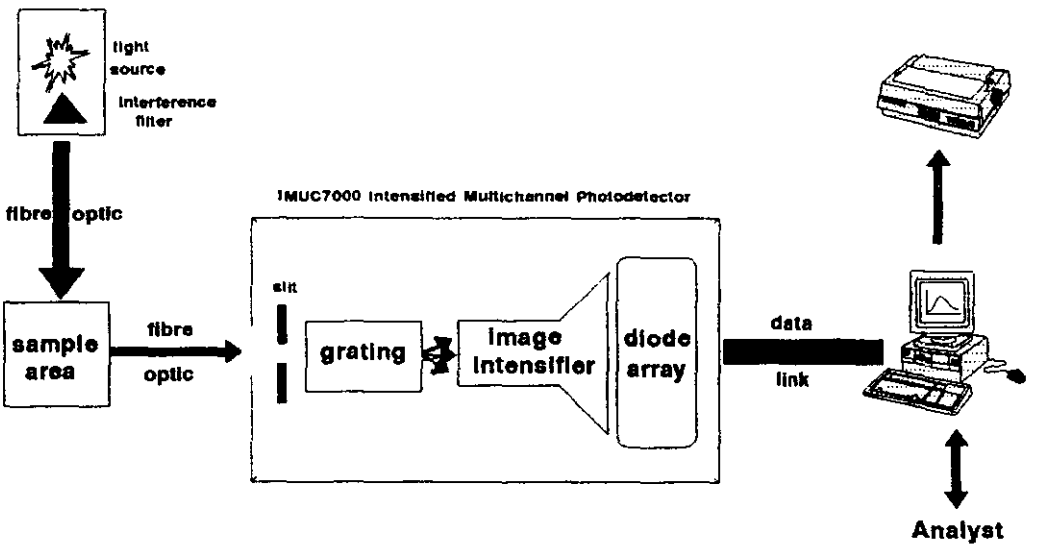

Figure 6.10: Schematic diagram of the experimental setup for the Otsuka IMUC-7000 image intensified photodiode array detector.

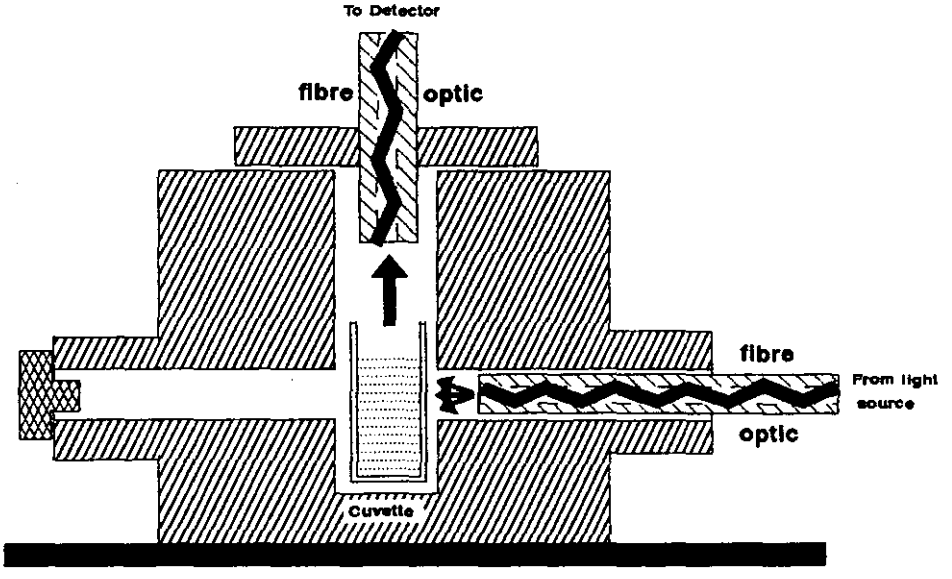

Figure 6.11: Transverse view of the sample area.

similar fibre optic, one and a half metre long, brought the light from the top of the sample compartment to the detector.

The three light sources used were a 50 watt tungsten-halogen lamp (Otsuka), an ozone free 275 watt xenon arc lamp (Applied Photophysics, London) and a two milliwatt LDM135 670 nm laser diode module (Imatronic Ltd., Newbury). The laser diode's peak wavelength measured by the IMUC-7000 was 677 nm. Some experiments using the other two light sources used excitation interference filters with bandwidths of five to ten nanometres. For comparison, some experiments were performed on a conventional Perkin-Elmer (Beaconsfield, UK) MPF-44B fluorescence spectrometer fitted with a 150 watt xenon arc lamp and a red sensitive Hamamatsu (Enfield, UK) R928 photomultiplier.

3.6.2 Results

Table 6.5: Relative fluorescence intensities for Nile Red [LIII], DOTC [XXII] and DTTC [XXIII] in methanol obtained by diode array detection. The figures in brackets are the peak wavelengths of the excitation filter, where used.

	Nile Red (546 nm)	DOTC (660 nm)	DTTC (730 nm)
275 W xenon arc lamp:			
with filter	0.5	5.6	<0.01
without filter	10.0	28.3	1.7
50 W tungsten halogen lamp:			
with filter			
without filter	0.1	5.1	0.1
2 mW 670 nm laser diode			
without filters	-	100.0[1]	-

Table 6.6: Limit of detection (ng/ml) comparison between diode array detection and a conventional fluorimeter. The figure in brackets are the peak wavelengths of the excitation filter, where used.

	Nile Red (546 nm)	DOTC (660 nm)	DTTC (730 nm)
Diode array spectrometer (without filters)			
275 W xenon arc lamp:	2.0	3.9	10.1
50 W tungsten halogen lamp:	4.0	9.0	11.5
2 mW 670 nm laser diode	-	1.6	-
MPF-44B Spectrofluorimeter			
150 W xenon arc lamp	0.1	1.1	0.5

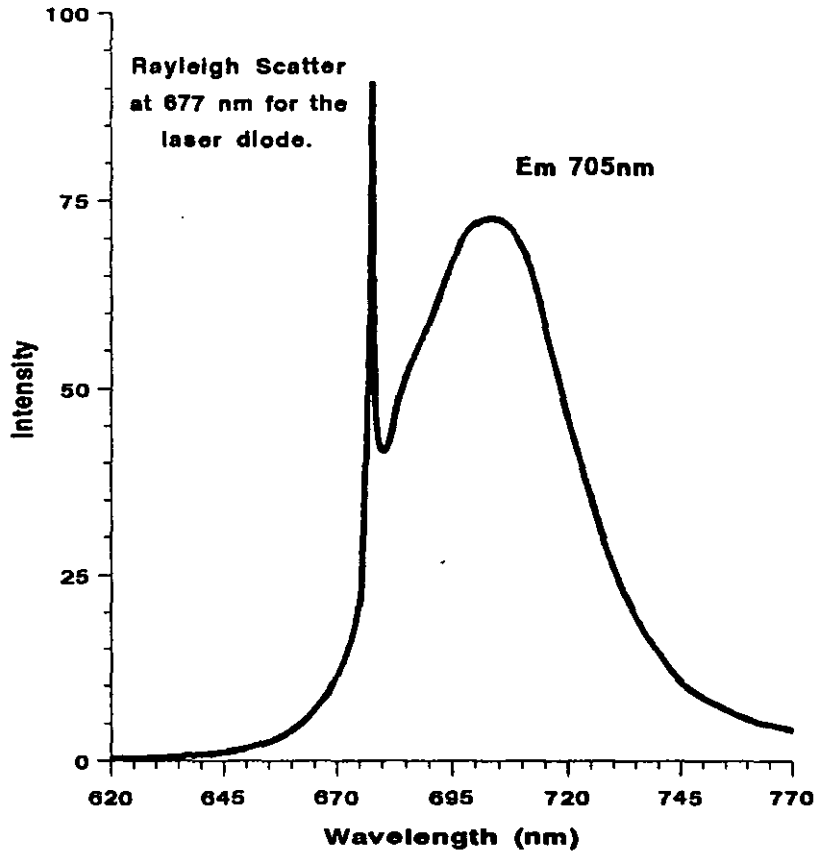

Figure 6.12: Image intensified diode array fluorescence spectrum of DOTC (100 ng/ml) in methanol excited using 2 mW 670 nm laser diode.

6.6.3 Discussion

Comparison of the relative fluorescent intensities measured at 638 nm for Nile Red [LIII] in methanol (Table 6.5) shows that the 275 watt xenon arc lamp was at least 2-3 times more effective as an excitation source than the 50 watt tungsten-halogen lamp. The fluorescent intensities of the higher absorbing DOTC [XXII] and DTTC [XXIII] are comparable since the xenon arc radiance falls off very sharply above 600 nm. So a tungsten-halogen lamp would seem to be a useful source for near infrared fluorescence.

The limit of detection for Nile Red [LIII] using no excitation filters were comparable for the two continuum light sources (Table 6.6). The conventional fluorescence spectrometer offered lower background signal and a far more sensitive detector in this region, resulting in the LOD being significantly better at 0.1 nanogram per millilitre.

DOTC [XXII] was most efficiently excited using the 670 nm laser diode, whose lasing output closely corresponds to the excitation wavelength of this dye. This combination of the light source and dye gave the highest fluorescence signal recorded in this work, and it is noteworthy that the very narrow emission bandwidth of the laser diode minimised scattered light interference despite the relatively small (30 nm) Stokes' shift (Figure 6.12). The LOD achieved was very similar to that determined using a conventional fluorescence spectrometer, and significantly better than when the diode array detector was used with broad-band light sources.

The relatively low emission of DTTC [XXIII] detected by the diode array system resulted in the LOD being twenty times poorer than on the conventional spectrofluorimeter. This was due to the poor performance of the image intensifier at the emission wavelength (788 nm) of DTTC [XXIII].

DTTC [XXIII] bound very rapidly to bovine serum albumin (BSA) with an initial enhancement of fluorescence of about four fold (Figure 5.2). The fluorescence intensity then fell by about 1.7% per minute: in a ten minute period more than forty complete emission spectra of the protein-fluorophore complex were determined with no significant change in the fluorescence emission wavelength. The fluorescence intensity of the Nile Red-BSA complex (Figure 5.1) fell by about 0.2% per minute. The blue shift of emission wavelength of Nile Red [LIII] from 663 nm (buffer) to 615 nm (Nile Red-BSA complex) may be attributed to a change from a hydrophilic to a hydrophobic environment (Sacket and Wolff, 1987). Nile Red [LIII] has also been used as a polarity probe in super critical fluid chromatography (Deye

and Berger, 1990).

These studies show that near infrared fluorescence spectrometry has excellent potential as a trace analysis technique, with LODs comparable to those achieved in conventional ultraviolet-visible fluorimetry, and obtainable using simple robust light sources. The very high irradiance and very narrow bandwidth of laser diodes make them particularly suitable light sources when their outputs match the fluorophore excitation wavelengths. The use of higher power laser diodes, and more efficient light collection systems than were used in this preliminary work, would further improve LODs. It has further been shown that the benefits of diode array detection can be used in the near infrared fluorimetry, with particular advantages in monitoring signals that rapidly change with time.

6.7 Avalanche Photodiode

The following experiments tested the potential usefulness and speed of response of an avalanche photodiode (EMI 70506) to light pulses from a 670 nm diode laser. Circuit diagrams of the modulated laser diode and the avalanche photodiode module from EMI (Cambridge, UK) are shown in Figure 6.13 and 6.14 respectively.

6.7.1 Method

The Imatronic (Newbury, UK) LDM135 2 milliwatt laser diode module was modulated using at various frequencies and the light was detected by the EMI 70506 (Cambridge, UK) avalanche photodiode module. The signal was displayed on an oscilloscope.

In the first experiment, light from the modulated laser diode was focused directly onto the active surface. The laser diode was modulated between 80 and 500 Herz. The limit to the pulse frequency was that of the pulsing unit. Laser diodes are routinely pulsed in excess of a Gigaherz (one million Herz). Figure 6.15 shows the response to modulating the laser diode at 100, 120 and 200 Herz.

In the second experiment, directly in front of the APD was placed a silica fluorescence cuvette containing various concentrations of dye dissolved in methanol. The dyes used were DOTC and Rhodamine 800 that have strong absorbances at 670 nm. The wavelength of the laser diode.

In the final experiment, the laser diode was placed at right angles to the laser diode. The fluorescence cuvette was filled with DOTC or Rhodamine 800 in methanol.

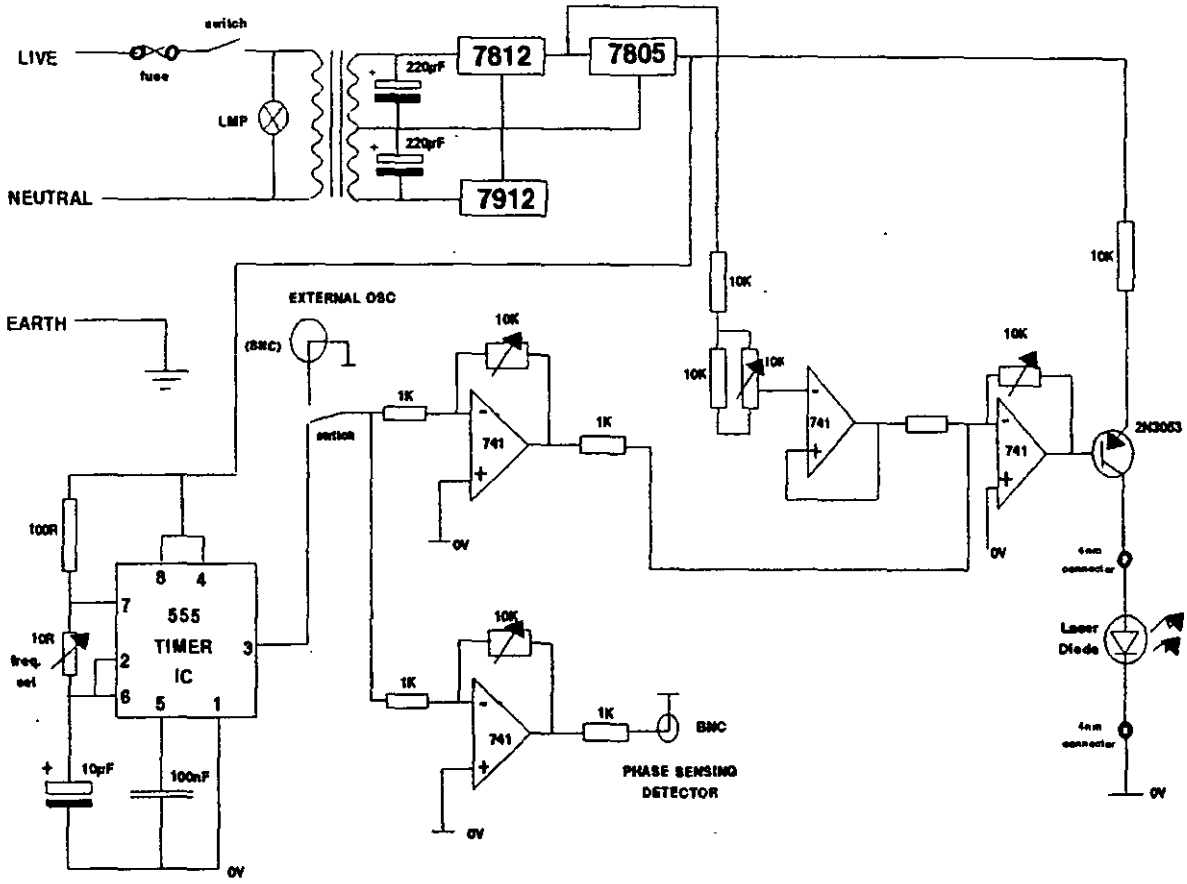

Figure 6.13: Circuit Diagram of the laser diode modulator.

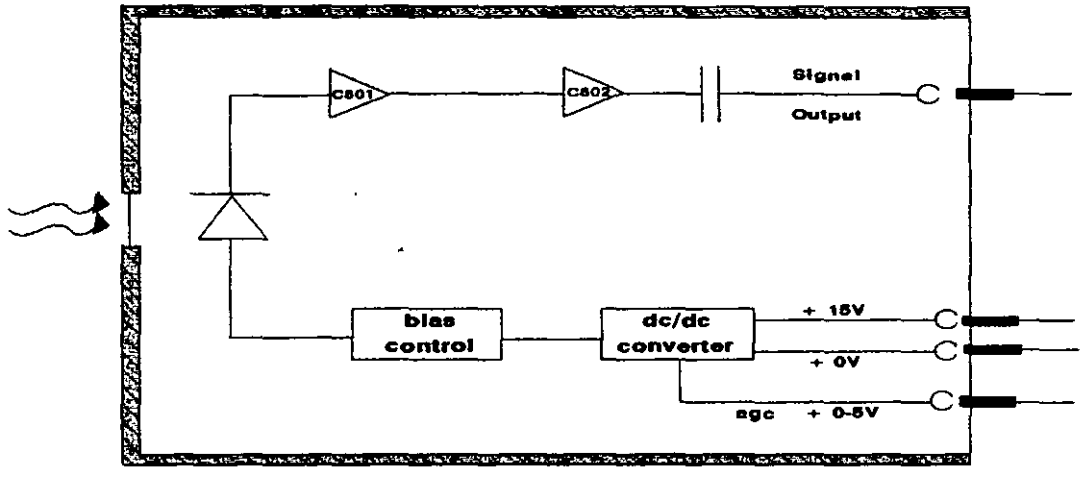

Figure 6.14: Circuit diagram of the EMI 70506 silicon avalanche photodiode. Active diameter = 0.5 mm: Bias Voltage = 160 to 240 V: Efficiency = 25%

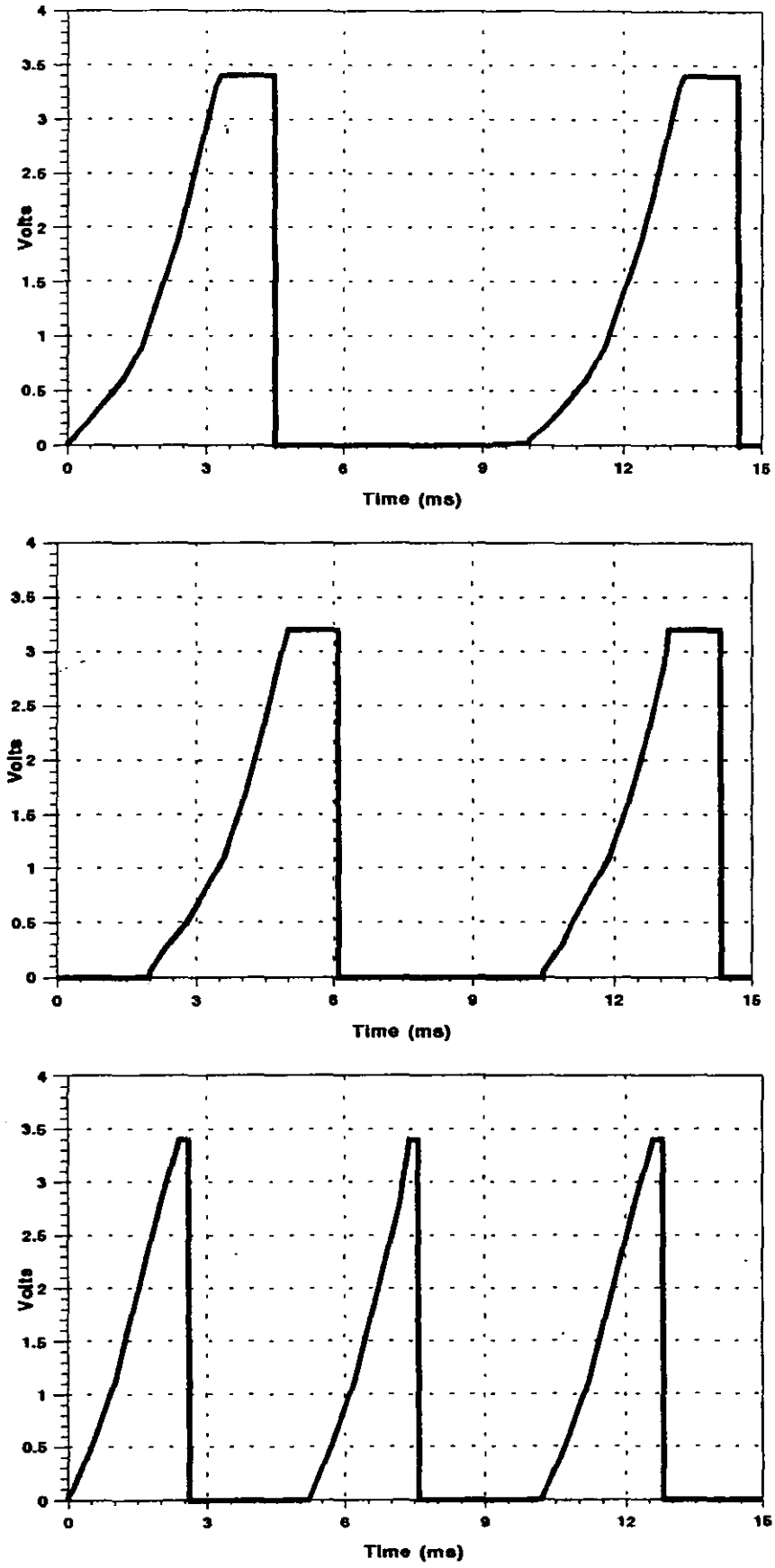

Figure 6.15: Laser diode modulated at 100, 120 and 200 Hz respectively detected by an EMI 70506 avalanche photodiode.

6.7.2 Results and Discussion

The APD allows the detection of fast changing levels of light. The real disadvantage of the APD was that its active area was only 0.25 mm^2. The setup could have been improved by using better optics and enclosing the electronics in a box to exclude light. Cooling of the APD by Peltier cooling would have reduced the dark current and hence increased the sensitivity of the APD. This was not possible without a great deal of modification of the APD module.

The signal output is a pulse rather than the usual continuous output. The conversion from a pulsed signal to a continuous output could be done using a Box Car Integrator or by gating.

The simple absorption spectrometer was assembled with the pulsed laser diode focused on the active area of the APD. Various concentrations of DOTC [XXII] and Rhodamine 800 [XI] were illuminated and attenuation of the signal followed when they were placed into the beam.

A simple fluorimeter was assembled with the pulsed laser diode as the light source and the APD as the detector at right angles to this. DOTC [XXII] was dissolved in methanol. Due to the scattering of the laser light and alignment problems intermittent signals were displayed. A few lenses were used to focus the beam of light on the very small active area but this was not very successful and a more intricate optical system was required.

6.8 Fluorimeter design

Light must be absorbed before fluorescence can occur and thus raises various problems in fluorimetry. The distance light travels is also very important. The Inverse Square Law states that the amount of power incident upon a surface is inversely proportional to the square of the distance from the source. For example, a 1W (watt) light source illuminates an object at 1m has a power of 1 Wm^{-2}, but at 2m the same power is ¼ Wm^{-2}.

The direction at which the incident beam is observed is also important. The Cosine Law states that the incident power upon a given area is the function of the cosine of the angle between the normal and the direction of incident flux.

For example.

1.0	x	1 W	=	1 W at 0°
0.707	x	1 W	=	0.707 W at 45°
0.5	x	1 W	=	0.5 W at 60°

There are four methods of illuminating and viewing the sample that have various advantages over each other. Only the first method is used very widely.

The *right angle method* (Figure 6.16a) is very efficient because none of the cuvette surfaces that are viewed by the emission monochromator are directly illuminated by the excitation beam. Therefore, no inherent cuvette fluorescence from the trace uranium content of glass or silica, or scattered radiation enters the emission monochromator.

Frontal method (Figure 6.16b) is used for semi-opaque materials or solids, or for solutions that are highly absorbing. The reflected radiation and residual fluorescence from the cuvettes is minimised by using the 37 degree angle.

Straight through (transmission) method is seldom used but has been used for the determination of uranium with lithium fluoride-sodium carbonate.

The *rotating cell method* (Figure 6.16c) was developed by Adamson and co-workers in 1982 as a method of correcting the fluorescent power for incident beam absorption and secondary emission beam absorption. The instrument incorporated an off-centre cuvette rotated so that different thickness of sample solution through which the excitation and emission beams penetrate. Knowledge of the transmittance as a function of pathlength along both the excitation and emission axes permits

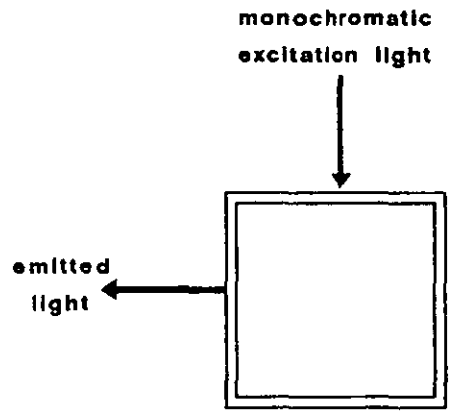

Figure 6.16a: 90° viewing for low absorbing samples (clear or reasonably clear solutions).

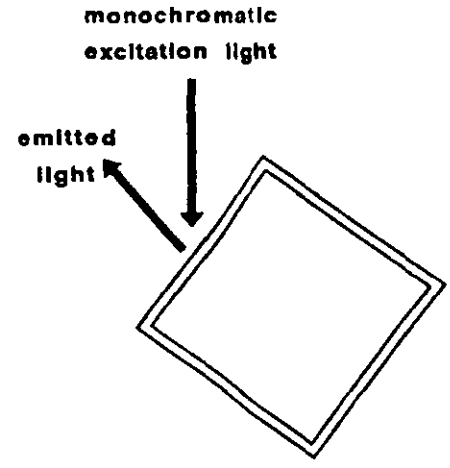

Figure 6.16b: Small angle viewing mode for high absorbances (turbid solutions and solids).

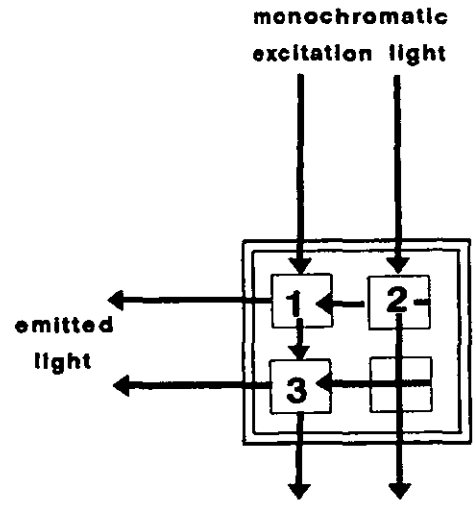

Figure 6.16c: Areas of sample in rotating cell method.

Figure 6.16: Viewing modes in fluorescence

determination of the fluorescence signal attenuation caused by sample absorption. The difference in power between position 1 and 2 (Figure 6.16c) gave a measure of the absorption of fluorescence radiation by the sample. The difference between positions 1 and 3 (Figure 6.16c) gave a measure of the absorption of exciting radiation by the sample. The corrections obtained by this method gave fluorescence-concentration graphs linear to an absorbance of 2.7.

6.8.1 Conventional Lasers

The three outstanding features that distinguish lasers from conventional light sources are their spatial coherence, high degree of monochromaticity, and narrow temporal pulse width, if pulsed. The use of lasers as excitation sources in fluorescence analysis has led to significant improvements in the detection limits of many compounds. Kessler and Wolfbeis in 1989 used the 632.8 nm helium-neon laser to excite fluorescence. An argon ion laser was used by Richardson and George in 1978 to obtain 0.5 pg/ml detection limits for Rhodamine B.

Another approach may be to use a small nitrogen-laser-pumped dye laser but the output power is unstable, and the pulse-to-pulse variation can be as high as 50%. Despite this sensitivities down to 1 pg/ml of Fluoranthene (Richardson and Ando, 1977) and 0.02 pg/ml (Ishibashi et al., 1979) or 1 picomolar (Bradley and Zare, 1976) of Fluorescein have been achieved.

However, the use of lasers in commercial spectrometers, other than Raman spectrometers, has not been widespread because of the problems of the reliability of lasers as compared with conventional light source, and lasers require additional maintenance (e.g. replacing the plasma tube). For example, a helium-cadmium (He-Cd) laser requires the replacing of the plasma tube every 4000 hours of use, at a cost of more than half the price of the original equipment.

6.8.2 Laser diodes

The near infrared laser diode, developed for telecommunications and data processing, has the advantage over conventional lasers of being cheaper, smaller and has a long life (approximately 10^5 hours). Unfortunately, a diode laser has the disadvantage of being restricted to the near infrared. Even though as far back as 1986 CW laser diode operation near 620 nm at 0°C (Kawata et al., 1986) and in the yellow spectral range (585 nm) at liquid nitrogen temperature (Hino et al., 1986) was demonstrated. As yet no commercial laser diodes in these regions are available and only in 1992 did Philips first market the 635 nm laser diode.

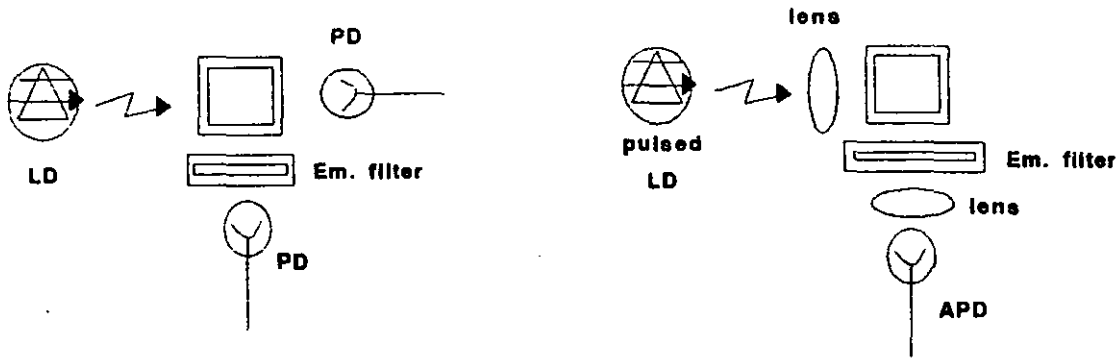

Figure 6.17: Proposed design of simple fluorimeters using laser diode (LD) excitation and either a silicon photodiode (PD) or avalanche photodiode (APD) detection. The emission (Em.) filter is either a bandpass or cutoff filter.

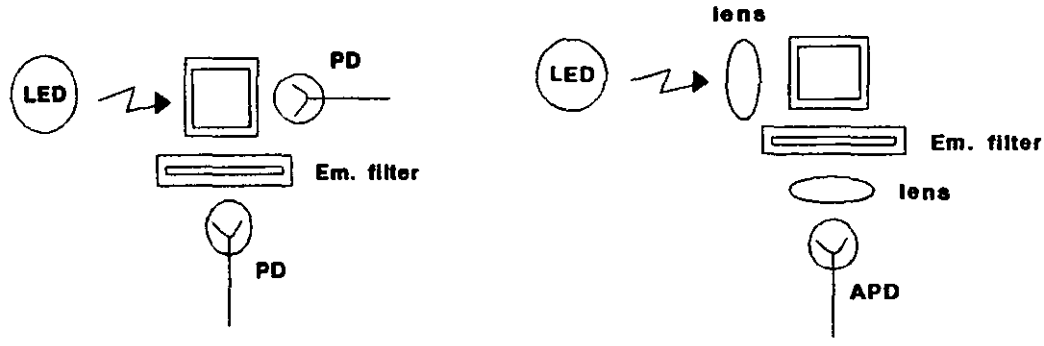

Figure 6.18: Proposed design of simple fluorimeters using light emitting diode (LED) excitation and either a silicon photodiode (PD) or avalanche photodiode (APD) detection. The emission (Em.) filter is either a bandpass or cutoff filter.

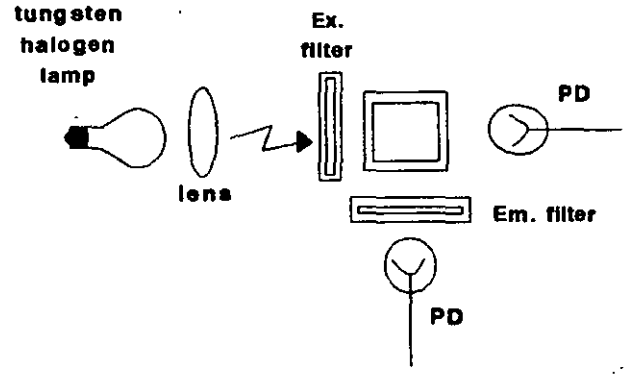

Figure 6.19: Proposed design of a simple fluorimeter with tungsten halogen lamp excitation and silicon photodiode (PD) detection. Excitation (Ex.) filter is a bandpass filter and the emission (Em.) filter is a cut-off filter.

A method of producing shorter wavelength light from a near-infrared laser diode is to produce second harmonic radiation in lithium niobiate ($LiNbO_3$) non-linear crystals grown and formed as waveguides. This frequency doubles the input radiation and hence halves the output wavelength (i.e. a 830 nm laser diode after frequency doubling emits at 415 nm). Higashijima et al. (1992) used a 50 microwatt 415 nm diode laser, which consisted of an 830 nm laser diode, a $LiNdO_3$ waveguide and collimating optics, as the light source for capillary zone electrophoresis by fluorimetric detection by a photomultiplier tube. The 40 mW power level of blue radiation has already been surpassed (Kozlovsky et al., 1990). The conversion efficiency of frequency doubling is low so to produce high output power pulsing of the laser diode is usually carried out.

A laser diode provides excellent sensitivity, when it is applied to fluorescence spectrometry (Imasaka and Ishibashi, 1990). In 1984, Imasaka and co-workers detected some polymethines down to a concentration of 5 picomolar. When an optical fibre and a capillary cell were used for light transmission and sample detection, the detection limit was reduced to 12 femtograms (Kawabata et al., 1986).

The introduction of the deep red laser diodes (635-680 nm) has extended the application to other dye groups that are more stable than the polymethines. These dye groups have already been discussed in Chapters 3. In 1989, Imasaka et al. used the water soluble bifunctional reagent carbodiimide to covalently bond phenoxazines and thiazines to albumin.

Figure 6.17 shows two possible optical arrangements for using a laser diode as an excitation source. Laser diodes do not require an excitation filter because of their monochromaticity. If the laser diode is mounted as close as possible to the cuvette then collimating optics may be dispensed with. The emission filter before the detector may either be a cut-off filter or a bandpass filter. The latter only allows approximately 40% throughput but is advisable if there are any overlapping fluorescent bands present. The photodiode at 180° to the laser diode detects light absorption at the lasing wavelength and so could allow the instrument to be used as an absorbance spectrometer as well. A laser diode with output of less than 1 mW is classed as a Class II laser, so the stringent precautions of using lasers of higher power could be dispensed with. Section 6.6 showed that a 2 mW laser diode had a higher output at 670 nm than either a 275 W xenon arc lamp or a 50 W tungsten halogen lamp. Laser diodes that have a lasing wavelength greater than 750

nm are invisible so are an extra hazard and also this causes alignment problems. These are other good reasons for using 635-680 nm laser diodes.

Pulsing a laser diode has the advantage of allowing easy discrimination of the fluorescent light from ambient light and pulsing is also required for operation of a avalanche photodiode, a very fast detector that was originally developed for fast data communications. The disadvantage of this is mainly one of cost and increasing the complexity of the instrument. With more elaborate electronics time-resolved fluorescence as demonstrated by Imasaka et al. in 1985 would be possible. The active area of an avalanche photodiode (APD) is very small, only 0.25 mm^2 in the case of the EMI 70506 APD, so good focusing optics are required which was the reason as already stated that the APD as a fluorescent detector was not developed further at the time.

Laser diodes emit polarised light so an instrument for polarisation fluorescence could be constructed by placing a polarising filter in front of the detector.

6.8.3 Light emitting diodes (LEDs)

Light emitting diodes are exceptionally stable light sources. LEDs are available in a wide variety of wavelengths ranging from 440 to 950 nm, with spectral bandwidth from 20 to 100 nm. The combination of extremely high stability, reasonably high intensity, small size, low cost (less than £1 per unit), and very long life (generally in excess of 10^6 hours) makes the LED an attractive source for spectroscopic measurements. Due to the wider spectral bandwidth of an LEDs than a laser diode, excitation bandpass or a cut-on filter may be required. If the LED is not collimated, the LED should be placed as close to the sample area as possible because the light from a LED is more divergent than that of a laser diode (see Figure 6.18). Collimation of the light from the LED would seem to be essential. As yet there does not seem to be any collimated visible LEDs commercially available.

A blue LED has been used as a source for a fibre optic based fluorimetric end-point detector for acid-base titrations (Wolfbeis et al., 1986). Imasaka and co-workers in 1983 determined phosphate using a LED-based absorption measurements. Smith et al. in 1988, who constructed a simple battery powered fluorimeter of a similar design to Figure 6.18a but without the photodiode at 180° to the LED, obtained a detection limit for Oxazine 720 of 20 ng/l as compared to 12 ng/l for a conventional fluorimeter. Wickliff and Wickliff (1991) measured in vivo

Chlorophyll fluorescence with a red LED excitation using the frontal optical arrangement and photodiode detection.

Brown et al. (1993) used an "ultra-bright" green LED (maximum emission at 565 nm) to determine Nile Red [L111] down to less than 1 micromolar using a photodiode as a detector.

6.8.4 Tungsten halogen lamps

For a 50 W tungsten halogen lamp, it has been shown that the photon flux at wavelengths longer than 600 nm is greater than that of a 275 W xenon arc lamp. Tungsten halogen lamps are small, cheap, robust, and can be operated using batteries unlike the conventional xenon arc lamp used in fluorimetry. The use of an excitation filter (see Figure 6.19) such as a laser line filter which gives a very narrow bandwidth (about 2 nm) and high efficiency (80-90%) compared to conventional interference filters (about 10-20nm and 40% efficiency) means that the tungsten halogen has similar advantages to lasers. The additional advantages being that lower excitation wavelengths are available. The main problem of using a tungsten halogen lamp is that a considerable amount of heat emitted from the lamp, which would seriously influence the other components and the fluctuation of intensity with a change in the current.

6.8.5 Photodiodes

Silicon photodiode with large active areas have the advantage over ones with smaller active areas in that there is a larger surface area to collect the emitted light. The disadvantage is that they have slower response times and suffer from higher dark currents. Large active area photodiodes are used if there are no colleting optics to focus the light on the detector.

Avalanche photodiodes can cope with fast changes in light levels and so lend themselves to time-resolved fluorescence measurements and as detectors for high pressure chromatography (HPLC).

Photodiode arrays allow spectra to be obtained without the use of mechanical components and fast acquisition time. Photodiode arrays such as that in the IMUC-7000 allow almost simultaneous acquisition of light (Summerfield and Miller, 1993). Simply by dispersing the light using a monochromator grating over the photodiode array.

6.8.6 Calibration of near infrared fluorimeters

Calibration of the monochromators is a problem especially in the near infrared region that has not been addressed fully. The problem of calibration of the monochromators was noticed when the emission wavelengths of three spectrofluorimeters were compared. A greater divergence of values was noted at at longer emission wavelengths when the same cuvette containing dye in methanol was measured on the three spectrofluorimeters (see Table 6.6).

Table 6.7: Emission wavelengths for various acrylic block standards (Starna, Essex, UK) and dyes made up in methanol (unless otherwise stated) using a Perkin Elmer LS-50, Perkin Elmer MPF-44B and a Shimadzu RF-5001PC spectrofluorimeter. The excitation and emission slits were either 2.5 or 5 nm.

	Ex. (nm)	Perkin Elmer LS-50 (nm)	Perkin Elmer MPF-44B (nm)	Shimadzu RF-5001PC (nm)
Acrylic blocks				
Block 1: Anthracene and	290	322/336	323/334	317/332
Naphthalene	340	380/401/424	378/400/426	ND
Block 2: Ovalene	350	462/482/503	463/468/501	459/478/500
Block 3: Perylene[1]	396	ND	ND	ND
Block 4: 7,8 Benzo-				
quinolinium	290	348/365/384	347/364/383	ND
Block 5: Coronene	340	427/438/445/	426/437/445/	
		454/474/484	453/474/507	ND
Block 6: Rhodamine B	490	573	568	563
Block 7: Triphenylene	290	354/362/371	354/361/370	ND
Block 8: Tetraphenyl-				
butadiene	360	420	422	ND
Dyes in methanol				
Cresyl Violet [XLVIII]	580	620	615	611
Nile Red [LIII]	550	628[e]	610[e]	609[e]
Styryl 11 [XXVII]	560	626	ND	610
Nile Blue A [XLIX]	620	670	660	ND
DTTC [XXIII]	730	785	780	ND
IR125 [XXV]	750	830	817	ND

[1] this fluorescent block showed no fluorescence as and hence the perylene was assumed to have decomposed.
[e] ethanol

The wavelengths longer than 650 nm could not be tested on the Shimadzu RF-5001PC because the spectrofluorimeter did not posses a red sensitive

photomultiplier tube unlike the two Perkin Elmer instruments. All measurements were carried out at either 2.5 or 5 nm excitation and emission slits. Larger slits were required at longer wavelengths due to the reduction in performance of the optics and photomultiplier tube.

No acrylic block standards of dyes beyond the longest wavelength acrylic block standard contains Rhodamine B (Ex. 490; Em. 568). These standards were produced by Starna (Essex, UK). Various dyes have been submitted to this company to produce standards but at the time of writing these have not been evaluated.

6.8.7 Automation

Automation is a major trend in instrument and technological development. The reasons for automation arise from the improved precision, saving in time and labour, saving in reagents and hence cost. The disadvantages of such automation relate to the high price of complicated instruments and reagents delivered as unit-dose packages. This produces a "black box" which requires little if any expertise. The ultimate dream is probably rather dangerous.

To optimise the operation of a flow infection or high pressure chromatography system, a different design of the sample area is required. Most fluorimeters used for flow injection analysis are modified conventional fluorimeters using a modified 1 cm cuvette configuration. This is rather inefficient and can cause the problems of dispersion. A simple straight silica tube set in an acrylic block as illustrated in Figure 6.20 would be better. The light from a laser diode is launched down a silica tube and the fluorescence is collected at right angles. The problems from light scattering is much reduced by having the detector placed further away from the light source. Light scattering is of little if any problem because the laser diode is collimated.

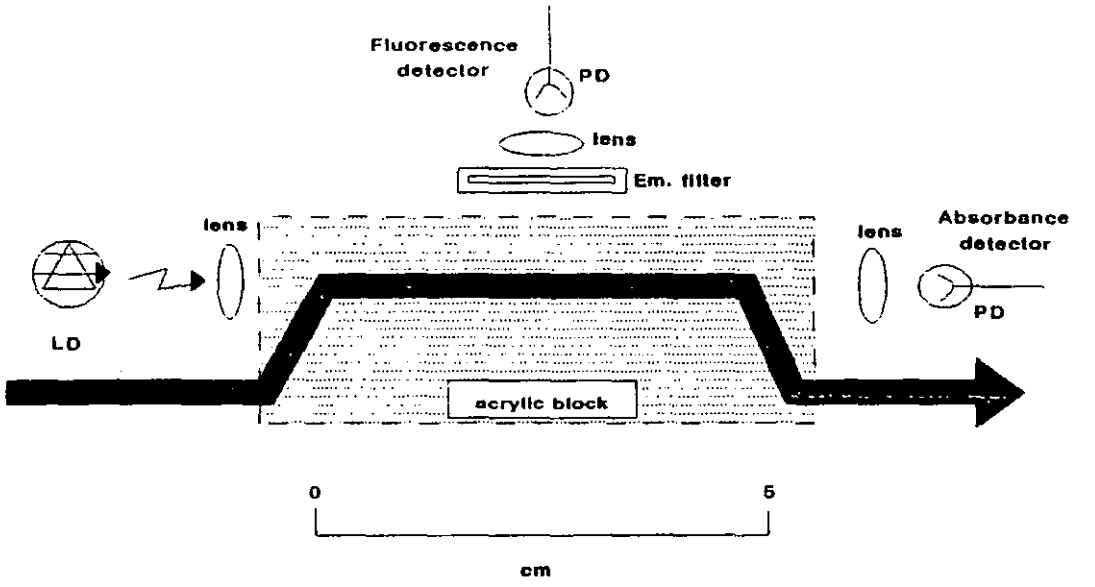

Figure 6.20: Proposed design of a flow cell with a laser diode (LD) and spectrometric and fluorimetric detection by silicon photodiodes (PD). Emission (Em.) filter is a cut-off filter.

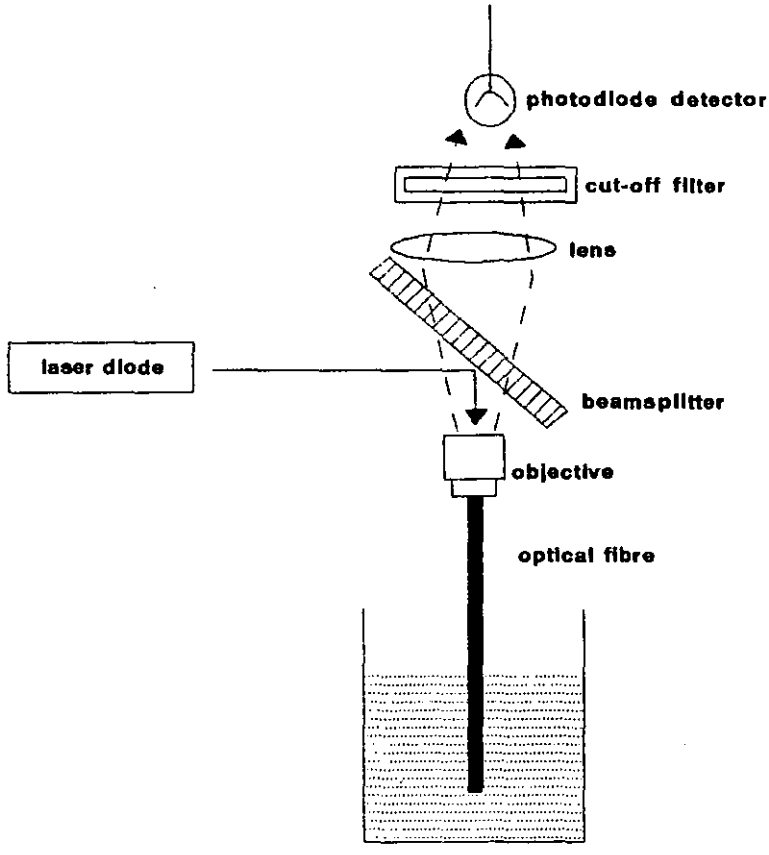

Figure 6.21: Configuration of a fibre-optic fluorimeter. Laser diode excitation (solid line) is reflected off the beam splitter (a dichroic mirror) into the objective, which launches it into the optical fibre. The fluorescence produced (dotted line from the sample travels back through the fibre, the objective, and the beamsplitter; the fluorescence is focused by the simple lens through the cut-off filter onto the photodiode detector.

6.8.8 Optical fibres

Optical fibres were developed mainly for telecommunication purposes, but they also provide a convenient means to conduct light in a fluorimeter (Seitz, 1988). By using fibres for excitation and transmitting emission, even a normal spectrofluorimeter, such as the Perkin Elmer LS-50, can be converted to detect fluorescence from well plates, TLC or gel plates.

The use of fibre optics in conjunction with fluorimetry is growing in fields as diverse as biophysics, remote sensing, immunodiagnostics, and chemical process monitoring (Thompson et al., 1990). The intrinsic wavelength difference between excitation and emission makes fluorescence well suited for use with fibre optics. The fundamental idea of fluorescence-based sensors is to detect an analyte by a change in the fluorescence of a susceptible molecule. Several instrumental configurations for performing fluorimetric measurements through fibre optics have been described in the literature (Angel, 1987; Chudyk et al., 1985; Milanovitch et al., 1986; Bright and Litwiler, 1989).

A typical system (Figure 6.21) couples a high intensity light source, such as a laser, laser diode or a xenon arc lamp, into a single large core quartz fibre. Light travels along the length of the fibre as a result of total internal reflections with little light loss. Upon exiting the fibre, the light impinges upon the sample that gives off fluorescence and is transmitted by either the same or another fibre back to the photodetector. Remote measurement is especially useful in a continuous industrial application where the fluorimeter would be too fragile.

The combination of optical fibres and solid state components (i.e. laser diodes, LEDs, photodiodes etc.) is advantageous in allowing great flexibility of the use of these components.. These could be used in various ways.

1. Light from a laser diode or a LED could be launched down a fibre;
2. Light from a tungsten halogen lamp launched down a fibre would isolate the heat of the lamp from the sample area and especially the detector;
3. Collection of the fluorescent light and then illuminating the detector;
4. Bifurcated fibre optics combines 2 and 3;
5. Remote sensing in hostile environments.

These have the advantage of being able to isolate the components from each other and hence reducing thermal and electrical interferences.

6.8.9 Read-out Systems

The final link is the conversion of the signal from the detector into a form that the analyst can use.

Moving coil meters are the lowest cost of the read-out system and usually incorporate a mirrored surface behind the pointer so that its position relating to the printed scale could be read without errors due to parallax. Greater accuracy in reading is achieved by increasing the length of the scale. Meters suffer from limited linearity and mechanical instability.

Digital displays are not subject to misreading errors and have greater linearity. These advantages usually outweigh the problems of a flickering display that is difficult to read. The display is usually a 7 bar red LED. It is robust, reliable and has low power supply consumption.

Chart recorders produce a permanent record of the analog signal but have rather long response time of the recorder pen (0.1-0.5 sec) that limits the speed at which an accurate spectrum can be swept. There are two types of chart recorders: The x-t or strip chart recorder displays fluorescent intensity on the x-axis and wavelength on the t (time) axis. The t axis is traversed at a constant rate and may be slaved to the monochromator drive motor. In x-y chart recorders, the x-axis displays the fluorescent intensity and the y-axis is continuously synchronized with the scanning speed of the monochromators. Thus the slowing of the monochromators scanning rate in the area of spectroscopic interest, or speeded up in a less interesting area. The spectrum may also be retraced by back scanning.

Computers are the most recent breakthrough in the man-machine interface to control the fluorimeter and display of instrument status and results. The setting up of such an instrument is usually just a matter of choosing modes from the display menu and inputting information or carrying out action in the sequence prompted by the instrument. Although computer programs vary slightly from one instrument to another, the commands fall into the following categories.
Instrument controls help the communication between instrument and the computer. These include setting the starting and ending wavelengths, data interval, slit width, scan speed and response time. During data collection, the information can be monitored on the monitor.

Library - Spectra can be stored on either a hard disk or diskette for future use and manipulation.

Graphics - The spectrum is displayed on the graphics monitor, normally either EGA, VGA, SVGA or XGA monitor. The xy scaling allows the expansion or compression of the spectrum displayed on the graphic monitor. A hard copy of the pertinent experimental information can then be made.

Data-extractive commands - These provide information from the spectrum, such as peak area, peak tables and intensity values. The wavelength and intensity can be found by using the movable cursor.

Data Manipulative - These are spectral subtraction, spectrum overlay routines that allows the analyst to compare two or more spectra and a smoothing function that fits a moving point polynomial to the data and averages the rapid transients that characterise noise.

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Chapter 7

7.1 The Prospects of Near Infrared Fluorescence

Near infrared fluorescence is still in its infancy but the prospects are very encouraging from the work that has been presented. The potential has also been demonstrated by the research groups lead by Imasaka (Imasaka et al. 1984 to 1990: Kawabata et al. 1989: Okazaki et al. 1988), Miller (Miller, 1990; 1993: Summerfield and Miller, 1993: Brown et al., 1993: Palmer and Miller 1993), Patonay (Patonay and Antione, 1991), Vo-Dinh (Cheng et al. 1990; Tromberg et al. 1987), Winfordner (Smith et al. 1988) and Wolfbeis (Kessler and Wolfbeis, 1989; Wolfbeis et al., 1986). The obstacles to be overcome are reasonably straight forward and require the application of expertise from various areas of science and technology, especially electronics and classical organic synthesis.

Conclusions relating to the areas addressed in the aims of the project are addressed below.

7.1.1 Near Infrared Fluorescence

The main advantages of near infrared fluorescence that have been demonstrated by experiment in this thesis are as follows.

Very few naturally occurring fluorophores in biological and environmental samples absorb and emit in near infrared region (600-1000 nm) so there are less likelihood of background fluorescence. Most biological compounds are fluorescent between 300 and 400 nm. The intensity of Rayleigh scattering at right angles to the direction of the incident light beam is inversely proportional to the forth power of the wavelength. This is demonstrated by the 100 fold reduction of background scattering and fluorescence from 1% human blood serum in the near infrared region as compared to the visible region (Figure 1.5 and 1.6). There are no stable fluorophores in the near infrared region beyond 1000 nm, this is due to thermal excitation to the triplet state (see Section 1.6).

The study of the comparative photostability of near infrared dyes (Section 3.8) showed that the phenoxazines (i.e. Nile Blue A [XLIX], Nile Red [LIII], and Oxazine 750 [LII]) were about an order of magnitude more stable than Fluorescein [IV], the most commonly used fluorescent label. This is mainly due to the longer absorption wavelengths, and hence lower energy of excitation for the phenoxazines compared with Fluorescein. There is also lower photodecomposition of the sample at

longer wavelengths because the lower incident radiation energy is not sufficient to excite the analyte, usually a protein.

7.1.2 Dyes

The dye groups that show fluorescence in the near infrared region are the phycobiliproteins (Section 3.6.2), the polyaromatic aromatic hydrocarbons (Section 3.6.4), the quinonoids (Section 3.6.3), the indigoids (Section 3.6.1), the higher conjugated forms of the xanthenes (Section 3.3), the polymethines (Section 3.4), the azines (Section 3.5) and the phthalocyanines (Section 3.6.5). The latter three dye groups were shown to have the most promising capabilities as labels and probes.

The most promising of those investigated were the phenoxazines (e.g. Nile Blue A [XLIX], Nile Red [LIII], Oxazine 750 [LII] etc.) and thiazines (e.g. Azur A [LIV], Azur B [LV], Methylene Blue [XLVII] etc.). This is due to their longer absorption/fluorescence wavelengths than the xanthenes and superior photostability over the xanthenes and polymethines. Their main disadvantage is their tendency to plate solid surfaces and to form aggregates especially in water (Section 3.7). These phenomena could be reduced by the use of disaggregating agents (e.g. surfactants or polar solvents) and the addition water solubilising groups to the dye (such as sulphonic acid groups.)

The tricarbocyanines (e.g. DOTC [XXII], DTTC [XXIII] and IR125 [XXV]) have the longest wavelength absorbance/fluorescence but the worst chemical and photochemical stability. Tricarbocyanines are difficult to derivatise because of the danger of altering or destroying the conjugation bridge if too severe conditions are used (see Section 4.2) and so if pursued, should be synthesised from scratch to produce a reactive dye. The rigidised carbocyanines (e.g. thiazolium squarylium [XXIX] and thiazolium croconium [XXX]), are the most promising for the synthesis of new near infrared fluorophores due to their superior chemical and photochemical stability compared to other polymethines. No reactive dyes of this type have appeared in the literature as far as the author is aware.

The phthalocyanines (Section 3.6.5) have outstanding chemical and photochemical stability. These promising fluorophores are being investigated by our laboratory for covalently labelling of proteins for biochemical analysis, specifically immunoassay. These dyes have a very small Stokes' shift of only 5 to 10 nm, and low stability in water. The latter can be overcome by the addition of sulphonic acid groups. Almost all the reactive dyes available in near infrared region are phthalocyanines.

7.1.3 Fluorescent probes

The initial studies of dye protein binding proved that some near infrared dyes bound to proteins (see Section 5.2). For example, the emission wavelength of Nile Red shifts from 663 to 615 nm on binding to bovine serum albumin. Nile Red [LIII] shows good probe characteristics with bovine serum albumin because the fluorescence of Nile Red [LIII] bound to BSA is stable and the unbound dye decays quickly. The fluorescence of unbound Nile Red [LIII] in aqueous buffer after 15 minutes falls off considerably due to dimerisation causing the dye to become insoluble and hence precipitate out of solution (see Section 3.7). This property lead to the suggestion that if a substance could be found to dislodge the dye, this could be used as an assay method for drugs. This has been followed up by other members of the research group who have investigated the number and type of binding sites, and the drugs that dislodge the bound dye.

From the work on the aggregation of near infrared dyes (Section 3.7), further work has been performed by the research group in enhancing their assays to reduce non specific binding. Ion pairing that occurs with compounds with opposite charges (such as Methylene Blue [XLVII] and sodium dodecyl sulphate, an anionic surfactant) has been used as an assay method for anionic drugs.

7.1.4 Reactive Dyes (Labels)

There are very few commercially available reactive dyes (labels) and these are confined to the phthalocyanines. These are very large dye molecules with molecular weights in excess of 1000 and suffer from the problems of lack of solubility, steric hindrance and an inclination to dimerise. A dye may be considered as being composed of an electron donor group connected by a conjugation bridge to an electron acceptor group. The absorbance maximum of dyes may generally be extended by:

a. Lengthening the conjugation bridge (i.e. the absorbance of the symmetrical cyanines is extended by approximately 100 nm for each ethylene group added, see Figure 3.11).

b. Increasing the strength of the electron donors and/or acceptors (i.e. the strength of the electron donors increase of going down Figure 3.11).

c. Substitution in the conjugation bridge of a carbon atom by a heteroatom (i.e. nitrogen).

d. cationic dyes with amino groups absorb at longer wavelength than the anionic dyes with hydroxyl substituents (see Table 3.7 and 3.25). For example, Nile Blue A

at 627 nm and its hydroxyl analog Nile Red [LIII] absorbs 565 nm.

e. Substitution para to the heteroatom causes a bathochromic shift. For example, the homologous series of azines in Figure 3.15. The bathochromicity increases on passing from phenazines (nitrogen) to phenoxazines (oxygen) to thiazines (sulphur).

All these features are identifiable in the dye groups investigated (Chapter 3). It is also desirable that the dye is water soluble, has reduced or no plating of solid surfaces, and be both chemically and photochemically stable. Water solubility can be achieved by adding sulphonic or carboxylic acid groups. These solubilising groups should be added before the final formation of the dye and should not be directly added to the conjugation bridge (e.g. to the aromatic rings) because sulphonic and carboxylic groups tend to destroy the conjugation or the planarity of molecules, hence destroy its fluorescence properties. Anionic dyes have almost no plating tendencies.

The synthesis of reactive near infrared dyes (labels) should be from scratch because of the scarcity of fluorophores that have groups that lend themselves to modification (i.e. hydroxyl, amine, carboxylic or sulphonic acid groups) and the difficulty of adding solubilising groups to an existing fluorophore. The properties required of a label can therefore be designed into the molecule. The most promising fluorophores in the near infrared region that could be modified are the phenoxazines, thiazines, rigidised polymethines and the phthalocyanines. The properties of can be found in Chapter 3 and in the Appendix. These are being further pursued with commercial collaboration at Loughborough University.

7.1.5 Biochemical Applications

There is virtually no background fluorescence, light scattering due is greatly reduced and photodecomposition of the analyte is much reduced in the near infrared region as compared with ultraviolet-visible fluorescence. These give near infrared labels (reactive dyes) great advantages over the usual ultraviolet-visible labels.

Near infrared fluorophores could be used in conjunction with visible fluorophores as labels in a dual assay where a number of analytes are customarily measured together in the same sample. Typical analyte pairs include the thyroid hormones, T_3 and T_4 (Denning et al., 1979), T_4 and TSH (Bluett et al., 1977), and in the screening of Hepatitis B antigens and HIV antibodies from blood collected for transfusion. The first pair of fluorophores used for simultaneous determination of two parameters was Fluorescein isothiocyanate (FITC [VI]) and Tetramethyl-rhodamine isothiocyanate (TRITC), in detecting IgD and IgM on lymphocyte surfaces

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(Loken et al., 1977) and for the identification of bacteria with a fluorescence microscope (Gillis and Thompson, 1978). Sidki et al. (1985) produced a double label simultaneous fluorescence immunoassay for primidone and phenobarbital, using FITC [VI] labelled phenobarbital and Rhodamine X isothiocyanate (XRITC) labelled primidone and is one of a few examples of this type of assays.

Another method of using near infrared labels is in singlet-singlet energy transfer homogeneous immunoassay (see Section 5.3.2). A potential pair of labels could be Texas Red [XIII] (Ex. 595 nm/ Em. 620 nm) and Nile Blue SS [CXVII] (Ex. 640 nm/ Em. 670 nm). These fit the criteria of the donor emission spectrum overlapping the excitation spectrum of the acceptor and that the acceptor does not absorb or fluoresce at same wavelength as the donor fluorophore and there is negligible fluorescence at the acceptor emission wavelength by the donor. This would be very useful method of producing an assay for a small molecular weight analyte, which would be labelled by the lower wavelength fluorophore and the long wavelength fluorophore would label the antibody.

As already noted near infrared labels are by nature large molecules and flow injection analysis could be a problem caused by their tendency to adhere to solid surfaces.

Fluorescent labelling could also be combined with other powerful tools to study several important parameters (e.g. surface organisation, physical state, dynamics, and cell function) of cell surfaces (Edidin, 1989). Most fluorescent labels that can react with proteins are suitable for such work. Relatively little if any work has been done on the application of near infrared labels.

7.1.6 Electronics

Only until recently fluorescence has been almost inaccessible to inexpensive instrumentation. This has changed rapidly with the introduction of solid state detectors based primarily on silicon semiconductors, such as photodiodes, diode arrays and avalanche photodiodes which operate predominately in the near infrared region rather than at lower wavelength. The design of fluorimeters was described in Section 6.8 and encompass simple, inexpensive portable fluorimeters to expensive research instruments with diode array detection (such as that discussed in Section 6.6). Only when specifically designed fluorimeters are available will the potential of near infrared fluorescence be realised.

The light source that has revolutionised near infrared fluorescence is the laser diode (Section 6.2.2). The three outstanding features that distinguish lasers

and laser diodes from conventional light sources are their spatial coherence, high degree of monochromaticity (1 to 2 nm bandwidths), and narrow temporal pulse width, if pulsed. The introduction of the deep red laser diodes (635-690 nm) has extended the application to other dye groups that are more stable than the polymethines. Pulsing a laser diode allows the simple discrimination of the fluorescent light from ambient light and is required for operation of a avalanche photodiode. Laser diodes emit polarised light so an instrument for polarisation fluorescence could be constructed by placing a polarising filter in front of the detector.

Light emitting diodes, the cousin of the laser diode, are available in a wider range of wavelengths (440-950 nm), with spectral bandwidth from 20 to 100 nm. The combination of extremely high stability, reasonably high intensity, small size, low cost and very long life (in excess of 10000 hours) makes the LED an attractive source for spectroscopic measurements. It has been proved in our laboratory that very simple unfocused and unoptimised instruments can be used.

The combination of optical fibres with a light source and a detector allows great flexibility of the use of the subsequent instrument. These could be used to transmit the light from a laser diode or a LED to the sample; isolating the large heat output from a tungsten halogen lamp that would seriously influence the other components; collecting the fluorescent light and then illuminating the detector; and the use of bifurcated fibre optics that both transmits the incident light to the sample and the subsequent fluorescence. All these allows remote sensing in hostile environments.

Dispersive elements (Section 6.3) for the near infrared region take the form of grating monochromators which should be blazed beyond 500 nm or interference filters. This latter for fixed wavelength instruments.

Detectors in the near infrared region (Section 6.5) are either photomultipliers (e.g. the R928 PMT is sensitive to 900 nm) or silicon photodiodes (peak sensitivity between 750-900 nm). The former are very delicate, prone to failure if daylight is allowed to fall directly on them and require an special power supply to keep the dynodes at the high potential required that is expensive. This makes instruments using a photomultiplier tube as the detector bulky, not very portable and expensive (in excess of £20000). On the other hand, silicon photodiodes are cheap, robust, and require low operating voltages so may be run off batteries, so can be used to construct a portable instrument. A single photodiode can be used for single wavelength detection or producing a spectrum by sweeping the spectrum across it

using a monochromator.

Avalanche photodiodes can cope with fast changes in light levels and so lend themselves to time-resolved fluorescence measurements and as detectors for transient signals from high pressure chromatography (HPLC) or flow injection analysis (FIA).

Photodiode arrays allow spectra to be obtained without the use of mechanical components and fast acquisition time by dispersing the light using a monochromator grating over the photodiode array (e.g. Otsuka IMUC-7000).

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Appendix

Physical and Spectroscopic Data of Dyes

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Part 2 gives the structural formula (where known) of the dyes, methods of manufacture, solubilities in mineral acids and alkali, and literature references, including patents.
Part 3 includes abbreviations of manufacturers names, Generic Names and commercial names

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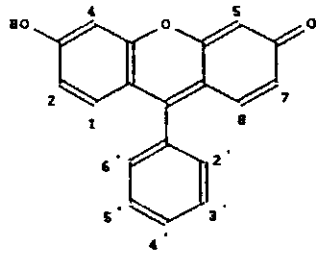
Glossary

Abs.	absorbance
Ac	acetone
alk.	alkaline
anhyd.	anhydrous
aq.	aqueous
bp	boiling point
Bu	butyl
BuAc	butyl acetate
CAS #	Chemical Abstracts Number (xxxxx-xx-x)
Chl	chloroform
CI	Colour Index Generic Name
CI No.	Colour Index Number (CI-xxxxx)
col.	colourless
conc.	concentrated
(dec)	decomposes (°C)
dil.	dilute
DMF	dimethylformamide
DMSO	dimethylsulphoxide
Docsan	docosanyl
Dodec	dodecyl
E	diethyl ether
EG	ethylene glycol
EGME	2-methoxyethanol: methyl celusolve
Em	fluorescence (emission) maximum
Et	ethyl
EtAc	ethyl acetate
EtOH	ethanol
Gl	glycerol
Hept	heptyl
Hex	hexyl
Hexdec	hexadecyl
H_2O	water
hr.	hour
H_2SO_4	concentrated sulphuric acid
hyd.	hydrated
I	insoluble
LOD	limit of detection
max.	maximum
Me	methyl
MeOH	methanol
min.	minimum
mp	melting point (°C)
mol wt	molecular weight
Neo	neopentylene
NaOH	aqueous sodium hydroxide
NCS	isothiocyanate
ND	not determined
Octdec	octadecyl
Pent	pentyl
Ph	phenyl or phosphorescence
ppt.	precipitate
pptd.	precipitated
Pro	propyl
Py	pyridine
s	soluble (around 1%)
satd.	saturated
soln.	solution
ss	slightly soluble (under 1%)
THF	tetrahydrofuran
TMS	tetramethylsilane
vs	very soluble (over 10%)
vss	very slightly soluble (only just partially soluble)
wh.	white
Xyl	xylene
yel.	yellow
ϵ	molar absorptivity (l mol^{-1} cm^{-1})
λ	wavelength
τf	fluorescence lifetime (ns)
τp	phosphorescence lifetime (ms)
Φf	fluorescence quantum yield
Φp	phosphorescence quantum yield

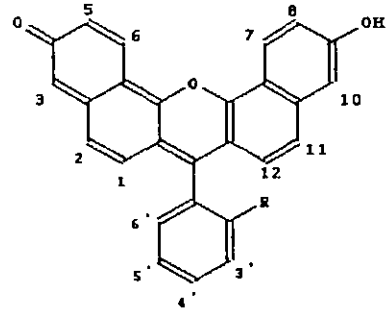
Appendix A

XANTHENES

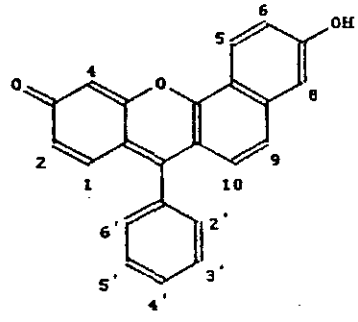
FLUORESCEINS



A1: Fluoresceins

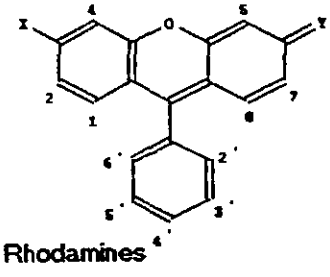


A2: Naphthalofluoresceins

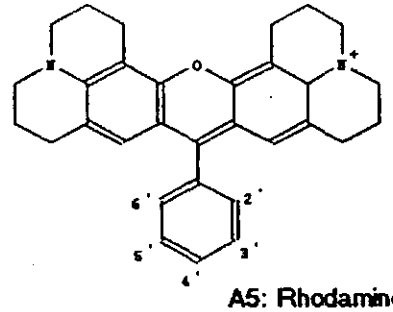


A3: Benzo[c]fluoresceins

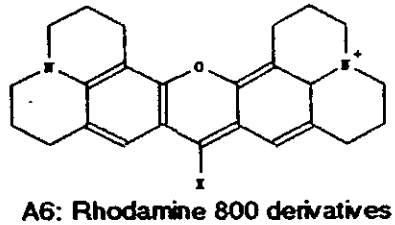
RHODAMINES



A4: Rhodamines

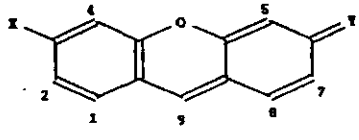


A5: Rhodamine 101 derivatives

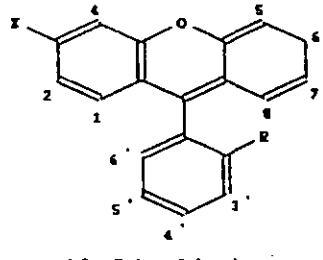


A6: Rhodamine 800 derivatives

OTHER XANTHENES



A7: Pyronines



A8: Other Xanthenes

A1: Fluoresceins (continued)

CI No	CAS #	Dye Name	Position 2'-	Other Position	Absorbance nm	ε x 10^4 ($M^{-1}cm^{-1}$	Fluorescence nm	Solvent	pKa	Solubility H_2O	Solubility EtOH	Solubility Other Solvents	Other Information
45370:1	596-03-2	CI Solvent Red 72 (free acid)	COOH	4,5-Br2	as 4,5-Dibromofluorescein					0.03%	3%	8% EGME vs Ac. s in BuAc & EtAc	as above but acid form mp 270-273 °C
		5(6)-carboxy-4',5'-dimethyl-fluorescein	COOH	4'(5')-COOH 4,5-Me2	504	6.3	536	pH 9	7.0	s		s in alkali	soluble pH>5
		4'5'-dimethoxycarboxyfluorescein	COOH	4',5'-COMe	512	7.82	ND	water					
	76-54-0	2,7-dichlorefluorescein Fluorescein 27 Fluorescein 548	COOH	2,7-Cl2	515 535 512 502	7.41 0.54 11.0	ND ND 530 ND	ethanol acetone ethanol/base water	3.7 & 4.9				pH 4.0 (blue green fluorescence) pH 6.6 (green fluorescence
		Hydroxy-hydroquinone-phthalein 2,7-Dihydroxyfluorescein	COOH	2,7-OH2	ND		ND						Metallochrome indicator
		5(6)-carboxy-2',7'-dichloro-fluorescein	COOH	2,7-Cl2 4'(5')-COOH	504	9.0	529	pH 8	5.1	s		s in alkali	soluble pH>5
45371		CI Solvent Orange 18	COOH	2,7,-I2 4,5-Br2	ND		ND			I	es	s in 5% Na_2CO_3	Yellow in H_2SO_4, on dil orange ppt Bright pink in 10% NaOH
45375		Phloxine N	COONa	4,5-Br2 3',6'-Cl2									Brominate 4,7-dichlorofluorescein with Br2, sodium chlorate & EtOH.
45380	17372-87-1 548-26-5	Eosin Y: CI Acid Red 87 Tetrabromofluorescein Eosin Yellowish (YS)	COONa	2,4,5,7-Br4	525 520 516	10.1 12.6 7.94	550 539 540	ethanol water pH 7.1	1.5	20% bluish red	2% bluish red	I in Xyl, E, D, vs in EG s in Py, Gl, & Chl	pHD (not fl.); pH3 (bluish red with yellowish green fl.) Yellow in H_2SO_4, on dil orange ppt ϕ_f=0.76 alkaline, ϕ_f=0.18 water, ϕ_f=0.68 EtOH; τ_f=0.9ns EtOH, Ph=550ns, ϕ_p=0.001 water Pink with lt green fl. in water 1% soln in H_2O previously adjusted to pH7 has a pH of 6.7
45380:2	15086-94-9	CI Solvent Red 43 (free acid) Eosin Acid	COOH	2,4,5,7-Br4	537 521		ND ND	water alkaline		0.05% in cold s hot	1%	s in Xyl, Py, EGME & EG vs in D & Ac ss in Chl	As Eosin Y (CI No.45380) Almost colourless in EtOH & xylene Acid-base indicator.
		Eosin-5-isothiocyanate	COONa	2,4,5,7-Br4 4'-NCS	524	10.1	548	methanol		pHD6	s	s in DMF	Isothiocyanates are unstable in water
45386	23391-49-3	Methyl Eosin CI Solvent Red 44	COOMe	2,4,5,7-Br4	525		ND	water		s in hot cherry red	1% red	1% in EGME	Yellow in H_2SO_4 on dil. brownish yellow ppt. Darker red soln. with green fl. in NaOH. Cherry red in hot water. Red with browny yellow fl. in EtOH

A1: Fluorescein

CI No	CAS #	Dye Name	Position 2'-	Other Position	Absorbance nm	ε x 10^4 $M^{-1}cm^{-1}$	Fluorescence nm	Solvent	pKa	Solubility H_2O	Solubility EtOH	Solubility Other Solvents	Other Information
45350	2321-07-5	Fluorescein (disodium salt) Fluorescein sodium; Uranin CI Acid Yellow 73	COONa		437 437 455,475 488 491 501 498	6.01 1.56 3.16 8.6 7.94 9.33 8.39	none none 510 514 518 531 518	pH 0 pH 3.3 pH 5.5 pH 8.2 pH 12 basic EtOH basic NaOH	2.2 & 4.4 & 6.7	50.02%	7.19%	>10% DMSO. i in Xyl, Chl. ss in D & Py. s in Ac & Gl.	No fluorescence when acidified LD50 for mice (oral) is 4738 mg/kg LD50 for rats (oral) is 6721 mg/kg pH4 (pink with green fluorescence) pH5.0 (yellowish red with green fluorescence); Merck 11-4085 Yellow with fluorescence in H_2SO_4, on dil. yellow with yellow ppt. Darker soln. with a dark green fluorescence in NaOH. ϕf=0.03 NaOH; ϕf=0.81 water ϕf=0.90 alk. EtOH; τf=4.5ns H_2O 1% soln. in H_2O previously adjusted to pH7 has a pH of 9.5
45350:1	1518-47-6	Fluorescein (free acid) CI Solvent Yellow 94	COOH		as Fluorescein sodium					0.03%	2.21%	s in dil. alkali E, Xyl & Ac. ss in D, Gl & Chl vs in Py.	Merck 11-4087; as above
		5-Fluorescein isothiocyanate FITC (isomer 1)	COOH	4'-NCS	494	7.2	520	pH 8		<0.01% pH>6	2.21%	s in DMF & EGME	mp 221-222.5 °C -NCS is unstable in water ϕf=0.3-0.5 on binding to protein
		6-Fluorescein isothiocyanate FITC (isomer 2)	COOH	5'-NCS	491	6.5	521	pH 8					
		5-(4,6-dichlorotriazinyl)amino-fluorescein (5-DTAF)	COONa	4'-DT	492	6.6	516	pH 8		pH>6		s in DMF	
	3326-34-9	Fluoresceinamine, isomer 1 4-Aminofluorescein	COOH	4'-NH2	493		519	pH 8		0.1%	6%	6% in EGME	mp 223 °C (dec)
	51649-83-9	Fluoresceinamine, isomer 2 5-Aminofluorescein	COOH	5'-NH2	491		520	pH 8		1%	2%	10% in EGME	mp 285 °C (dec)
45630		CI Acid Yellow 74	COOH	2-Me-Ph	ND		ND			s			Brown with green fl. in water Yellow in H_2SO_4, on dilution brown yellow ppt.
45385	76-54-0	4,5-dichlorofluorescein CI Solvent Orange 32	COONa	4,5-Cl2	509 503	11.0	ND ND	basic EtOH water	4.6	<0.01%	3%	s in Ac, EGME vs in THF ss in Gl, E, EG i in oils, fats & waxes.	Yellow in H_2SO_4 on dilution brown yellow ppt. Red in NaOH. Orange & yellowy green fl. in water & EtOH pH4 (weakly green) to pH8 (more intense green); Merck 11-3056
45386		CI Solvent Red 42	COOH	2,4,5,7-Cl4	ND		ND			i		s in 10% NaOH	
45370	4372-02-5	4,5-dibromofluorescein CI Acid Orange 11 Eosin H	COONa	4,5-Br2	530 520 450	2.40	ND ND ND,	ethanol water MeOH/HCl		ss orange	s orange	s in Ac, Gl, D, Chl & Py. vs in THF & Ac. ss in Xyl.	Orange with faint yellow fl in H_2O Orange with pea green fl. in EtOH Pink with yellow fl. in acetone Eosin red in NaOH. Red-yellow in H_2SO_4, on dil. yellowish brown with orange ppt. Merck 11-3005

A1: Fluoresceins (continued)

CI No	CAS #	Dye Name	Position 2'-	Other Position	Absorbance nm	ε x 10^4 $l\,M^{-1}cm^{-1}$	Fluorescence nm	Solvent	pKa	Solubility H_2O	Solubility EtOH	Solubility Other Solvents	Other Information
45370:1	596-03-2	CI Solvent Red 72 (free acid)	COOH	4,5-Br2	as 4,5-Dibromofluorescein					0.03%	5%	8% EGME vs Ac. s in BuAc & EtAc	as above but acid form mp 270-273 °C
		5(6)-carboxy-4',5'-dimethyl-fluorescein	COOH	4'(5')-COOH 4,5-Me2	504	6.3	535	pH 9	7.0	s		s in alkali	soluble pH>5
		4'5'-dimethoxycarboxyfluorescein	COOH	4',5'-COMe	612	7.82	ND	water					
	76-54-0	2,7-dichlorofluorescein Fluorescein 27 Fluorescein 548	COOH	2,7-Cl2	515 535 512 502	7.41 0.64 11.0	ND ND 530 ND	ethanol acetone ethanol/base water	3.7 & 4.9				pH 4.0 (blue green fluorescence) pH 6.6 (green fluorescence
		Hydroxy-hydroquinone-phthalein 2,7-Dihydroxyfluorescein	COOH	2,7-OH2	ND		ND						Metallochrome indicator
		5(6)-carboxy-2',7'-dichloro-fluorescein	COOH	2,7-Cl2 4'(5')-COOH	504	9.0	529	pH 8	6.1	s		s in alkali	soluble pH>6
45371		CI Solvent Orange 16	COOH	2,7,-I2 4,5-Br2	ND		ND			i	ss	s in 5% Na_2CO_3	Yellow in H_2SO_4, on dil orange ppt Bright pink in 10% NaOH
45375		Phloxine N	COONa	4,5-Br2 3',6'-Cl2									Bromination 4,7-dichlorofluorescein with Br2, sodium chlorate & EtOH.
45380	17372-87-1 548-26-5	Eosin Y: CI Acid Red 87 Tetrabromofluorescein Eosin Yellowish (YS)	COONa	2,4,5,7-Br4	525 520 518	10.1 12.6 7.94	550 539 540	ethanol water pH 7.1	1.5	20% bluish red	2% bluish red	i in Xyl, E, D, vs in EG s in Py, Gl, & Chl	pH0 (not fl.); pH3 (bluish red with yellowish green fl.) Yellow in H_2SO_4, on dil orange ppt ϕ_f=0.76 alkaline, ϕ_f=0.19 water, ϕ_f=0.66 EtOH; τ_f=0.9ns EtOH, Fl=550nm, ϕ_p=0.001 water Pink with it green fl. in water 1% soln in H_2O previously adjusted to pH7 has a pH of 6.7
45380:2	16086-94-9	CI Solvent Red 43 (free acid) Eosin Acid	COOH	2,4,5,7-Br4	537 521		ND ND	water alkaline		0.06% in cold s hot	1%	s in Xyl, Py, EGME & EG vs in D & Ac ss in Chl	As Eosin Y (CI No.45380) Almost colourless in EtOH & xylene Acid-base indicator.
		Eosin-5-isothiocyanate	COONa	2,4,5,7-Br4 4'-NCS	524	10.1	548	methanol		pH>5	s	s in DMF	Isothiocyanates are unstable in water
45385	2321-49-3	Methyl Eosin CI Solvent Red 44	COOMe	2,4,5,7-Br4	525		ND	water		s in hot cherry red	1% rad	1% in EGME	Yellow in H_2SO_4 on dil. brownish yellow ppt. Darker red soln. with green fl. in NaOH. Cherry red in hot water. Red with browny yellow fl. in EtOH

A1: Fluoresceins (continued)

CI No	CAS #	Dye Name	Position 2'-	Other Position	Absorbance nm	ε x 10^4 $l\,M^{-1}cm^{-1}$	Fluorescence nm	Solvent	pKa	Solubility H_2O	Solubility EtOH	Solubility Other Solvents	Other Information
45386	6359-05-3	Ethyl Eosin CI Solvent Red 45	COOEt	2,4,5,7-Br4	520 532	12.6	545 ND	pH7 ethanol		0.03% in cold s in hot cherry red	1.13% red	ss in Xyl, D, & Chl. s in Ac, Gl, Py vs in EG	Yellow in H_2SO_4 on dil. brownish yellow ppt. Cherry red with faint greenish yellow fl. in hot water Red and browny yellow fl. in EtOH ϕ_f=0.19 water
45390		Chromoxane Brilliant Red RD	COOH	2,4,5,7-Br4 4'-CH, 6'-COOH									
45395		Orange for Lipstick	COOH	2,7-(NO2)2	ND		ND						Nitate Fluorescein with conc. H_2SO_4 & 98% nitric acid at 0°C
45396		CI Solvent Orange 18	COOH	4,6-(NO2)2	ND		ND						
45400	528-24-3	Eosin B Eosine I Bluish CI Acid Red 91	COONa	2,7-(NO2)2 4,5-Br2	530 515	4.6	557 ND	pH7 water		5%	.75%	I in Xyl, ss in D, Chl vs in Ac, EG & Py	pH 2.6 (not fluorescent) pH 4.0 ((light green fluorescence) Merck 11-3554: ϕ_f=0.01 water
	56360-46-4	Eosin B, spirit soluble	COOH	2,7-(NO2)2 4,5-Br2		as Eosin B				0.01%	0.4%	5% EGME	as above
45405		Phloxine New Pink Erythrosine B CI Acid Red 98	COONa	2,4,5,7-Br4	ND		ND			50.9% cherry red	9.02%		Brownish yellow in H_2SO_4 unaltered by heating, on dilution brownish yellow ppt. Bluish red in NaOH. Cherry red with greeny yellow fluorescence in water.
45410	18472-87-2	Phloxine B; CI Acid Red 92 Cyanine; Eosin 10B Eosin S extra bluish	COONa	2,4,5,7-Br4 3',4',5',6'-Cl4	535 548	7.7	556 ND	pH7 ethanol	3.7	10%	5%	I in E & Xyl vss in D & Chl ss in Ac s in Py, Gl vs in EG	pH 2.6 (no fl.); pH4 (bluish red with faint dark green fl. Yellow in H_2SO_4, on dil. yellowish red ppt; ϕ_f=0.16 water. 1% soln. in H_2O previously adjusted to pH 7.0 has a pH of 7.5
45410:1	2134-15-8	Phloxine B (free acid) CI Solvent Red 48	COOH	2,4,5,7-Br4 3',4',5',6'-Cl4		as Phloxine B							
45415		Cyanosine (Spirit Soluble)	COONa	2,4,5,7-Br2 3',6'-Cl2	ND		ND			I	s		Bluish red with a reddish yellow fl. in ethanol. Yellow in H_2SO_4, on dilution reddish brown ppt. Fluorescence disappears in HCl(aq)
45420		Cyanosine B	COOEt	2,4,5,7-Br2 3',4',5',6'-Cl4	ND		ND			ss	s		Bluish red with a reddish yellow fl. in ethanol. Yellowish brown in H_2SO_4, on dil. brownish red ppt.
45425		4,6-diiodofluorescein CI Acid Red 95 Erythrosine Extra Yellowish Erythosine Y	COONa	4,6-I2	510 522	6.6	ND ND	water methanol		vs cherry red	s	I in Xyl s in Ac, G ss in Pyridine vss in D & Chl	Brownish yellow in H_2SO_4, on dil. brownish yellow ppt. Soluble red ppt in NaOH Cherry red without fl. in water

A1: Fluoresceins (continued)

CI No	CAS #	Dye Name	Position 2'-	Other Position	Absorbance nm	ε x 10^4 $l.mol^{-1}.cm^{-1}$	Fluorescence nm	Solvent	pKa	Solubility H2O	Solubility EtOH	Solubility Other Solvents	Other Information
45425:1	31395-16-1	4,5-diiodofluorescein CI Solvent Red 73	COOH	4,5-I2	ND		ND			0.1%	3%	s in alkali & MeOH, 20% EGME	mp 240°C (dec) as above.
45430	16423-68-0 568-63-8	Erythrosine B CI Acid Red 51, CI Food Red 14	COONa	2,4,5,7-I4	530 525 528 531	8.4	550 550 554 ND	pH3 pH7 pH10 ethanol	1.8	11.1% cherry red	1.67%	i in Xyl vs in Py & EG s in Ac & Gl as in D vss in Chl	LD50 for rat (oral) 1900 mg/kg LD50 for mice (oral) 2891 mg/kg Soluble red ppt in NaOH Yellowish brown ppt in dil. HCl Brownish yellow in H_2SO_4, on dilution brownish yellow ppt. Cherry red without fl. in water 1% soln in H_2O previously adjusted to pH7 has a pH of 7.3 ϕ_f=0.015, ϕ_p=0.003, Ph=690nm, τ_p=270ms water. Merck 11-8242
45430:2	15905-32-5	CI Solvent Red 140 (free acid), tetraiodofluorescein		as Erythrosine B						0.07%	0.2%	s EGME, Gl & EG as in E	as above but acid form mp 303°C (dec)
		Erythrosine-5-isothiocyanate	COONa	2,4,5,7-I4 4'-NCS	535	10.1	558	methanol		pH>6		s in DMF	Phosphorescence @ 690nm τ_f = 0.11 ns; τ_{ph} = 0.35 ms
45435		Rose Bengal G Rose Bengal GTO CI Acid Red 93	COONa	2,4,5,7-I4 3',6'-Cl2	ND		ND			s cherry red	s		Brownish yellow in H_2SO_4, on dilution brownish red ppt Soluble crimson red ppt in NaOH Cherry red without fl. in water
45435:1		CI Solvent Red 47	COOH	2,4,5,7-I4 3',6'-Cl2	ND		ND						as Rose Bengal G
45440	632-69-9	Rose Bengal CI Acid Red 94	COONa	2,4,5,7-I4 3',4',5',6'-Cl4	562 550 554 559	7.76	605 590 591 598	water ethanol methanol DMSO		36.3% bluish red	7.5%	i in E, H, Xyl vss in D & Chl s in Gl & Ac vs in Py & EG	LD50 for mouse (oral) 6.5g/kg pH>6 to have max. fluorescence Brown in H_2SO_4, on dilution gives flesh pink ppt: Merck 11-8242 Bluish red without fl. in water. ϕ_f=0.05 (EtOH)
45440:1		Rose Bengal (free acid) CI Solvent Red 141	COOH	2,4,5,7-I4 3',4',5',6'-Cl4	as Rose Bengal								as Rose Bengal
45445	2103-64-2	4,5-dihydrofluorescein Calcein, Calcocyanine Pyrogallophthalein CI Mordant Violet 25	COOH	4,5-OH2	526		ND	water	5.2 & 11.8	i as in hot scarlet red	s reddish brown	i in B, Xyl & Chl s in D as in E s in Ac & alkali	pH 3.8 brownish yellow pH 6.6 rose red; pH 13 violet Loses water of crystallisation @180 °C & blackens above this temp but does not melt even above 300°C Reddish yellow in H_2SO_4, on dil. flocculent reddish yellow ppt. Blue in NaOH. Soluble above pH8. Merck 11-4250. Metallochrome Indicator

A1: Fluoresceins (continued)

CI No	CAS #	Dye Name	Position 2'-	Other Position	Absorbance nm	ε x 10⁴ l·mol⁻¹·cm⁻¹	Fluorescence nm	Solvent	pKa	Solubility H2O	Solubility EtOH	Solubility Other Solvents	Other Information
45450		Chromoxane Red B	Cl	4,5-Me 2,7-(COOH)2	ND		ND						
45455		Chromoxane Red Violet 1358	Cl	4,5-Me, 6'-Cl 2,7-(COOH)2	ND		ND			I		s in 5% NaOH	
45456		CI Solvent Orange 17	COOH	2,7-COOH 4,5-Br2, 6'-Br	ND		ND			I		s in 5% Na2CO3	
45457		CI Solvent Orange 46	COOH	1,4,5,6-Br4 2,7-COOH, 6'-Br	ND		ND			I		ss in 5% Na2CO3	
		Nitrofluorescein	COOH	NO2	456	0.29	ND	ethanol					
	72088-94-9	5(6)-Carboxyfluorescein	COOH	4'(5')-COOH	490	7.2	515	pH 8	6.4			s in 0.1N NaOH	φf=0.87 water
		2'-Methoxycarboxyfluorescein	CoMe	4'(5')-COOH	500		534	water					φf=0.78 water
	6262-21-1	3',4',5',6'-Tetrachloro-fluorescein	COOH	3',4',5',6'-Cl4	ND		ND			3%	1%	3% in BCME s in alkali	Intermediate for Phloxine B
		4',5'-Dimethoxycarboxy-fluorescein	COOH	4',5'-COOH	ND		ND						φf=0.0004 water
	976-17-7	Fluorane Black 9-Phenyl-2,3,7-trihydroxy-6-fluorone.	-	2,7-OH2	ND		ND			0.2%	0.3%	0.4% in BCME s in EtAc, Ac alkali.	Soluble in hot water or hot EtOH mp >300°C
	129-16-8	Mercurochrome 220 Merbromin	COONa	2,7-Br2 4-HgOH	508		ND	water		2% carmine red	1%	2% in MeOH vss in Ac, Chl & Py I in D & Xyl vs in BG	pH of a 0.5% soln. is 8.8 Very dilute solution (1:2000) has yellow green fluorescence Merck 11-5757
		Sulfonefluorescein	SO3H	-	497	7.6	517	pH9.5	6.23				φf=0.92 pH9.5
		Merafein sodium Merodicein	SO3Na	2,7-I2 4-HgOH	ND		ND			s			Aqueous soln. slightly fluorescent
		Pyrogallol Red Pyrrolesulphonaphthalein	SO3H	4,5-OH2	ND		ND		3, 6.5, 9.8, 12	0.1%	0.4%	0.3% in BCME	Metallochrome indicator
		Bromopyrogallol Red	SO3H	4,5-OH2 2,7-Br2	ND		ND		0.16, 4.4 9.13 & 11.27				Metallochrome indicator

A2: Napthofluoresceins

CI No	CAS #	Dye Name	Position 2'-	Other Position	Absorbance nm	ε x 10^4 $M^{-1}cm^{-1}$	Fluorescence nm	Solvent	pKa	Solubility H2O	Solubility EtOH	Solubility Other Solvents	Other Information
45460		Napthofluorescein	COOH		594	4.6	663	pH10	7.6	s pH>6		s in alkali	ϕ_f=0.14 pH9.5
		5(6)-carboxynaphthofluorescein	COOH	4'-COOH	598	4.9	660	pH10	7.6	s pH>6		s in alkali	
		3,10-Dibromonapthofluorescein	COOH	3,10-Br_2	606	3.2	682	pH9.5	7.45	s pH>6			ϕ_f=0.05 pH9.5
		Vita Blue	SO_3H		609 624	3.6 1.3	685 670	pH9.5 pH5.2	7.55	s pH>5			ϕ_f=0.15 pH9.5; ϕ_f=0.05 pH5.4

A3: Benzo[c]fluoresceins

CI No	CAS #	Dye Name	Position 2'-	Other Position	Absorbance nm	ε x 10^4 $M^{-1}cm^{-1}$	Fluorescence nm	Solvent	pKa	Solubility H2O	Solubility EtOH	Solubility Other Solvents	Other Information
		Carboxy SNAFL-1	COOH	4'(5')-COOH	479,508 537	2.9 5.2	543 623	pH 1-7 pH 10	7.8	s pH>6		s in DMSO	
		Carboxy SNAFL-2	COOH	2-Cl 4'(5')-COOH	525 547	2.6 4.8	546 630	pH 1-7 pH 10	7.6	s pH>6			
		SNAFL calcein, ammonium salt	$COO^-NH_4^+$	4,8-[Me-N-(acetate)2]2	506 538	2.7 4.95	535 620	pH 1-7 pH10	7.2	s pH>6		s in DMSO	Metal complexing agent

A4: Rhodamine

CI No	CAS #	Dye Name	X	Y	Other Position	Absorbance nm	ε x 10^4 $l\,mol^{-1}cm^{-1}$	Fluorescence nm	Solvent	pKa	Solubility H_2O	Solubility EtOH	Solubility Other Solvents	Other Information
45090		Benzorhodamine, Rosamine, Rosindamine, Tetramethylrosamine.	NMe_2	NMe_2		552	9.9	582	methanol		s	s	s in DMSO & NaOH	Bluish red with bright yellowish red fluorescence in water and ethanol. Orange yellow in H_2SO_4, on dil. red Bluer and less fluorescent in NaOH.
45095		Sulphorein	NEt_2	NEt_2	2'-SO_3H	ND		ND			s	s		Bluish red with yellowish red fluorescence in water and ethanol. Yellowish red in H_2SO_4, on dilution bluish red.
45100	2609-88-3 3520-42-1	Sulforhodamine B, Kiton Red 620, Xylene Red B, CI Acid Red 52, Acid Rhodamine B, Phloxine Rhodamine, Kiton Rhodamine B	NEt_2	NEt_2	2'-SO_3H 4'-SO_3Na	566 564	11.1	575 575	ethanol water		2%	0.5%	l in Xyl vss in D ss in Chl s in Gl & Ac. vs in EG	ϕf = 0.68 (ethanol) Bluish red with yellow fluorescence in water and ethanol. Orange yellow in H_2SO_4, on dil. red Bluish red in NaOH.
		Sulforhodamine B sulphonyl chloride, Lissamine Rhodamine B200 Lissamine Rhodamine B200,	NEt_2	NEt_2	2'-SO_3H 4'-SO_2Cl	567 560	8.0 8.0	584 580	methanol water			s	s in DMF & MeCN	ϕf = 0.04, τf = 1ns (water)
	62796-29-6	Sulforhodamine B sulphonyl fluoride	NEt_2	NEt_2	2'-SO_3H 4'-SO_2F	570	9.6	589	methanol					
45105		Rhodamine 5G	NHEt	NHEt	2'-Cl 2,7-Me_2	ND		ND			s	s		Golden yellow in H_2SO_4, on dilution orange red to pink ppt. Magenta soln. with yellow green fluorescence & ppt in HClaq. Red with yellow Fl. in water & EtOH
45150		CI Basic Red 8	NHEt	NHEt	2'-COOH	530		560	ethanol		s	s		Pale yellow in H_2SO_4, on dilution red Fl. soln. Yellow in HClaq. Reddish violet with red fluorescence in both water and ethanol.
45160	989-38-8 13161-28-1 54854-14-7	Rhodamine 6G, CI Basic Red 1 Rhodamine 590	NHEt	NHEt	2'-COOEt 2,7-Me_2	530 528 540 524	9.7 10.4 19.7	556 553 ND 550	ethanol methanol DMSO pH7		2% scarlet red	5%	vss in Xyl s in Gl, Chl, & Py. ss in Ac,	Yellow in H_2SO_4, on dilution red. Red ppt in NaOH. ϕf=0.95 (ethanol) Crimson red in water and scarlet red with green fluorescence in ethanol. 1% soln. in deionised H_2O previously adjusted to pH7 has a pH of 3.8
	62669-66-3	Rhodamine 19	NHEt	NHEt	2'-COOH 2,7-Me_2	525 518 540	12.0 9.49	543 ND ND	ethanol/HCl ethanol/NaOH DMSO					ϕf= independent of pH Similar to Rhodamine 6G
45165		Rhodine 20	NHEt	NHEt	2'-COOEt	530		550	ethanol					Similar properties to Rhodamine 6G

A4: Rhodamines (continued)

CI No	CAS #	Dye Name	X	Y	Other Position	Absorbance nm	ε x 10^4 $(M^{-1}cm^{-1})$	Fluorescence nm	Solvent	pKa	Solubility H2O	Solubility EtOH	Solubility Other Solvents	Other Information
45166		Rhodamine 3B, Rhodamine 4G	NEt_2	NH_2	2'-COOEt	541	20.0	ND	ethanol		s	s		Greenish yellow in H_2SO_4 on dil. red & browny red Fl. Yellow in dil. HCl.
						555	11.7	ND	ethanol/HCl		red	scarlet		
						566		ND	DMSO					
45170	81-88-9	Rhodamine B, Rhodamine 610, CI Basic Violet 10, Pilot 578, Rhodamine O, Rosazaine B, Brilliant Pink B.	NEt_2	NEt_2	2'-COOH	500	1.26	ND	HCl		0.78%	1.47%	ss in Xyl, Chl, HCl, NaOH & D	LD50 (iv. for rats) 89.5mg/kg
						545	10.2	565	methanol				s in Ac, Gl & EG	mp = 165°C; Merck 11-8181
						552	1.00	575	water					Yellowish brown with strong green fluorescence in H_2SO_4, on dilution scarlet then bluish red and orange. Rose red ppt in NaOH on heating. Bluish red with strong green fluorescence in water & ethanol. ϕ_f=0.4 (EtOH/HCl) & 0.6 (basic EtOH)
						554	12.6	580	ethanol/HCl					
						552	10.7	568	ethanol					
						555	23.3	581	pH7					
						543	ND	ND	basic EtOH					
	36877-69-7	Rhodamine B isothiocyanate (RBITC)	NEt_2	NEt_2	2'-COOH	545		565	methanol					ϕ_f = 0.70, τ_f = 3ns (water)
					4'(5')-NCS	550	10.3	565	water					
	37299-86-8	5(6)-Carboxyrhodamine B Rhodamine WT	NEt_2	NEt_2	2'-COOH	500	5.7	556	methanol					ϕ_f = 0.28 (water)
					4'(5')-COOH	554		572	water					
45175		Rhodamine 3B CI Basic Violet 11	NEt_2	NEt_2	2'-COOEt	ND		ND			s	s		Violet red with browny red Fl in H_2O Red & vermilion fluorescence in EtOH Greeny yellow in H_2SO_4, on dil. red.
45210		Rhodamine 3G, CI Basic Red 3	NH_2	NMe_2	2-Me 2'-COOEt	535		550	water		s	s		Crimson red with brown fluorescence in water. Scarlet red with green Fl. in ethanol. Scarlet red ppt in NaOH. Yellow in H_2SO_4, on dil. red.
45215		Rhodamine 3GO CI Basic Red 4	NH_2	NEt_2	2-Me 2'-COOEt	ND		ND			s	s		Similar to Rhodamine 3G (above)
45220		Sulfohodamine G, CI Acid Red 50	NEt_2	NEt_2	2,7-Me_2 2',4'-SO_3^-	532		558	water		2%	0.2%	0.4% EGME	Bluish red with yellow Fl. in water ethanol. Orange yellow in H_2SO_4, on dil. red. Bluish red in NaOH. Similar to Sulphorhodamine B.
		Tetramethylrhodamine	NMe_2	NMe_2	2'-COOH	548	10.0	ND	ethanol			s	s in DMSO, MeOH	
		Tetramethylrhodamine-6(6)-isothiocyanate (TRITC)	NMe_2	NMe_2	2'-COOH	548		ND	ethanol					τ_f = 2ns (water)
					4'(5')-NCS	541	8.2	572	methanol					
						550	5.0	565	water					
		TRITC (isomer G)	NMe_2	NMe_2	2'-COOH	537	10.7	556	methanol					
					4'-NCS	540	10.7	575	water					
		TRITC (isomer R)	NMe_2	NMe_2	2'-COOH	538	10.3	555	ethanol					
					5'-NCS	540	10.3	575	water					

A4: Rhodamines (continued)

CI No	CAS #	Dye Name	X	Y	Other Position	Absorbance nm	ε x 10^4 $l\,mol^{-1}cm^{-1}$	Fluorescence nm	Solvent	pKa	Solubility H_2O	Solubility EtOH	Solubility Other Solvents	Other Information
		Tetramethylrhodamine methyl ester	NMe_2	NMe_2	2'-COOMe	548	10.1	573	methanol			s	s in DMSO, MeOH	
		Tetramethylrhodamine ethyl ester	NMe_2	NMe_2	2'-COOEt	549	10.0	574	methanol			s	s in DMSO, MeOH	
		5(6)-Carboxytetramethylrhodamine	NMe_2	NMe_2	2'-COOH 4'(5')-COOH	544		571	methanol					
	13558-31-1	Rhodamine 110, Rhodamine 560	NH_2	NH_2	2'-COOH	510 501 510 506 496 518	8.71 8.99 8.9 	ND 526 535 ND 520 ND	ethanol/HCl ethanol/NaOH ethanol chloroform methanol DMSO					ϕ_f=0.85 (methanol) ϕ_f= independent of pH
	62669-77-6	Rhodamine 116	$NHMe$	$NHMe$	2'-COOH	525	8.71	ND	ethanol					
	62669-70-9	Rhodamine 123	NH_2	NH_2	2'-COOH	505	9.8	534	methanol			s	s in E & DMF	
		Chromoxane Brilliant Red BL	NEt_2	NEt_2	4'-OH 2',5'-COOH	549		ND	water	s	s s	s s	l in Xyl ss in D & Ac vss in Py s in Chl & EG	ϕ_f = 0.28 (water) Not pH sensitive.
		Rhodamine S	NMe_2	NMe_2	2'-EtCOOH	544		569	methanol					

A5: Rhodamine 101 derivatives

CI No	CAS #	Dye Name	Position	Absorbance nm	ε x 10^4 $l·mol^{-1}·cm^{-1}$	Fluorescence nm	Solvent	pKa	Solubility H_2O	Solubility EtOH	Solubility Other Solvents	Other Information
	64339-18-0	Rhodamine 101	2'-COOH	664	9.5	ND	ethanol			s	s in DMF & DMSO	ϕ_f=1.00 (glycerol)
		Rhodamine 640		575		594	methanol/HCl					ϕ_f= independent of pH
				566	10.6	583	methanol					
				577		ND	ethanol/HCl					
				586		ND	DMSO					
		Rhodamine 101 methyl ester	2'-COOMe	576		600	methanol				s in DMF & DMSO	
		5(6)-Carboxy-X-rhodamine	2'-COONa	575	6.6	604	pH 8		pH>6		s in DMF	
			4'(5')-COOH	568	8.6	595	methanol					
		Rhodamine X isothiocyanate	2'-COONa	578	8.0	604	pH 8		pH>6		s in DMF &	-NCS are unstable in water.
		(XRITC)	4'(5')-NCS	575	8.4	603	water				DMSO	
	14-73-6	Sulphorhodamine 101	2',4'-SO3H	557	10.6	602	ethanol		2%	3%	s in DMF	ϕ_f = 0.90 (ethanol)
	123333-78-6	Sulforhodamine 640		575	13.9	605	methanol				0.8% in EGME	
				595		615	water					
	82354-19-6	Texas Red	2'-SO3H	578	8.3	ND	ethanol		ss	s	s in DMF & MeOH	ϕ_f = 0.50 (water)
		Sulfohodamine 101 sulphonyl chloride	4'-SO2Cl	596	8.6	615	pH 8					
				595	8.5	617	water					

A6: Rhodamine 800 derivatives

CI No	CAS #	Dye Name	X	Absorbance nm	ε x 10^4 $l·mol^{-1}·cm^{-1}$	Fluorescence nm	Solvent	pKa	Solubility H_2O	Solubility EtOH	Solubility Other Solvents	Other Information
		Rhodamine 700 perchlorate	CF3	643	9.25	666	ethanol		ss	s	i in E & Xyl	
		LD700									s in DMSO & DMF	
		Rhodamine 800 perchlorate	OH	685	8.95	700	ethanol	~11	ss	s	i in E & Xyl	pH >11 no fluorescence @ 707 nm
		LD 800		676		704	methanol				s in DMSO	ϕ_f=0.90 (water): Dimer Abs. @ 605nm
				693		717	ethylene glycol					Mauve (526) in H_2SO_4, on dil. orange
				669		690	acetone					then blue. Orange (462/495/526) in
				684		703	50% methanol					5M HCl. Pale yellow (nm)in NaOH
				695	8.95	700	water					Turquoise (605/668) in water

A7: Pyronines

CI No	CAS #	Dye Name	X	Y	Other Position	Absorbance nm	ε x 10^4 $l·g^{-1}·cm^{-1}$	Fluorescence nm	Solvent	pKa	Solubility H2O	Solubility EtOH	Solubility Other Solvents	Other Information
45000		Acridine Red 3B	NHMe	NHMe		525		535	ethanol		s red	s red		Yellow with green Fl. in H_2SO_4, on dil. orange then red. Red ppt in NaOH. Red with greeny yellow Fl. in water and ethanol
45005	92-32-0	Pyronine Y, Pyronine G	Me2	NMe2		548	6.7	557	pH7		SX red	0.6% red	I in E & Xyl ss in Chl s in Ac & Py. vs in Gl	Red with yellow Fl. in water. Bright orange in dil. HCl. Reddish yellow in H_2SO_4, on dilution red. Merck 11-8017: ϕ_f = 0.30 water
45010	2150-48-3	Pyronine B	NEt2	NEt2		555 550 552	11.2	562 560 562	50% ethanol water ethanol		vs red	s red	ss in Xyl & Py s in Ac, Chl & D vs in Gl	Merck 11-8016. mp 176-178 °C Reddish yellow in H_2SO_4, on dil. red. Bright orange in dilute HCl
45015		Rhodamine Scarlet G	NHMe	NHMe	2,7-Me2	528		540	ethanol		s orange red	s orange red		Light lemon yellow in H_2SO_4, on dil. orange. Decolorised to faint pink in NaOH. Orange red with strong yellow green Fl. in water & ethanol
		Pyronine 20	NHEt	NHEt	2,7-Me2	527		540	ethanol		s red	ss red		Red with intense yellow Fl. in water & ethanol. Blue violet in conc. HCl. Brownish yellow (strong green Fl.) in H_2SO_4, on dilution rose red. Slowly decolorised in aqueous NaOH.

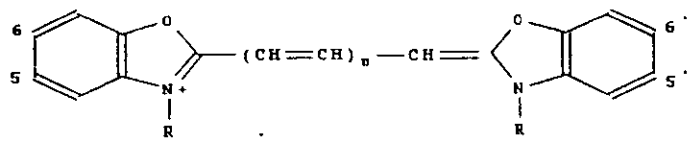
A8: Other Xanthenes

CI No	CAS #	Dye Name	Pos. 3-	Other Pos.	R	Absorb-ance nm	ε x 10^4 ($M^{-1}cm^{-1}$	Fluor-escence nm	Solvent	pKa	Solubility H2O	Solubility EtOH	Solubility Other Solvents	Other Information
45300		CI Mordant Red 77	NMe2	6-O 7-SO3H	COOEt	ND		ND			s	s		Cherry red with a strong yellow Fl. in water. Lemon yellow with green Fl. in H_2SO_4, on dil. orange red then pink. No Fl. in HClaq
45310		Rhodamine 12GM Rhodine 12GM	NMe2	6-COOH	COOEt	ND		ND			s	s		Yellowish red in water & ethanol Yellow in H_2SO_4, on dil. yellowy red Light red ppt in NaOH.
45315		Rhodamine 12GF Rhodamine 12G extra	NMe2	6-COOH	COOMe	ND		ND			s	s		Yellowish red in water & ethanol Yellow in H_2SO_4, on dil. yellowy red Light red ppt in NaOH.
	25319-73-7	Thiofluorescein	SH	6-SH	COOH									
	4091-99-0	2,7-dichlorofluorescein diacetate	COOMe	6-COOMe 2,7-Cl2	COOH			none					s in Ac	mp 210 °C
	630-88-6	3,6-dichlorofluoran	Cl	6-Cl	COOH									
	596-09-8	diacetylfluorescein fluorescein diacetate	COOMe	COOMe	COOH			none						mp 207 °C
		3-diethylamino-6-methyl-7-chloro-fluoran	NEt2	6-Me 7-Cl	COOH	497	3.02	ND	acetic acid					Pressure- and thermo-sensitive dyes
		3-diethylamino-6-methylamino-fluoran	NEt2	7-NHMe	COOH	594	1.32	ND	acetic acid					Pressure- and thermo-sensitive dyes
		3-diethylamino-6-phenylamino-fluoran	NEt2	7-NHPh	COOH	601	1.66	ND	acetic acid					Pressure- and thermo-sensitive dyes
		3-diethylamino-7-(N-methyl-N-phenyl)aminofluoran	NEt2	7-NMePh	COOH	607	1.55	ND	acetic acid					Pressure- and thermo-sensitive dyes
		3-diethylamino-6-methyl-7-phenyl) aminofluoran	NEt2	6-Me 7-NHPh	COOH	592	1.55	ND	acetic acid					Pressure- and thermo-sensitive dyes
		3-diethylamino-7-benzylamino-fluoran	NEt2	7-Bz	COOH	592	1.66	ND	acetic acid					Pressure- and thermo-sensitive dyes
		Rhodol	NMe2	6-O	COOH	522	6.7	545	water	6.6				ϕ_f=0.20 pH9.6

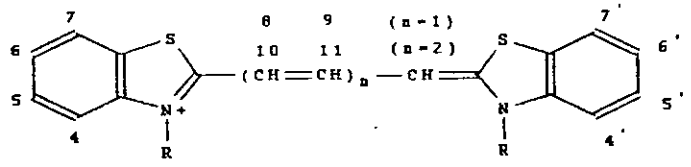
Appendix B

POLYMETHINES

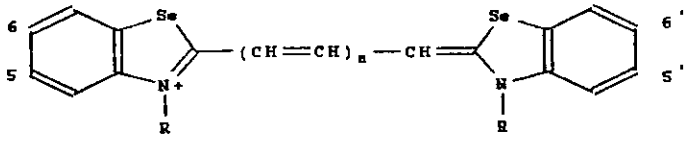
B1: Oxacarbocyanine



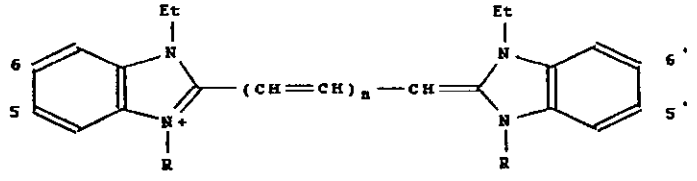
B2: Thiacarbocyanine



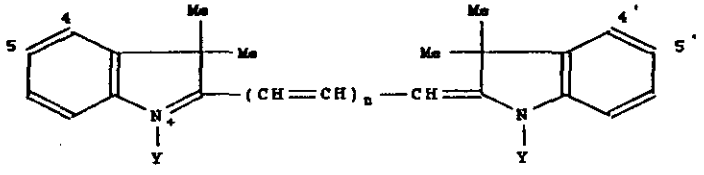
B3: Selenocarbocyanine



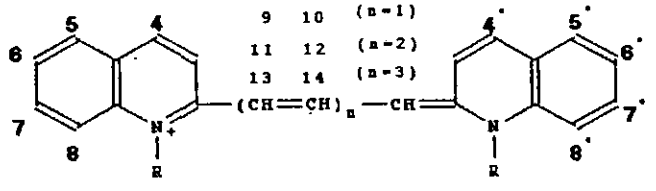
B4: Benzaimidazolocarbocyanine



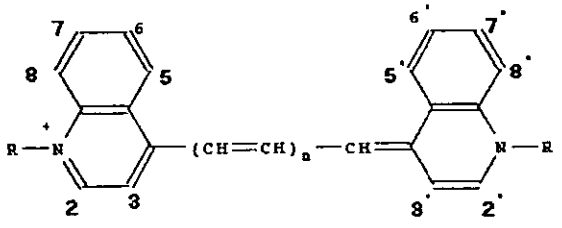
B5: Indocarbocyanine



B6: 2..2'-Quinocarbocyanine



B7: 4,4'-Quinocarbocyanine



B1: Oxacyanine

CAS #	Abbrev-iation	Dye Name	n	Pos. 3,3'	Other Pos.	Absorb-ance nm	ε x 10^4 $M^{-1}cm^{-1}$	Fluor-escence nm	Solvent	Solubility H_2O	Solubility EtOH	Solubility Other Solvent	Other Information
		3,3'-diethyloxacyanine iodide	0	Et		377		none	EG				not fluorescent
						376		none	ethanol				
						372		none	methanol				
		3,3'-diethyl-5,5'-diphenyloxacyanine iodide	0	Et	5,5'-Ph2	387		none	methanol				not fluorescent
		3,3'-dimethyloxacarbocyanine iodide	1	Me		487		507	ethanol				mp 284°C (dec)
905-96-4	DOC DIOC2(3)	3,3'-diethyloxacarbocyanine iodide	1	Et		479		498	ethanol			s in DMSO	mp 270-271°C (dec)
						482	14.7	512	methanol				ϕ_f=0.04, τ_f<0.35ns, ϕ_p=0.01 (MeOH)
						485		515	water				ϕ_f=0.58 (DMF); τ_f=2.2ns (glycerol)
						487		507	EG				ϕ_f=0.75, τ_f=2.9ns (gelatin); Ph=620nm θ-196°C (EtOH) Ph=614nm θ-196°C (EPA)
53213-79-9		3,3'-dipropyloxacarbocyanine iodide	1	Pro		480		506	ethanol			s in DMSO	mp 283°C (dec) Potential sensitive.
	DIOC5(3)	3,3'-dipentyloxacarbocyanine iodide	1	Pent		484	14.7	509	methanol			s in DMSO	Potential sensitive.
	DIOC6(3)	3,3'-dihexyloxacarbocyanine iodide	1	Hex		484	15.4	509	methanol			s in DMSO	Potential sensitive, organelle probe. mp 219-221°C (dec)
	DIOC7(3)	3,3'-diheptyloxacarbocyanine iodide	1	Hept		484	15.4	509	methanol			s in DMSO	Cationic membrane probe
	DIOC16(3)	3,3'-dihexadecyloxacarbocyanine perchlorate	1	HexDec		486	12.7	510	methanol		s	s in DMF	Cationic membrane probe
60711-74-4	DIOC18(3)	3,3'-dioctadecyloxacarbocyanine perchlorate	1	OctDec		484	13.0	507	methanol		s	s in DMF	Cationic membrane probe Probe for lipid bilayer membranes
		3,3'-diethyl-5,5'-diphenyloxacarbocyanine iodide	1	Et	5,5'-Ph2	494		511	methanol				ϕ_f=0.11, τ_f=0.45ns, ϕ_p=0.01 (MeOH)
		"Rigidised" 3,3'-diethyl-5,5'-diphenyl-oxacarbocyanine iodide	1	Et	5,5'-Ph2	507		520	methanol				ϕ_f=0.63, τ_f=3ns, ϕ_p=0.03 (MeOH)
						536		545	gelatin				τ_f=2.3ns (gelatin)
		3,3',9-trimethyl-5,5'-diphenyloxacarbo-cyanine iodide	1	Me	9-Me 5,5'-Ph2	494		511	methanol				ϕ_f=0.11, ϕ_p=0.01 (MeOH)
		"Rigidised" 3,3',9-triethyl-5,5'-diphenyl-oxacarbocyanine iodide	1	Et	9-Et 5,5'-Ph2	511		523	methanol				ϕ_f=0.77, ϕ_p=0.03 (MeOH)
		3,3'-diethyl-4,5,4',5'-dibenzoxacarbo-cyanine iodide	1	Et	4,5,4',5'-dibenzo	513		ND	methanol				S_{max}=555nm

B1: Oxacyanine continued

CAS #	Abbrev-iation	Dye Name	n	Pos. 3,3'	Other Pos.	Absorb-ance nm	ε x 10^4 $l\,M^{-1}cm^{-1}$	Fluor-escence nm	Solvent	Solubility H_2O	Solubility EtOH	Solubility Other Solvent	Other Information
		3,3'-diethyl-6,6,4',5'-dibenzoxacarbocyanine iodide	1	Et	6,6,5',6'-dibenzo	508		ND	methanol				S_{max}=550nm
		3,3'-diethyl-6,7,6',7'-dibenzoxacarbocyanine iodide	1	Et	6,7,6',7'-dibenzo	515		ND	methanol				
14806-50-9	DODC DIOC2(5)	3,3'-diethyloxadicarbocyanine iodide	2	Et		582	24.0	610	ethanol			ε in DMSO	mp 232°C (dec): S_{max}=620nm
						579	22.4	603	methanol				ϕ_f=0.44, τ_f=1.2ns, ϕ_p<0.01 (EtOH)
						585		620	water				
						586		614	EG				
		3,3'-diethyl-5,5'-diphenyloxadicarbocyanine iodide	2	Et	5,5'-Ph2	593		ND	methanol				
		3,3'-diethyl-4,5,4'5'-dibenzoxadicarbocyanine iodide	2	Et	4,5,4'5'-dibenzo	606		ND	methanol				
		3,3'-diethyl-4,5,4'5'-dibenzoxadicarbocyanine iodide	2	Et	6,7,6',7'-dibenzo	606		ND	methanol				
19764-95-5	DMOTCI	3,3'-dimethyloxatricarbocyanine iodide Methyl-DOTCI	3	Me		682	19.8	718	ethanol				mp 175°C (dec): S_{max}=735nm
						682		ND	methanol				
15185-49-0	DOTC DOTCI DEOTC	3,3'-diethyloxatricarbocyanine iodide	3	Et		687	25.1	725	ethanol				mp 185-186°C (dec): ϕ_f = 0.93 EG
						678		703	methanol				ϕ_f = 0.49 EtOH:
						695	18.93	719	DMSO				Fluorescent (708nm) pH 3-10
						660		706	60% methanol				Not fluorescent pH <3 and pH >10
						692		712	acetone				L_{max}=771nm (EG)
						705		720	DOX				
						690		715	acetonitrile				
						689		720	EG				
						697		714	propan-1-ol				
						684		695	glycerol				
						660		740	water				
		3,3'-diethyl-5,5',6,6'-tetramethyloxatricarbocyanine iodide	3	Et	5,5',6,6'-Me4	703		731	EG				ϕ_f=0.49 (EG): L_{max}=787nm (EG)

B2: Selenacyanine

CAS #	Abbreviation	Dye Name	n	Pos. 3,3'	Other Pos.	Absorbance nm	ε x 10⁴ M⁻¹cm⁻¹	Fluorescence nm	Solvent	Solubility H2O	Solubility EtOH	Solubility Other Solvent	Other Information
		3,3'-diethylselenacyanine iodide	0	Et		429		none	ethanol				
1049-38-3		3,3'-diethylselenocarbocyanine iodide	1	Et		573		ND	ethanol				mp=270-271°C (dec)
						570		ND	methanol				ϕ_f=0.40 acetone
		3,3'-diethyl-9-methyl-selenocarbocyanine iodide	1	Et	9-Me	565		ND	methanol				mp=262°C (dec)
		3,3'-diethylselenodicarbocyanine iodide	2	Et		660		ND	methanol				mp=235°C (dec)
		3,3'-diethylselenotricarbocyanine iodide	3	Et		778		816	EG				mp=225°C (dec); S_{max}=825nm
						770		ND	methanol				ϕ_f=0.40 (EG); L_{max}=725nm (EG)
						770		ND	ethanol				

B3: Benzimidazolocyanine

CAS #	Abbreviation	Dye Name	n	Pos. 3,3'	Other Pos.	Absorbance nm	ε x 10⁴ M⁻¹cm⁻¹	Fluorescence nm	Solvent	Solubility H2O	Solubility EtOH	Solubility Other Solvent	Other Information
		1,1',3,3'-tetraethylbenzimidazolocyanine iodide	0	Et		396		ND	methanol				
						386		ND	acetic acid				
		1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide	1	Et		496		ND	methanol				
						500		ND	acetic acid				
		1,1',3,3'-tetraethyl-5,5',6,6'-tetrachlorobenzimidazolocarbocyanine iodide	1	Et	5,5',6,6'-Cl4	515		ND	methanol		s		mp 297°C (dec)
		1,1',3,3'-dimethylbenzimidazolodicarbocyanine iodide	2	Me		638			acetonitrile				
						683			CH2Cl2				
		1,1',3,3'-dimethylbenzimidazolotricarbocyanine iodide	3	Me		740		ND	methanol		s		
						685		ND	CH2Cl2				
		1,1',3,3'-diethylbenzimidazolotricarbocyanine iodide	3	Et		741		ND	methanol		s	s in MeOH vs in CH2Cl2	I⁻ 0.5% in MeOH; >10% in CH2Cl2 ClO4⁻ 5%I⁻ in MeOH; 10% in CH2Cl2

B4: Thiacyanines

CAS #	Abbreviation	Dye Name	n	Pos. 3,3'	Other Pos.	Absorbance nm	ε x 10^4 $l·mol^{-1}·cm^{-1}$	Fluorescence nm	Solvent	Solubility H2O	Solubility EtOH	Solubility Other Solvent	Other Information
2197-01-6		3,3'-diethylthiacyanine iodide	0	Et		423	8.5	none	methanol				mp 311°C (dec)
						428		none	acetone				
						425		none	ethanol				
						428		none	EG				
		3,3'-dicarboxyethylthiacyanine bromide	0	EtCOOH		424		none	methanol				mp 210-211°C (dec)
	DMTCI	3,3'-dimethylthiadicarbocyanine iodide	1	Me		556	13.6	580	ethanol			ε in DMSO	Potential sensitive
	$DISC_1(3)$					555	10.3	579	methanol				
905-97-5	DTCI	3,3'-diethylthiacarbocyanine iodide	1	Et		557	14.6	571	ethanol				mp 269°C (dec)
	$DISC_2(3)$					558	13.5	580	methanol				Potential sensitive
						551		586	acetone				
						556		ND	nitromethane				
						560		590	water				
						561		586	EG				
						ND		586	glycerol				
905-97-5	DTCI	"Rigidised" 3,3'-diethylthiacarbocyanine iodide	1	Et		571		581	methanol				
						565		595	gelatin				
53336-12-2	DIOC7(3)	3,3'-dipropylthiacarbocyanine iodide	1	Pro		557	14.6	ND	ethanol				mp 288°C (dec)
	DIOC7(3)	3,3'-diheptylthiacarbocyanine iodide	1	Hept		550		572	ethanol			ε in DMF & DMSO	Organelle probe
	$DISC_{18}(3)$	3,3'-dioctadecylthiacarbocyanine perchlorate	1	OctDec		546	12.7	575	methanol			ε in DMF MeOH	Cationic membrane probe
		3,3'-dicarboxymethylthiacarbocyanine inner salt	1	MeCOOH		559		ND	methanol/ triethylamine				mp 182°C (dec)
		3,3'-di(3-trimethylammoniumpropyl)thiacarbocyanine tribromide	1	ProMe_3N^+		556	13.2	577	methanol	ε			Polar tracer
		3,3'-diethyl-5-cyano-thiacarbocyanine iodide	1	Et	5-CN	523		ND	nitromethane				
		3,3'-diethyl-5-fluoro-thiacarbocyanine iodide	1	Et	5-F	557		ND	nitromethane				
		3,3'-diethyl-9-cyanothiacarbocyanine iodide	1	Et	9-CN	610		ND	nitromethane				
		3,3'-diethyl-9-methylthiacarbocyanine iodide	1	Et	9-Me	610,649		ND	methanol				mp 276°C (dec)
						643		ND	nitromethane				
3055-79-0	DMETC	3,3'-dimethyl-9-ethylthiadicarbocyanine iodide	1	Me	9-Et	540	10.6	ND	ethanol				mp 290°C (dec)
						543		557	methanol				

B4: Thiacyanines (continued)

CAS #	Abbrev-iation	Dye Name	n	Pos. 3,3'	Other Pos.	Absorb-ance nm	ε x 10^4 $l·M^{-1}cm^{-1}$	Fluor-escence nm	Solvent	Solubility H2O	EtOH	Other Solvent	Other Information
		3,3',9-triethylthiacarbocyanine iodide	1	Et	9-Et	547		ND	methanol				mp 235-236°C (dec)
		3,3'-diethyl-9-fluoro-thiacarbocyanine iodide	1	Et	9-F	523		ND	nitromethane				
		3,3'-diethyl-9-phenylthiacarbocyanine bromide	1	Et	9-Ph	552 560		ND ND	methanol nitromethane				mp 267°C (dec)
		3,3'-dicarboxyethyl-6,5',9-trimethyl-thiacarbocyanine, inner salt	1	EtCOOH	5,6',9-Me3	548		ND	methanol/ triethylamine				mp 232°C (dec)
		3,3'-dihydroxyethyl-6,5'-dimethylthia-dicarbocyanine bromide	1	EtOH	9-Et 6,5'-Me	579		ND	methanol				
3028-94-2	DBTC	3,3'-dimethyl-9-ethyl-4,4',6,5'-dibenzo-thiacarbocyanine bromide Stains all (bromide)	1	Me	9-Et 4,5,4',5'-dibenzo	576		ND	methanol	0.1%	0.3%	0.5% EGME	pH 2.4 (colourless) pH 3.4 (purple)
	DDTCI	3,3'-diethyl-4,4',6,5'-dibenzothia-carbocyanine iodide	1	Et	4,6,4',6'-dibenzo	597	19.6	625	ethanol				mp 236°C (dec)
3028-94-2		3,3',9-triethyl-4,5',4,5'-dibenzothia-carbocyanine bromide Ethyl stains all	1	Et	9-Et 4,6',4,5'-dibenzo	579		ND	methanol				Differential stain of phospholipids & glycoproteins on on gels & tissue sections
		3,3'-dimethyl-6,6,6',6'-dibenzothia-carbocyanine iodide	1	Me	6,6,6',6'-dibenzo	570		ND	methanol				
		3,3'-dimethyl-6,7,6',7'-dibenzothia-carbocyanine iodide	1	Me	6,7,6',7'-dibenzo	550,594 593		ND ND	methanol ethanol				mp 274°C (dec)
		3,3'-dimethylthiadicarbocyanine chloride	2	Me		633 650	25.1	ND ND	ethanol methanol				
		3,3',9-trimethylthiadicarbocyanine iodide	2	Me	9-Me	650	17.6	ND	ethanol				
514-73-8	DTDC DTDCI DISC2(5)	3,3'-diethylthiadicarbocyanine iodide Dithiazanine iodide	2	Et		653 649 662 659 659 660 ND	25.1 20.7 20.0	ND 660 679 656 657 690 691	ethanol methanol DMSO acetone EG water glycerol	1		e in DMSO	mp 247°C (dec) Merck 11-3376 ϕ_f = 0.66 EG: λ_{max}=728nm (EG)
		3,3'-diethyl-6,5'-dimethoxythiadicarbo-cyanine iodide	2	Et	6,6'-MeO	666		690	EG				ϕ_f = 0.36 EG: λ_{max}=756nm (EG)
		3,3'-diethyl-6,6'-dimethoxythiadicarbo-cyanine iodide	2	Et	6,6'-MeO	660		ND	methanol				ϕ_f = 0.36 EG: λ_{max}=756nm (EG)

B4: Thiacyanines (continued)

CAS #	Abbreviation	Dye Name	n	Pos. 3,3'	Other Pos.	Absorbance nm	ε x 10^4 $M^{-1}cm^{-1}$	Fluorescence nm	Solvent	Solubility H_2O	Solubility EtOH	Solubility Other Solvent	Other Information
		3,3'-diethyl-9-phenylthiacarbocyanine iodide	2	Et	9-Ph	668	25.7	ND	methanol				
		3,3'-diethyl-10-bromothiacarbocyanine iodide	2	Et	10-Br	645 649		ND 673	methanol EG				
		3,3'-diethyl-10-chlorothiacarbocyanine iodide	2	Et	10-Cl	652		674	EG				
		3,3'-diethyl-10-fluorothiacarbocyanine iodide	2	Et	10-F	666		ND	nitromethane				
		3,3'-diethyl-10-methylthiacarbocyanine iodide	2	Et	10-Me	640		ND	methanol				
		3,3'-diethyl-10-phenylthiacarbocyanine iodide	2	Et	10-Ph	651		ND	methanol				
		3,3'-diethyl-4,4',5,5'-dibenzothiadicarbocyanine iodide	2	Et	4,5,4',5'-dibenzo	691		ND	ethanol				
		3,3'-dimethyl-6,7,6',7'-dibenzothiadicarbocyanine iodide	2	Me	6,7,6',7'-dibenzo	657		ND	methanol				
		3,3'-diethyl-10-chloro-4,5,4',5'-dibenzothiadicarbocyanine iodide	2	Et	4,5,4',5'-dibenzo 10-Cl	666		ND	ethanol				
		3,3'-diethyl-10-chloro-6,6,6',6'-dibenzothiadicarbocyanine iodide	2	Et	6,6,6',6'-dibenzo 10-Cl	690		ND	ethanol				
		3,3'-dimethylthiatricarbocyanine iodide	3	Me		762	24.0	ND	methanol				mp 223°C (dec)
3071-70-3	DTTC	3,3'-diethylthiatricarbocyanine iodide	3	Et		763	20.8	816	ethanol	ss	ss in MeOH,		mp 211°C (dec); ϕf = 0.61 EG
22268-66-2	DTTCP	perchlorate				760		782	methanol		CH_2Cl_2 &		pHC3 no fl; pHD3 fl. @ 784nm
23178-66-6	DTTCI	iodide				772	19.3	818	DMSO (ClO_4^-)		DMSO		I^- 0.12% in MeOH; 0.11% in CH_2Cl_2
						772	18.6	820	DMSO (I^-)		I in E		ClO_4 0.03% in MeOH; 0.3% in CH_2Cl_2
						764		800	acetone				Violently decomposed by H_2SO_4.
						760		784	60% methanol				Turquoise then purple in NaOH.
						769		791	propan-1-ol				λ_{max}=650nm (EG)
						768		786	acetonitrile				
						765		810	water				
						785		803	chloroform				
						783		796	DCM				
						764		800	EG				
						ND		803	glycerol				

B4: Thiacyanines (continued)

CAS #	Abbrev-iation	Dye Name	n	Pos. 3,3'	Other Pos.	Absorb-ance nm	ε x 10^4 $M^{-1}cm^{-1}$	Fluor-escence nm	Solvent	Solubility H_2O	Solubility EtOH	Solubility Other Solvent	Other Information
	IR116	3,3'-diphenylthiatricarbocyanine iodide	3	Ph		ND		ND					
		3,3'-diethyl-5,5'-dimethoxythiatricarbo-cyanine iodide	3	Et	5,5'-$(MeO)_2$	769		818	EG				ϕf = 0.19 EG: λ_{max}=874nm (EG)
		3,3'-diethyl-6,6'-dimethoxythiatricarbo-cyanine iodide	3	Et	6,6'-$(MeO)_2$	777		816	EG				ϕf = 0.66 EG: λ_{max}=869nm (EG)
		3,3'-diethyl-5,6,6',6'-tetramethoxythiatri-carbocyanine iodide: Temo-DTTC	3	Et	5,6,5',6'-$(MeO)_4$	803 793		842 ND	EG methanol				ϕf = 0.35 EG: λ_{max}=859nm (EG)
	DDTTCI	3,3'-diethyl-4,5,4',5'-dibenzothiatri-carbocyanine iodide Hexadibenzocyanin 45	3	Et	4,5,4',5'-dibenzo	798 803 797	19.8	825 842 ND	ethanol EG methanol				ϕf = 0.35 EG
17094-98-5		3,3'-diethylthiatetracarbocyanine iodide	4	Et		850 870	16.0	ND ND	methanol ethanol				S_{max}=940nm
		3,3'-diethyl-12-acetoxy-2,2'-thiatetra-carbocyanine	4	Et	12-acetoxy	872		ND	methanol				
15979-16-7		3,3'-diethylthiapentacarbocyanine iodide	5	Et		980 995	4.0	ND ND	methanol ethanol				S_{max}=1050nm

B5: Indocyanine

CAS #	Abbreviation	Dye Name	n	Pos. Y	Other Pos.	Absorbance nm	ε x 10^4 $M^{-1}cm^{-1}$	Fluorescence nm	Solvent	Solubility H_2O	Solubility EtOH	Solubility Other Solvent	Other Information
		1,1',3,3,3',3'-hexamethylindocarbocyanine chloride; Indolenine Yellow	0	Me		434 439		none none	acetic acid EG	s yellow		s in DMSO	
	DiIC1(3)	1,1',3,3,3',3'-hexamethylindocarbocyanine iodide Basic Red 12 CI-48070	1	Me		541 545 548 537	14.2	564 ND 569 ND	methanol acetic acid EG water	s	s pink	s in DMSO, Gl, Py & EG vs in Chl & Ac	Potential sensitive Yellowish in H2SO4, on dilution bluish pink; Bluish pink in NaOH. Same colour in dil HCl & dil NaOH
	DiIC6(3)	1,1'-dipentyl-3,3,3',3'-tetramethylindocarbocyanine iodide	1	Pent		546	14.2	576	methanol				
	DiIC12(3)	1,1'-didodecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate	1	Dodec		548	ND	570	methanol		s	s in DMF	Cationic membrane probes
	DiIC16(3)	1,1'-dihexadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate	1	Hexdec		547	12.8	565	methanol		s	s in DMF	Cationic membrane probes
	DiIC18(3) DiI	1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate	1	Octdec		547	11.7	571	methanol		s	s in DMF	Cationic membrane probes
	DiIC22(3)	1,1'-didocosenyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate	1	Doc5en		549	11.0	573	methanol			s in DMF & DMSO	Cationic membrane probes
	HIDC	1,1',3,3,3',3'-hexamethylindodicarbocyanine	2	Me		641	22.5	ND	ethanol		s	s in DMSO	Potential sensitive
36536-22-8	HIDCI	Iodide				635	22.0	662	methanol				mp 264°C (dec)
	DiIC1(5)	Hexacyanine 2 Basic Red 12				648 646	22.6	666 660	DMSO EG				
						642 650		ND 690	acetic acid water				
	HITC	1,1',3,3,3',3'-hexamethylindotricarbocyanine	3	Me		750	19.0	780	DMSO (ClO_4^-)	ss	s	s in DMSO	Potential sensitive
18595-48-6	HITCP	perchlorate				743	21.6	778	ethanol				mp 198°C (dec)
19764-96-6	HITCI	Iodide				739	24.2	765	methanol				ϕf = 0.50 (EG); λ_{max}=836nm
	DiIC1(7)	Hexacyanine 3				750	20.3	780	DMSO (I^-)				
						749		782	EG				
						757		795	chloroform				
						740		ND	acetic acid				
						765		810	water				
23178-67-8	HDITC	1,1',3,3,3'3'-hexamethyl-4,4',5,5'-dibenzo-2,2'-indotricarbocyanine perchlorate	3	Me	4,5',4',5'-dibenzo	780 776 780 771	17.4 23.1 	828 824 ND 805	DMSO chloroform ethanol methanol		s	s in DMSO	
3599-32-4	IR125	Indocyanine Green, Fox Green, Cardio Green	3	Bu-SO3-	4,5',4',5'-dibenzo	795 800 780 775 780	17.3	833 836 812 ND 830	DMSO chloroform 50% methanol water methanol	s	ss	I in E vs in DMSO	pH does not effect fluorescence Merck 11-4868: dimer Abs @ 690 nm & Fl @ 825 nm.

B6: 2,2'-quinocyanine

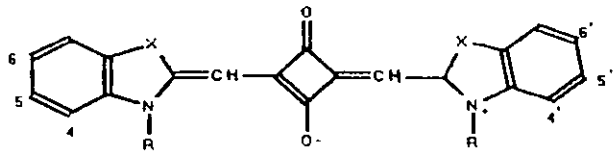
CAS #	Abbreviation	Dye Name	n	Pos. Y	Other Pos.	Absorbance nm	ε x 10^5 $l\,g^{-1}cm^{-1}$	Fluorescence nm	Solvent	Solubility H_2O	Solubility EtOH	Solubility Other Solvent	Other Information
977-95-8		1,1'-diethyl-2,2'-quinocyanine iodide Pseudoisocyanine	0	Et		524 526 527	8.5	none none none	methanol ethanol EG				mp 278°C (dec)
2768-90-3	DCI	1,1'-diethyl-2,2'-quinocarbocyanine iodide Pinacyanol iodide, Quinaldine Blue, Chinaldinblau, Sensitol Red	1	Et		605 606 526,563 610 605	17.0	ND ND ND 666 641	methanol ethanol water EG glycerol	0.7% violet red	2% blue	4% EGME	mp 297°C (dec); Merck 11-8056 Solution are dichroic alkaline (blue); acid (colourless) λ_{max}=570-660nm
		1,1'-diethyl-3,4,3',4'-dibenzo-2,2'- quinocarbocyanine iodide	1	Et	3,4,3',4'- dibenzo	613		ND	ethanol				
		1,1'-diethyl-5,6,5',6'-dibenzo-2,2'- quinocarbocyanine iodide	1	Et	5,6,5',6'- dibenzo	635		ND	ethanol				
14187-31-6	DDI	1,1'-diethyl-2,2'-quinodicarbocyanine iodide	2	Et		710 715 ND 706	23.0	745 745 760 ND	ethanol EG glycerol methanol				ϕf = 0.04 (EG); λ_{max}=812nm (EG)
		1,1'-diethyl-11-chloro-2,2'-quinodi carbocyanine bromide	2	Et	11-Cl	697		ND	methanol				mp 239°C (dec);
		1,1'-diethyl-11-bromo-2,2'-quinodi carbocyanine bromide	2	Et	11-Br	692 694		ND ND	ethanol methanol				mp 216°C (dec); λ_{max}=815nm (GI)
		1,1'-dimethyl-11-bromo-2,2'-quinodi carbocyanine bromide	2	Me	11-Br	691		ND	methanol				λ_{max}=745nm (GI)
		1,1'-diethyl-5,6,5',6'-dibenzo-2,2'- quinodicarbocyanine iodide	2	Et	5,6,5',6'- dibenzo	736		ND	ethanol				
		1,1'-diethyl-2,2'-quinocarbocyanine iodide	3	Et		808 ND 811 810		ND 867 860 ND	methanol glycerol acetone ethanol				mp 235°C (dec); λ_{max}=936nm (EG) I^- 0.01% in MeOH; 0.7 in CH_2Cl_2 Br^- 0.5% in MeOH; 0.3 in CH_2Cl_2 ClO_4^- 0.01% in MeOH; 0.7 in CH_2Cl_2
		1,1'-diethyl-13-acetoxy-2,2'-quinotetra carbocyanine bromide	4	Et	11-Br	843		ND	DMF				mp 185°C (dec); λ_{max}=1100nm (DMSO)

B7: 4,4'-quinocyanine

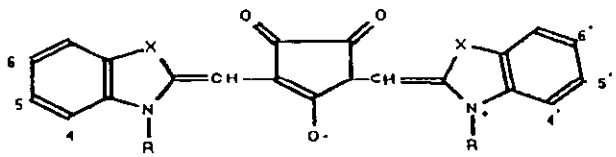
CAS #	Abbreviation	Dye Name	n	Pos. Y	Other Pos.	Absorbance nm	ε x 10^4 $M^{-1}cm^{-1}$	Fluorescence nm	Solvent	Solubility H2O	Solubility EtOH	Solubility Other Solvent	Other Information
862-57-7		1,1'-diethyl-4,4'-quinocyanine iodide Williams' cyanine	0	Et		550,590 595		none none	methanol EG				mp 280°C (dec)
	48380	1,1'-dipentyl-4,4'-quinocyanine iodide Cyanine Blue	0	Pe		564,592		none	ethanol				S_{max}=560-610nm
		1,1'-dimethyl-4,4'-quinocarbocyanine iodide	1	Me		711 709		725 ND	EG methanol				ϕ_f = 0.058 (EG); L_{max}=749nm (GI)
4727-50-6	DCI-4	1,1'-diethyl-4,4'-quinocarbocyanine iodide Crytocyanine, Kryptocyanine Rubrocyanine	1	Et		710 717 708 711 711	25.6 21.8	720 729 ND 747 728	ethanol DMSO methanol acetone EG	0.1%	0.2%	0.9% in EGME ε in EG	mp 257°C (dec); ϕ_f = 0.10 (EG) Greenish blue in ethanol S_{max}=520nm; L_{max}=747 (EG)
		1,1'-dibutyl-4,4'-quinocarbocyanine iodide	1	Bu		712		727	EG				ϕ_f = 0.096 (EG)
	DDCI-4	1,1'-diethyl-4,4'-quinodicarbocyanine iodide DDC-4 Jodid: NK 1144	2	Et		816 820 815	23.6	850 ND 849	ethanol methanol EG				ϕ_f = 0.12 (EG); L_{max}=930nm (EG) poor shelf life
		1,1'-diethyl-11-bromo-4,4'-quinodicarbocyanine iodide	2	Et	11-Br	795 793		ND 824	methanol EG				mp 237-238°C (dec); ϕ_f = 0.17 (EG) L_{max}=830nm (MeOH)
		1,1'-diethyl-11-chloro-4,4'-quinodicarbocyanine iodide	2	Et	11-Cl	797		834	EG				ϕ_f = 0.14 (EG)
7846-54-8		Neocyanine	2	Et	Structure I								Three quinoline endgroups joined by the conjugation bridge.
		1,1'-diethyl-4,4'-quinotricarbocyanine iodide; Xenocyanine	3	Et		923 924 932		ND 957 ND	methanol acetone ethanol				S_{max}=900-1100nm; L_{max}=1013nm (EG)
		1,1'-diethyl-6,6'-dichloro-4,4'-quinodicarbocyanine iodide	3	Et	6,6'-Cl2	948			methanol				mp 216-218°C (dec)

B8: Rigidised symmetrical cyanines

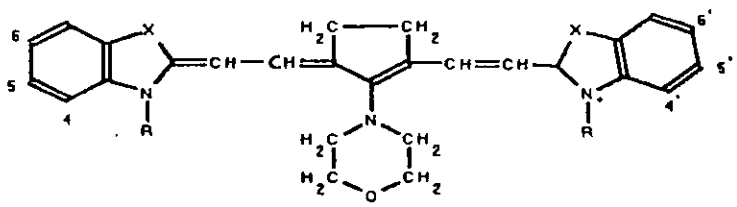
B8a: croconiums



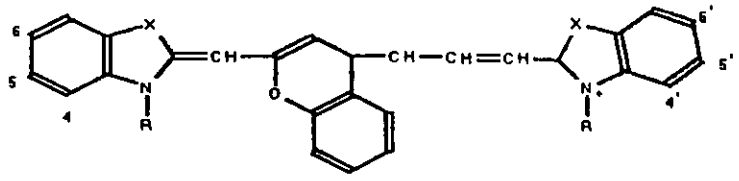
B8b: squaryliums



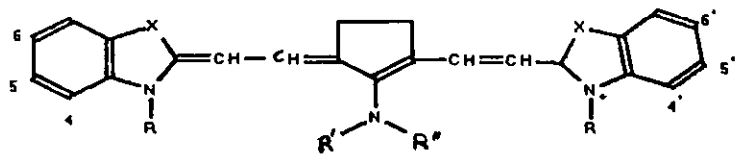
B8c: IR123 type



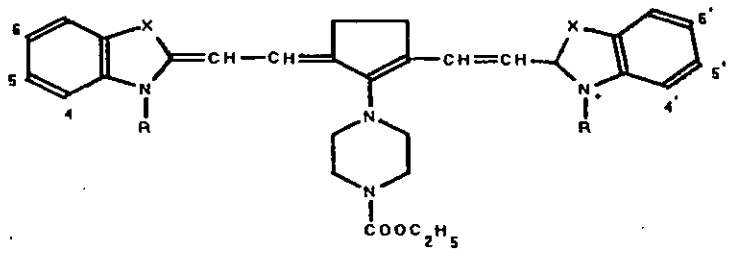
B8d: IR109 type



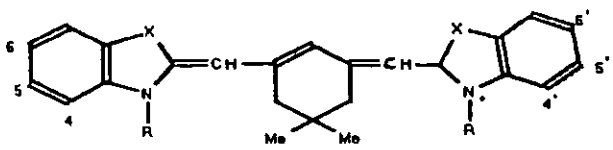
B8e: IR132 type



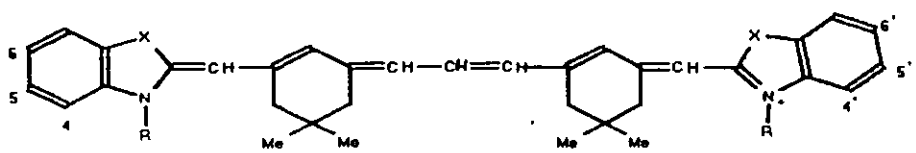
B8f: IR134 type



B8g: neopentylene



B8h: dineopentylene



B8: Rigidised symmetrical cyanines

Abbreviation	Dye Name	n	X	R	R'	R''	Other Pos.	Absorbance nm	e x 10^4 $M^{-1}cm^{-1}$	Fluorescence nm	Solvent	Solubility	Other Information
B8a: croconiums													
	3,3'-diethyl-9,11-croconium-dicarbocyanine iodide	2	CH=CH	Et				632 807		ND ND	acetonitrile methanol		
	3,3'-diethyl-9,11-croconium-thiadicarbocyanine iodide	2	S	Et				771		ND	acetonitrile		
	3,3'-diethyl-9,11-croconium-indodicarbocyanine iodide	2	CMe_2	Et				764		ND	acetonitrile		
B8b: squaryliums													
	3,3'-diethyl-9,11-squarylium-dicarbocyanine iodide	2	CH=CH	Et				724 706 730		ND ND ND	acetonitrile methanol chloroform		
	3,3'-diethyl-9,11-squarylium-thiadicarbocyanine iodide	2	S	Et				663 670		ND ND	acetonitrile chloroform		
	3,3'-diethyl-9,11-squarylium-indodicarbocyanine iodide	2	CMe_2	Et				629 630		ND ND	methanol chloroform		
B8c IR123 type													
IR123	3,3'-diethyl-9,11-(oxy-o-phenylene) thiatricarbocyanine iodide	3	S	Et				ND		ND			
B8d IR109 type													
IR109	3,3'-diethyl-10,12-ethylene-11-morpholinothiatricarbocyanine perchlorate	3	S	Et				ND		ND			λ_{max}=675nm DMSO
B8e IR132 type													
IR132	3,3'-di(3-acetoxypropyl)-11-diphenylamino-10-12-ethylene-6,6,5',6'-dibenzothiatricarbocyanine perchlorate CAS# 62669-62-9	3	S	$(PrOCOMe)_2$		Ph	Ph 5,6,5',6'-dibenzo	830 ND 764	15.9	905 861 824	DMSO chloroform methanol		λ_{max}=811nm DMSO
IR137	3,3'-diethyl-10,12-ethylene-11(N-methylanilino)thiatricarbocyanine	3	S	Et	Me	Ph		ND		ND			
IR139	11-dimethylamino-3,3'-diethyl-10,12-ethylene-4,5,4',5'-dibenzothiacarbocyanine perchlorate	3	S	Et	Me	Me	4,5,4',5'-dibenzo	ND		ND			λ_{max}=683nm DMSO
IR140	5,5'-dichloro-11-diphenylamino-3,3'-diethyl-10,12-ethylenethiacarbocyanine iodide: CAS# 53655-17-7	3	S	Et	Ph	Ph	5,5'-Cl	825 810 803	5.19 15.0	882 860 821	DMSO ethanol methanol		λ_{max}=834nm DMSO

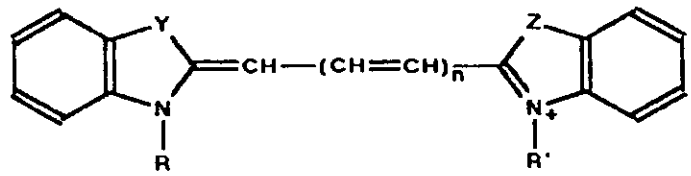
B8: Rigidised symmetrical cyanine continued

Abbreviation	Dye Name	n	X	R	R'	R''	Other Pos.	Absorbance nm	Absorbance x 10^4 $M^{-1}cm^{-1}$	Fluorescence nm	Solvent	Solubility	Other Information
IR141	5,5'-dichloro-3,3'-diethyl-10,12-ethylene-10,12-ethylene-11-(N-methyl anilino)thiacarbocyanine iodide	3	S	Et	Me	Ph	5,5'-Cl	ND		ND			λ_{max}=946nm DMSO
IR143	3,3'-diphenyl-3,3'-diethyl-10,12-ethylene-4,5,4',5'-dibenzothiatricarbocyanine perchlorate	3	S	Et	Ph	Ph	4,5,4',5' dibenzo	ND		ND			λ_{max}=970nm DMSO
B8f: IR134 type													
IR134	11-(4-ethoxycarbonylpiperidino)-3,3'-diethyl-10,12-ethylene-4,5,4',5'-dibenzothiatricarbocyanine perchlorate	3	S	Et			4,5,4',5'-dibenzo	ND		ND			λ_{max}=888nm DMSO
IR144	anhydro-11-(4-ethoxycarbonyl-1-piperazinyl)-10,12-ethylene-3,3,3',3'-tetramethyl-1,1'-di(3-sulphopropyl)-4,5,4',5'-dibenzoindotricarbocyanine hydroxide, triethylammonium salt CAS# 54849-69-3	3	CMe_2	$(Pr^SO_3H)_2$			6,7,6',7'-dibenzo	750 ND 745 696	14.1 12.4 	ND 848 825 708	ethanol chloroform DMSO methanol		λ_{max}=863nm DMSO
B8g: neopentylenes													
DNTDCI	3,3'-diethyl-9,11-neopentylenethiadicarbocyanine iodide	2	S	Et				650		ND	methanol		
DNTTCI	3,3'-diethyl-9,11-neopentylenethiatricarbocyanine iodide	3	S	Et				765	22.5	ND	ethanol		
B8h: dineopentylene													
DNTPC	3,3'-diethyl-9,11,15,17-dineo pentylene thiapentacarbocyanine perchlorate	5	S	Et				1060		ND	DMSO		λ_{max}=1124nm DMSO
DNDTPC	3,3'-diethyl-9,11,15,17-dineo pentylene (5,6,5',6'-tetramethoxy) thiapentacarbocyanine perchlorate	5	S	Et			5,6,5',6' $(MeO)_4$	1060		ND	DMSO		λ_{max}=1172nm DMSO
DNDTPC	3,3'-diethyl-9,11,15,17-dineo pentylene (6,7,6'7'-dibenzo)thiapentacarbocyanine perchlorate	5	S	Et			6,7,6',7' dibenzo	1060		ND	DMSO		λ_{max}=1100nm DMSO

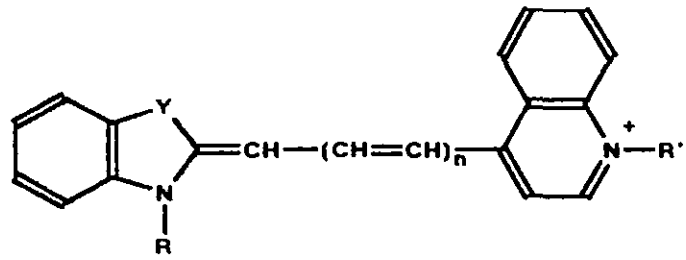
Appendix C

ASYMMETRICAL CYANINES

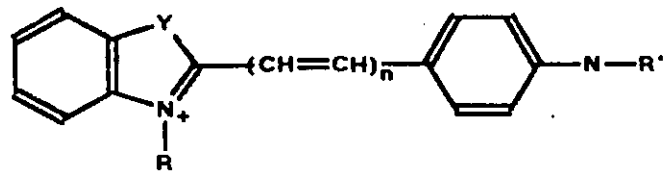
C1: 2,2'-assymetrical cyanines



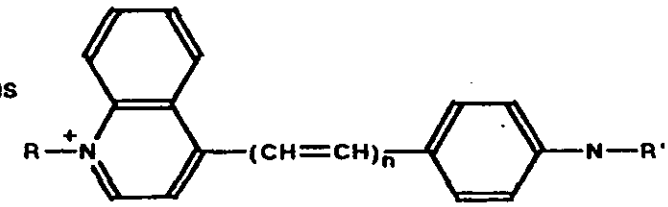
C2: 4-quinolium cyanines



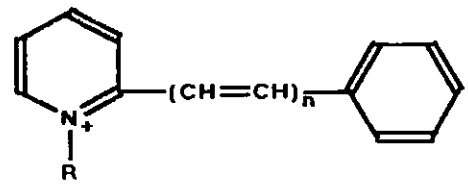
C3: Styryls



C4: Styryl-4-quiniolinium



C5: Pyridinium cyanines



C1 2,2'-asymmetrical cyanines

CAS #	Dye Name	n	Y	Z	R	R'	Absorbance nm	ε x 10^4 $l·g^{-1}·cm^{-1}$	Fluorescence nm	Solvent	Solubility	Other Information
	1,3'-Diethyl-2,2'-quinoylthiacyanine iodide	0	S	CH=CH	Et	Et	483		ND	methanol		
	1,3'-Diethyl-2,2'-quinoylselena cyanine iodide	0	Se	CH=CH	Et	Et	493		ND	methanol		
	1,3'-Diethyl-2,2'-quinoylindocarbocyanine perchlorate	1	CMe_2	CH=CH	Et	Et	551		ND	methanol		mp=247°C (dec)
	1,3'-Dimethyl-2,2'-quinoylindocarbocyanine iodide	1	CMe_2	CH=CH	Me	Me	540	6.16	ND	ethanol		
	1-Ethyl-3',3'-dimethyl-1'-phenyl-2-quinoyl-2'-indocarbocyanine iodide	1	CMe_2	CH=CH	Ph	Et	554		ND	methanol		
	1,3'-Diethly-2,2'-quinoyloxacarbocyanine iodide	1	O	CH=CH	Et	Et	545 542		ND ND	methanol ethanol		mp=248°C (dec); λ_{max}=580nm
	1,3'-Diethyl-2,2'-quinoylselenacarbocyanine iodide	1	Se	CH=CH	Et	Et	552		ND	methanol		
	1,3'-Diethyl-2,2'-quinoylthiacarbocyanine iodide	1	S	CH=CH	Et	Et	578		ND	methanol		mp=247°C (dec); λ_{max}=610nm
	1,3'-Diethyl-2-quinoyl-2'-(4',5'-benzothiacarbocyanine iodide	1	S 4',5'-benzo	CH=CH	Et	Et	587		ND	methanol		mp=247°C (dec); λ_{max}=610nm
	1-Ethyl-3,3-dimethyl-2-indo-2'(3'-propyloxyselena)-carbocyanine perchlorate	1	CMe_2	Se	Me	EtCOOH	550		ND	methanol		
	1-Ethyl-3,3-dimethyl-2-indo-2'(3'-ethyloxa)-carbocyanine iodide	1	CMe_2	O	Et	Et	508		ND	methanol		mp=266°C (dec); λ_{max}=550nm
	1-Ethyl-3,3-dimethyl-2-indo-2'(3'-ethylthia)-carbocyanine iodide	1	CMe_2	S	Et	Et	542		ND	methanol		mp=252°C (dec)
	1-Ethyl-3,3-dimethyl-1-phenyl-indothia carbocyanine iodide	1	CMe_2	S	Ph	Et	542		ND	methanol		
	1,3'-Diethyl-3,3-dimethyl-4',5'-benzoindothia carbocyanine iodide	1	CMe_2 4',5'-benzo	S	Et	Et	560		ND	methanol		
	1-Ethyl-3,3-dimethyl-1-phenyl-4',5'-benzoindothia carbocyanine iodide	1	CMe_2 4',5'-benzo	S	Ph	Et	557		ND	methanol		

C1 2,2'-asymmetrical cyanines continued

CAS #	Dye Name	n	Y	Z	R	R'	Absorbance /nm	ε x 10^4 ($l·g^{-1}cm^{-1}$	Fluorescence nm	Solvent	Solubility	Other information
	3,3'-Diethyl-2,2'-oxaselenacarbocyanine iodide	1	O	Se	Et	Et	527		ND	methanol		mp=257°C (dec); S_{max}=570nm
	3,3'-Diethyl-2,2'-oxathiacarbocyanine iodide	1	O	S	Et	Et	520		ND	methanol		mp=260°C (dec)
	3,3'-Diethyl-4',5'-benzooxathia carbocyanine iodide	1	O	S 4',5'-benzo	Et	Et	536		ND	methanol		
	3,3'-Diethyl-2,2'-oxaselenacarbocyanine iodide	1	O	Se	Et	Et	562		ND	methanol		
	3,3'-Diethyl-2,2'-selenathiacarbocyanine iodide	1	Se	S	Et	Et	562		ND	methanol		mp=254°C (dec); S_{max}=650nm
	3,3'-Diethyl-4,5-benzothiacarbocyanine iodide	1	S 4,5-benzo	S	Et	Et	577		ND	methanol		
	1,3'-Diethyl-2,2'-quinoylselenadicarbocyanine iodide	2	CH=CH	Se	Et	Et	675		ND	methanol		
	1,3'-Diethyl-2,2'-quinoylthiadicarbocyanine iodide	2	CH=CH	S	Et	Et	676		ND	methanol		mp=234°C (dec); S_{max}=715nm
	3,3'-Diethyl-2,2'-selenathiadicarbocyanine iodide	2	Se	S	Et	Et	659		ND	methanol		mp=236°C (dec); S_{max}=715nm

C2: 4-quinolium cyanines

CAS #	Dye Name	n	Y	R	R'	other position	Absorbance nm	ε x 10^4 $l\,mol^{-1}\,cm^{-1}$	Fluorescence nm	Solvent	Solubility	Other information
	1,1'-Diethyl-2,4'-quinocyanine iodide; Ethyl Diquinoline Red	0	CH=CH	Et	Et		520,560 559		ND ND	methanol ethanol		mp=160°C (dec); pKa 4.9 pH 4 colourless; pH 5.8 red
	1,1'-Diethyl-6,6'-diethoxy-2,4'-quinocyanine iodide	0	CH=CH	Et	Et	6,6'-(OEt)2	575		ND	methanol		mp=192°C (dec)
20391-23-5	6-Ethoxy-2[3-ethoxy-1-ethyl-2-methyl-4-quinolylidene)]propenyl-1-ethyl-4-methyl-quinolinium iodide; Dicyanine A	0	CH=CH	Et	Et	6,6'-(OEt)2 2',4-Me	575		ND	methanol		mp=192°C (dec)
	1,1'-Diethyl-2,4'-quinocarbocyanine iodide	1	CH=CH	Et	Et		606,657		ND	methanol		mp=240°C (dec); S_{max}=690nm
	1-Ethyl-4-quinoyl-2'(1'-ethyl-3',3'-dimethylindo)-carbocyanine iodide	1	CMe2	Et	Et		604		ND	methanol		mp=226°C (dec)
	1-Ethyl-4-quinoyl-2'(1'-phenyl-3',3'-dimethylindo)-carbocyanine iodide	1	CMe2	Et	Ph		602		ND	methanol		
	1-Methyl-4-quinoyl-2'(1'-ethyl-3',3'-dimethylindo)-carbocyanine iodide	1	CMe2	Me	Me		570	6.17	ND	ethanol		
	1,3'-Diethyl-4,2'-quinoyloxacarbocyanine iodide; DQOCI	1	O	Et	Et		592 596	13.6	ND ND	ethanol methanol		mp=261°C (dec)
	1,3'-Diethyl-4,2'-quinoylselenacarbocyanine iodide	1	Se	Et	Et		632 632		ND ND	ethanol methanol		
	1,3'-Diethyl-4,2'-quinoylthiacarbocyanine iodide; DQTCI	1	S	Et	Et		629 630	11.3	ND ND	ethanol methanol		mp=175°C (dec)
	1,1'-Diethyl-2,4'-quinodicarbocyanine iodide	2	CH=CH	Et	Et		761		ND	methanol		mp=240°C (dec); S_{max}=820nm
	1,3'-Diethyl-4,2'-quinoylselenadicarbocyanine iodide	2	Se	Et	Et		722		ND	ethanol		
	1,3'-Diethyl-4,2'-quinoylthiadicarbocyanine iodide	2	S	Et	Et		725		ND	ethanol		

C3 Styryls (hemicyanines)

CAS #	Dye Name	n	Y	R	R'	other position	Absorbance nm	ε x 10^4 $l\,mol^{-1}cm^{-1}$	Fluorescence nm	Solvent	Solubility	Other Information
	2-(-p-Diethylaminostyryl)-1'-ethyl-2'-quinolium Iodide: Quinaldine Red	1	CH=CH	Et	Et		530	6.6	ND	methanol	se in H_2O s in EtOH	pH1.0 colourless, pH2.2 red pKa 2.8 & 11.25: Merck 11-8057
	2-(-p-Diethylaminostyryl)-3-ethyl-benzoxazolium Iodide	1	O	Et	Et		500		ND	methanol		
	2-(-p-Dimethylaminostyryl)-3-ethyl-benzoxazolium Iodide	1	O	Et	Me		492	7.1	ND	methanol		
	2-(-p-Dimethylaminostyryl)-3-ethyl-benzothiazolium Iodide: DASBTI	1	S	Et	Me		525 530	7.9 6.49	ND ND	methanol ethanol		S_{max}=500nm: mp=255°C (dec) L_{max}=589-605nm
	2-(-p-Diethylaminostyryl)-3-ethyl-benzothiazolium Iodide	1	S	Et	Et		525	7.9	ND	methanol		
	2-(-p-Diethylaminostyryl)-3-ethyl-benzoselenazolium Iodide	1	Se	Et	Et		541 538		ND ND	nitromethane methanol		
	2-(-p-Diethylaminostyryl)-1,3-diethylindolinium Iodide	1	CMe_2	Et	Et		545		ND	methanol		
	2-(-p-Diethylaminostyryl)-3-ethyl-benzimidazolium Iodide	1	NEt	Et	Et		514		ND	nitromethane		
23238-76-4	Styryl 6: LDS 730 2-(4-(p-Dimethylaminophenyl)-1,3-butadienyl)-1,3,3-trimethyl-3H Indolinium perchlorate: CI Basic Violet 16: CI-48013	2	CMe_2	Me	Me		615 603	7.36	ND ND	ethanol methanol		L_{max}=670-760nm (EG)
8903-71-1	Styryl 7: LDS 750 2-(4-(4-Dimethylaminophenyl)-1,3-butadienyl)-3-ethylbenzothiazolium p-Toluenesulphonate	2	S	Et	Me		618 650 695	4.07	ND 690 704	1,2-dClEt water methanol		
	Styryl 8: LDS 751 2-(4-(4-dimethylaminophenyl)-1,3-butadienyl)-3-ethylbenzothiazolium perchlorate	2	S	Me	Me		670 660 642	6.16 3.48	ND 704 700	ethanol methanol methanol		L_{max}=703-724nm (methanol)
	Styryl 9: LDS 820 2-(6-(p-dimethylaminophenyl)-1,3,5-hexatrienyl)-3-methylbenzothiazolium perchlorate	2	S	Me	Me		670		760	methanol		Lower photochemical stability than Styryl 9M
	Styryl 9M: LDS 821 2-(6-(p-dimethylaminophenyl)-2,4-neopentylene-1,3,5-hexatrienyl)-3-methylbenzothiazolium perchlorate	2	S	Me	Me	2,4-neo	695 672	6.06	ND 750	ethanol methanol		L_{max}=783-860nm (methanol)

C4 Styryl-4-quinolinium

CAS #	Dye Name	n	Y	R	R'	other position	Absorbance nm	ε x 10^4 $M^{-1}cm^{-1}$	Fluorescence nm	Solvent	Solubility	Other information
	4-(4'-Dimethylaminostyryl) quinoline	1		-	Me		401		ND	methanol		S_{max}=450nm; mp=142°C (dec)
52968-09-3	Styryl 11; LDS 798 1-ethyl-4-(4-(p-dimethylaminophenyl)-1,3-butadienyl)-quinolinium perchlorate	2	CH=CH	Et	Me		576 555 558	4.77	ND 665 638	ethanol water methanol		L_{max}=770-840nm (EG)

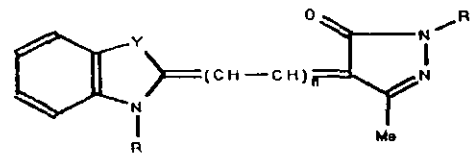
C5 Pyridinium cyanines

CAS #	Dye Name	n	Structure	Absorbance nm	ε x 10^4 $M^{-1}cm^{-1}$	Fluorescence nm	Solvent	Solubility	Other information
51325-91-8	4-dicyanomethylene-2-methyl-6-(p-dimethylaminostyryl)-4H-pyran DCM	1	V	472 480	4.25 3.77	ND 627	ethanol DMSO		L_{max}=604-672nm (methanol)
	DASPI; 2-(pDimethylaminostyryl)-pyridylmethyl iodide	1	VI	472	3,83	ND	ethanol		
57004-02-2	Pyridin 1; LDS 698 1-ethyl-2-(4-(p-dimethylaminophenyl)-1,3-butadienyl)-pyridium perchlorate	2	VI	480 476	3.80	ND ND	ethanol methanol		L_{max}=660-730nm (methanol)
89648-21-9	Pyridin 2; LDS 722 1-ethyl-4-(4-(p-dimethylaminophenyl)-1,3-butadienyl)-pyridium perchlorate	2	VII	500 494	4.22	ND ND	ethanol methanol		L_{max}=657-765nm (methanol)

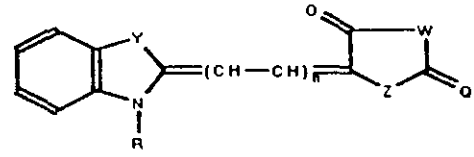
Appendix D

MEROCYANINES

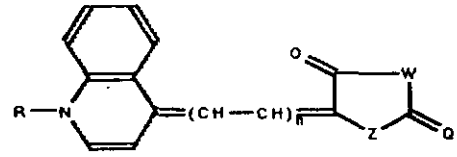
D1: Merocyanines



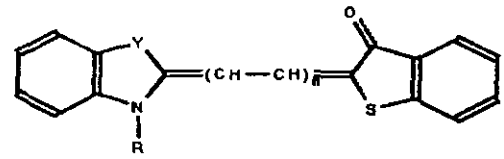
D2: Rhodanines



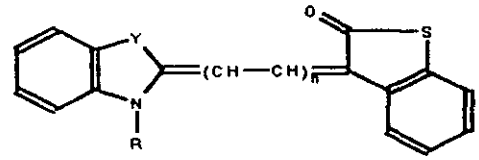
D3: 4-quinolium rhodanines



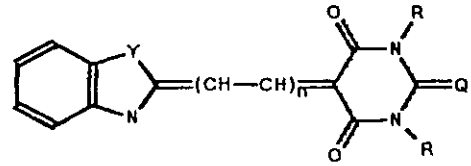
D4: Merocyanines



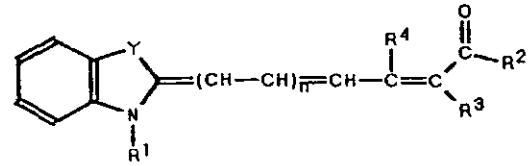
D5: Merocyanines



D6: Merocyanines



D7: Merocyanines



D1: Merocyanines

CAS #	Dye Name	n	Y	Other Pos.	R	R'	Absorbance nm	ε x 10^4 $(M^{-1}cm^{-1}$	Fluorescence nm	Solvent	Solubility	Other Information
		0	S		Me	Ph	ND		ND			
		1	CMe2		Me	Ph	476		ND	methanol		
		3	S		Me	Ph	ND		ND			

D2: Rhodanine

CAS #	Dye Name	n	Y	W	Q	Z	R	Absorbance nm	ε x 10^4 $(M^{-1}cm^{-1}$	Fluorescence nm	Solvent	Solubility	Other Information
	3-Ethyl-5-(3-ethyl-2-benzothiazolylidene) rhodanine	0	S	N-Et	S	S	Et	432		none	pyridine:H2O		
								426		none	methanol		
								421		none	cyclohexane		
		0	CH=CH	N-Et	S	S	Et	ND		ND	methanol		
		0	S	N-Et	NCOMe	Et		404		ND	methanol		
		0	CH=CH	S	S	N-Et	Me	552		ND	methanol		
		0	S	S	S	N-Et	Me	411		ND	methanol		
	3-Ethyl-5-[2-(3-ethyl-2-benzothiazolylidene)ethylidene]rhodanine	1	S	N-Et	S	S	Et	524		560	methanol		
								492		548	cyclohexane		
								540		563	pyridine:H2O		
								ND		550	acetone		
		1	CH=CH	N-Et	S	S	Et	578		ND	methanol		
		1	Se	N-Et	S	S	Et	526		ND	methanol		
		1	O	N-Et	S	S	Et	490		ND	methanol		
		1	CMe2	N-Et	S	S	Et	504		ND	methanol		
	3-Ethyl-5-[4-(3-ethyl-2-benzothiazolylidene)butenylidene]rhodanine	2	S	N-Et	S	S	Et	605		644	methanol		
								537		633	cyclohexane		
								633		651	pyridine:H2O		
								ND		643	acetone		
	3-Ethyl-5-[4-(3-ethyl-2-benzothiazolylidene)-2-hexenylidene]rhodanine	3	S	N-Et	S	S	Et	635		765	methanol		
								640	7.6	ND	chloroform		
								710		783	pyridine:H2O		
								570		744	cyclohexane		
								ND		774	acetone		

D3: 4-quinoline rhodanines

CAS #	Dye Name	n	Q	W	Z	R	Absorbance nm	ε x 10^4 $M^{-1}cm^{-1}$	Fluorescence nm	Solvent	Solubility	Other Information
	3-ethyl-5-(1-ethyl-4(1)-quinolylidene)-rhodanine	0	N-Et	S	S	Et	515 499 ND		577 574 561	methanol cyclohexane pyridine:H_2O		
	3-ethyl-5-(1-ethyl-4(1)-quinolylidene)-ethylidene rhodanine	1	N-Et	S	S	Et	617 548 ND		658 642 663	methanol cyclohexane pyridine:H_2O		
	3-ethyl-5-(1-ethyl-4(1)-quinolylidene)-butylidene rhodanine	2	N-Et	S	S	Et	716 585		ND ND	methanol cyclohexane		
	3-ethyl-5-(1-ethyl-4(1)-quinolylidene)-hexylidene rhodanine	3	N-Et	S	S	Et	820 607		ND ND	methanol cyclohexane		

D4: Merocyanines

CAS #	Dye Name	n	Y	Other Pos.	R	R'	Absorbance nm	ε x 10^4 $M^{-1}cm^{-1}$	Fluorescence nm	Solvent	Solubility	Other Information
		0	S		Et	Et	447		ND	methanol		
		1	S		Et	Et	558		ND	methanol		
		2	S		Et	Et	640		ND	methanol		
		3	S		Et	Et	ND		ND	methanol		

D5: Merocyanines

CAS #	Dye Name	n	Y	Other Pos.	R	R'	Absorbance nm	ε x 10^4 $M^{-1}cm^{-1}$	Fluorescence nm	Solvent	Solubility	Other Information
		0	S		Et	Et	418		ND	methanol		
		1	S		Et	Et	531		ND	methanol		
		2	S		Et	Et	619		ND	methanol		
		3	S		Et	Et	644		ND	methanol		

D6: Merocyanines

CAS #	Dye Name	n	Y	Q	R	R'	R''	Absorbance nm	ε x 10^4 $M^{-1}cm^{-1}$	Fluorescence nm	Solvent	Solubility	Other Information
		1	CMe2	S	Me	Et	Et	495		ND	pyridine		
		1	NEt	S	Et	Et	Et	ND		ND			
	Merocyanine 540 1,3-dibutyl-5-[4-[3-(3-sulphopropyl)-2-benzoxazolinylidene]-2,4-butylidene]-2-thiobarbituric acid sodium salt	1	O	S	Et	Et	Et	540 533		560 572	methanol water		
		2	CMe2	S	Et	Et	Et	595		ND	pyridine		
		2	NEt	S	Et	Et	Et	ND		ND			
		3	CMe2	S	Et	Et	Et	698		ND	pyridine		
		3	NEt	S	Et	Et	Et	ND		ND			

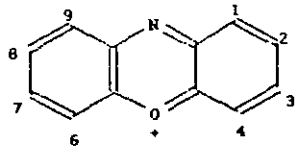
D7: Merocyanines

CAS #	Dye Name	n	R^1	R^2	R^3	R^4	Absorbance nm	ε x 10^4 $M^{-1}cm^{-1}$	Fluorescence nm	Solvent	Solubility	Other Information
		0	Et	OEt	CN	Et	ND		ND			
		0	Et	OEt	CN	-CH=CHPh	ND		ND			
		0	Me	OEt	CN	SEt	ND		ND			
		0	Et	OEt	CN	OEt	ND		ND			
		0	Me	OEt	CN	NHEt	ND		ND			
		1	Et	OEt	CN	Me	ND		ND			
		1	Et	OEt	CN	-CH=CHPh	ND		ND			
		1	Me	OEt	CN	SEt	ND		ND			
		1	Et	OEt	CN	OEt	ND		ND			
		1	Me	OEt	CN	NHEt	ND		ND			

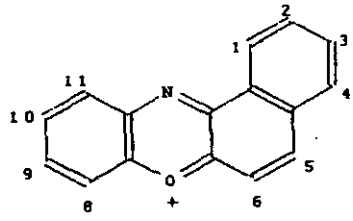
Appendix E

AZINES

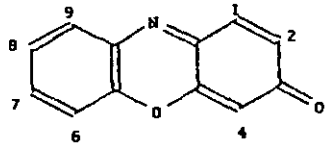
PHENOXAZINES



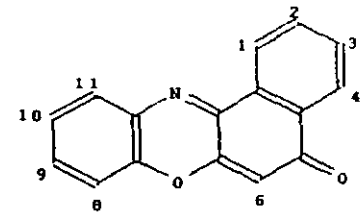
E1: Phenoxazines



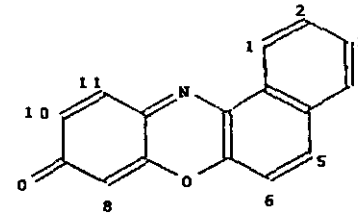
E2: Benzo[a]phenoxazines



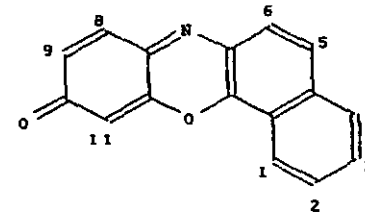
E3: Phenoxazin-3-one



E4: 5H-Benzo[a]phenoxazin-5-one

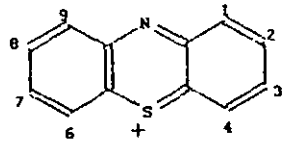


E5: 9H-Benzo[a]phenoxazin-9-one

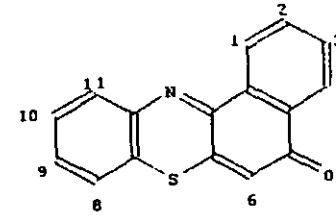


E6: 10H-Benzo[a]phenoxazin-10-one

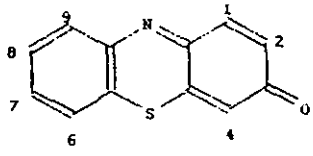
THIAZINES



E7: Thiazines



E9: 5H-Benzo[a]phenothiazin-5-one



E8: Thiazin-3-one

C1: Phenoxazine

CI No	CAS #	Dye Name	Pos. 3-	Pos 7-	other Pos.	Absorbance nm	ε x 10^4 $l\,M^{-1}cm^{-1}$	Fluorescence nm	Solvent	pKa	Solubility H_2O	Solubility EtOH	Other Solvents	Other Information
51000		Methyl Capri Blue	NMe2	NMe2	-	640		ND	water		s	s		Reddish-brown in H_2SO_4, on dilution paler but no colour change
		Capri Blue GN				645		ND	methanol		blue	blue		Reddish brown in dil HCl
						666		ND	0.01M NaOH					
51004	24796-94-9	Oxazine 725	NEt2	NEt2		643	12.3	658	methanol		3%	0.5%	0.6% EGME	ϕf = 0.15, τf= 1.02ns (EtOH)
	33203-62-6	Oxazine 1				646	13.0	650	ethanol			blue		
	55640-62-9	Ethyl Capri Blue				651		677	ethylene-glycol					
		CI Basic Blue 3												
51010	10127-36-3	Brilliant Cresyl Blue	NH2	NEt2	2-Me	595		630	acetone	10.74	3%	1%	I in E & B	τ = 3.6ns (EtOH)
		Cresyl Blue BBS				595		628	methanol	(9.9)	blue	blue	s in Ac & AA	Dichroic (green by reflected and
		Brilliant Blue C				600	11.6	625	ethanol					violet red by transmitted light) in
		Cresyl Blue ZRN				605		633	hexanol					H_2SO_4,on dil brown. Brown ppt in NaOH.
						606		642	pyridine					Spectrum concentration dependent,
						610		638	nitrobenzene					@ 40mg/l λ=625nm & @ 1g/l λ=575nm.
														Reduced (colourless) by dithionite.
														Reoxidised by air at pH>7 but not
														below pH6.4: mp 233-236°C
51015		Capri Blue GON	NEt2	NMe2	2-Me	ND		ND			s	s		Green in thin layers, red in thick
		Serron NF									blue	blue		layers in H_2SO_4, on dilution red.
														Red in dilute HCl.
	41830-81-3	Oxazine 4	NHEt	NHEt	2,8-Me	610	10.3	625	methanol		s	s	I in E & Xyl	Deep pink (460nm) in NaOH
		LD690				615	10.9	640	ethanol		blue	blue	s in DMSO	Olive green (432/510/580nm) in H_2SO_4
						612		635	water					Blue with red fluorescence in water.
														Dimer @ Abs 595nm
														Temperature Dependence -0.01% per °C.
		3,7-diaminophenoxazine	NH2	NH2		498	2.5	591	ethanol/base		s	s	s in Gl, Chl, A	pH indicator
		Oxonine				575	8.8	605	ethanol				se in D	
													vs in Py	
		Zapon Fast Blue 3G	NEt2	NEt2	1-OEt	655		ND	water			s		Red in NaOH. Blue violet with red
														fluorescence in ethanol.
		Phenoxazine	H	H		ND		ND				s	s in most organic solvents	Blue green in alcoholic ferric chloride solution. Violet red in H_2SO_4
														mp 156°C

62: Benzo[a]phenoxazine

CI No	CAS #	Dye Name	Pos. 6-	Pos. 9-	other Pos.	Absorbance nm	ε x 10^4 $l\,mol^{-1}cm^{-1}$	Fluorescence nm	Solvent	pKa	Solubility H_2O	Solubility EtOH	Solubility Other Solvents	Other Information
51175	7057-57-0 (chloride)	Meldola's Blue New Blue R CI Basic Blue 6	H	NEt2		573		ND	water	-	2% bluish violet	0.1% blue	I in Xyl, ss in Ac vss in D vs in Gl & EG	Brown flocculent ppt in NaOH Blackish green in H_2SO_4, on dil blue 1% soln in deionised water previously adjusted to pH7 has a pH of 4.6
51180	53340-16-2 (ClO_4^-) 2361-66-3 (chloride)	Nile Blue A CI Basic Blue 12 Nile Blue 690	NH_2	NEt2		627 633 636 628 627 626 630	7.75 7.66 3.96	660 672 670 655 666 670 676	methanol ethanol water chloroform hexan-1-ol acetone pyridine	1.6 9.7	0.2% vs hot blue	0.2% blue	s in Gl, Ac DMSO & EG I in D, CH, T, E & H. ss in Ac, Py, Chl, MeCN, D & Xyl.	τ_f= 1.62ns EtOH; τ_f= 1.19ns MeOH τ_f= 0.38ns H_2O; τ_f= 0.93ns G τ_f= 1.27ns EG; ϕ_f = 0.23 (EtOH) ϕ_f = 0.47 (MeOH); mp > 300°C (dec) Saturated conc. 2.8 x 10^{-4}M (EtOH) Molecular dimension 3.9 x 9.4 x 16.8 Å Pink & yellow fluorescence in Xyl. Mauve in Chl, Py & G. Orange in Ac. Orange/brown (430,519) in H_2SO_4, on dilution green (430) then blue (638) Brownish red ppt (505) in NaOH, that is soluble in ether (brownish orange with green fluorescence). Dimer Abs @ 590nm & Fl. @ 685nm Blue pH<9, Red pH>10 (no fluorescence @ 670 nm observed) Temperature Dependence -0.15% per °C
51185		Nile Blue 2B	NH-Ba	NEt2		640		ND	water	7.9	0.16% blue green	0.62% blue green		Pink (alkaline), Blue (acidic) Brownish red in H_2SO_4, on dilution green then blue. Brownish red ppt in NaOH, soluble in ether (orange yellow with green fluorescence).
51190		Basic Blue 10	NH-Ph-NMe3	NMe3		ND		ND			s blue	s blue		Dirty green in H_2SO_4, on dil. dirty violet then blue. Green ppt in NaOH Green in HClaq
51195		New Methylene Blue GG	NMe2	NMe2		ND		ND		-	s blue	s greenish-blue		Green ppt in NaOH, Reddish brown in H_2SO_4, on dil. green then blue. Green in Conc HCl
51200		New Fast Blue F, R	NMe2	N(Ph-pNMe2)2		ND		ND			s			Blue or violet in water Greenish blue in H_2SO_4, on dilution blue to violet or green. Dark flocculent ppt in NaOHaq. Reddish blue or green in HClaq.
51205		Muscarine	NMe2	-	3-OH	ND		ND			s in hot			Bluish green in H_2SO_4, on dilution blue to violet wih violet ppt Yellowish brown in aqueous NaOH.

E2: Benzo[a]phenoxazine continued

CI No	CAS #	Dye Name	Pos. 6-	Pos. 9-	other Pos.	Absorb-ance nm	ε x 10^4 $M^{-1}cm^{-1}$	Fluor-escence nm	Solvent	pKa	Solubility H_2O	Solubility EtOH	Other Solvents	Other Information
51210		Fast Green M	NH-Ph	NMe2		513	3.23	623	ethanol/base		s in hot	i	s in AA, conc HCl	Greenish blue in acetic acid
		9-dimethyl-6-phenylimino benzo[a]phenoxazine hydrate				650	7.18	ND	ethanol					Yellow brown solution in conc HCl. Browny violet in H_2SO_4, on dil orange mp 256 °C
	62669-69-7	Oxazine 170	NHEt	NHEt	10-Me	616		651	acetone			s		τf = 2.12ns H_2O: τf = 1.19ns G
	(ClO_4^-)	Oxazine 720				620	8.30	637	methanol			blue		τf = 3.40ns EtOH: τf = 3.32ns MeOH
						627	8.30	650	ethanol					τf = 2.84 EG
						623	9.12	650	ethanol					Molecular dimen. 3.9 x 11.5 x 17.0 Å
						630		660	pyridine					
						632		652	nitrobenzene					
						633		650	hexan-1-ol					
	41830-60-2	Cresyl Violet	NH2	NH2		593	8.30	615	methanol		0.38%	0.25%	i in E & Xyl	Φf = 0.59 (EtOH): Φf = 0.70 (MeOH)
	(ClO_4^-)	Oxazine 9				600	8.74	632	ethanol			violet	s in DMSO	τf = 3.23ns EtOH: τf = 3.23ns MeOH
		Cresyl Violet 690				590		628	water					τf = 2.32ns H_2O: τf = 2.80ns G
						595		630	acetone					τf = 2.77ns EG: mp 140-143°C
						606		642	pyridine					Molecular dimension 3.9 x 9.4 x 14.6 Å
						605		633	hexan-1-ol					Violet with red fluorescence in water.
						610		636	nitrobenzene					Orange (432/508nm) in H_2SO_4
														Golden Orange (470nm) in NaOH
														Dimer Abs @ 570nm.
	15391-69-0	Darrow Red	NH2	NHCOMe		ND		ND			0.1%	0.5%	0.5% EGME	Aqueous & alcoholic solutions are red by transmitted lt & muddy yellow by reflected lt. Precursor for Cresyl Violet. Metachromic dye.
		Nile Blue EX	NEt2	NEt2		ND		ND						
		Oxazine 750	NHEt	t-amine		662		705	methanol		s	s	i in E & Xyl	Saturated conc. 1.5 x 10^{-4}M (ethanol)
						667	8.25	680	ethanol				s in DMSO	Dimer Abs @ 610nm & Fl. @ 705nm
						680	8.25	716	water					Pink (522nm) in H_2SO_4
														Turquoise (674nm) in water
														Pink (510nm) in NaOH

§3: Phenoxazin-3-one

CI No	CAS #	Dye Name	Pos. 4-	Pos 7-	other Pos.	Absorbance nm	ε x 10^4 $(M^{-1}cm^{-1}$	Fluorescence nm	Solvent	pKa	Solubility H_2O	Solubility EtOH	Other Solvents	Other Information
51020		Resorcin Blue	H	NMe_2		ND		ND						Blue in H_2SO_4, on dil. unaltered Brown in NaOH
51025	1326-24-6	Modern Violet N	OH	NH_2		620,645 530,660 610	1.2,1.5 4.0,4.0 4.0	ND ND ND	ethanol methanol methanol/HCl		s blue	s		Oxidised to a dark violet red in NaOH Pale brownish violet in H_2SO_4, on dil dilution reddish
51030	1562-85-2	Gallocyanine Alizarin Navy Blue AT CI Mordant Blue 10	OH	NMe_2	1-COOH	636		ND	water	10.1	I ss in hot	0.07% blue-violet	s in AA 0.2% EGME	pH<4 blue-violet, pH5-6 blue, pH>8 red Cornflower blue in H_2SO_4, on dilution magenta red. Reddish violet in NaOH
51040	6416-51-9	Prune Galle Blue ET Violet PDH Solochrome Prune A CI Mordant Violet 54	OH	NMe_2	1-COMe	650		ND	water	3.6 & 8.05	s reddish violet	s blue		pH<4 magenta red, pH4-8 deep blue pH>8 purple, pH>10 decomposes Brown ppt in NaOH, s in xs (violet)
51045	1563-02-6	Gallamin Blue CI Mordant Blue 45	OH	NMe_2	1-$CONH_2$	651		ND	water		s			Red & dichroic in H_2SO_4. Reddy violet in NaOH, on dil. red flocculent ppt. Greenish in water
51050	1562-90-9	Celliestine Blue B CI Mordant Blue 14 Galle Sky Blue B Coreine 2R Celliestine Blue By	OH	NEt_2	1-$CONH_2$	655		ND	water		2% reddish violet	1.5% blue	0.005% Xyl 6.5% EG 2.3% EGME s in Py, Ac & D vs in Gl	Bluish violet with violet ppt in NaOH Cornflower blue in H_2SO_4, on dilution magenta red. Reddish violet in water. Blue in ethanol. mp 227-230°C
51055		CI Mordant Blue 60 CI Mordant Blue 36 Modern Violet	OH	NMe_2 NMe_2	1-$CONH_2$ 1-COMe	ND		ND			s s			Pale & dichroic in aqueous soln, blue when oxidized. Violet in HCl. Rapidly oxidized to bluish violet in NaOH Violet in conc HCl.
51060		Aminogallasine Blue (leuco form)	OH	OH	1-$CONH_2$ 2-NH_2	ND		ND			s			Brownish red in water. Browny red in H_2SO_4, on dil. unaltered Oxidized to greyish blue ppt in NaOHaq
51065		Galanil Violet B,BS,R	OH	NMe_2	1-NH-Ph 2-NH-Ph	ND		ND			ss	s		Blue in water and ethanol Greyish red in H_2SO_4, on dil. claret red ppt. Bright bluish violet in NaOH
51070		Galanil Blue Galanil Indigo F	OH	NMe_2	1-NH-Ph 2-NH-Ph	ND		ND			s			Indigo blue in water Brownish violet in H_2SO_4, on dilution brownish ppt. Bluish vioet in NaOHaq
51080		CI Mordant Blue 35 (leuco form)	OH	NR_2	1-COR' 2-NH-Ph-pNR_2	ND		ND			s			R=Me or Et, R'=NH_2 or OCH3 Lauco form: Pale bluish green in water Pale reddish brown (violet + MnO_2), on dilution brownish. Oxidised (reddy violet) by NaOHaq

E3: Phenoxazin-3-ones continued.

Dye Name		Pos. 2-	Pos 7-	other Pos.	Absorbance nm	ε x 10^4 ($M^{-1}cm^{-1}$)	Fluorescence nm	Solvent	pKa	Solubility H_2O	Solubility EtOH	Solubility Other Solvents	Other Information
51110	Pyrogallocyanine sulphonic acid	OH	NMe_2	1 = SO_3Na	ND		ND			s			Violet blue in water. Blue in H_2SO_4, on dil. red to violet Red ppt. in $NaOH_{aq}$
51120	CI Mordant Blue 56	OH	NMe_2	1-NH-$PhSO_3Na$	ND		ND			s			Bluelsh violet in water Reddish violet in H_2SO_4, on dil. dark blue ppt. Reddish ppt in $NaOH_{aq}$
51125	CI Mordant Blue 59	OH	NMe_2	2-NH-$PhSO_3Na$	ND		ND			s	s		Deep blue in water & violet in EtOH Wine red in H_2SO_4, on dil. brownr Violet in $NaOH_{aq}$
51130	Ultracyanine B,R (Leuco form)	OH	NMe_2	4-OH 2-O-Ph-αOH	ND		ND			s	s		Leuco form: yellow green or pale blue in water and ethanol Colourless or pale blue in H_2SO_4, on dilution colourless or faint yellow. Colourless; rapidly oxidised to blue or violet on addition of NaOH
51135	Modern Azurine	OH	NMe_2	1-COMe 2-OH	ND		ND			s			Yellow in water Green in H_2SO_4, on dilution yellow. Oxidised (blue) + NaOH
51165	CI Mordant Blue 66 (Leuco form)	OH	NHEt	1-$CONH_2$ 7-Me	ND		ND			s			Leuco form: pale green in water Pale violet red in H_2SO_4, (violet blue + MnO_2), on dil. pale yellowish red. Oxidation to turbid violet red in NaOH
51170	Meta Celestine Blue	OH	NEt_2	1-$CONH_2$ 8-Me	ND		ND			s	s		Reactions and solubilities similar to Celestine Blue B
51400	Iris Blue B	=	OH	8-=	628		ND	HCl		ss	s	ss in E	The exact structure is in doubt
	Fluorescent Blue				637		ND	KOH				s in MeOH, AA	pH4.4 (red), pH 6.4 (blue)
	Lacmoid				608	4.40	ND	methanol/HCl				Ac & PhOH	
	Resorcin Blue		O^-		574	7.80	ND	methanol/KOH				I in B, PE & Chl	
	Chromazurine	OH	NMe	2-NPh(SO_3)	ND		ND			s	ss	I in Xyl & Chl ss in D & Ac vs in G, EG & Py	Blue in pH7 water, olive in acid soln and violet in alkaline solution.
635-78-9	Resorufin	H	OH		571	6.6	584	pH9	6.83	2%	0.2%	s in DMF & dil NaOH	Molecular dimen. 2.4 x 7.0 x 12.0 Å
550-82-3	Resazurin				571	6.7	589	pH9					Acid (pink), alkaline (blue)
	Resazoin				573	7.76	593	methanol					pH3.8 (orange), pH 8.8 (dark violet)
					573	7.94	ND	ethanol					Resorufin is a reduced form Resazurin
					578		598	hexan-1-ol					
					595		602	pyridine					
					600		ND	water					

3: Phenoxazin-3-one (continued)

Dye Name	Pos. 4-	Pos. 7-	ether Pos.	Solubility H_2O	Solubility EtOH	Other Solvents	Other Information	Solubility H_2O	Solubility EtOH	Other Solvents	Other Information
Resorufin acetate	H	OCOMe		442	1.3	ND	methanol/HCl			s in DMSO & DMF	
				445	1.22	ND	methanol				
Resorufin methyl ether Methoxyresorufin	H	OMe		462	2.2	553	methanol			s in DMSO & DMF	Microsomal dealkylase substrates
Resorufin ethyl ether Ethoxyresorufin	H	OEt		464	2.2	554	methanol			s in DMSO & DMF	Microsomal dealkylase substrates
				550	1.0	ND	ethanol				
Resorufin benzyl ether	H	O-benzyl		464	2.2	554	methanol			s in DMSO & DMF	Microsomal dealkylase substrates
Resorufin pentyl ether	H	O-C_5H_{11}		463	2.2	550	methanol			s in DMSO & DMF	Microsomal dealkylase substrates
Phenoxaz-3-one-3H-phenoxazin-3-one	H	H		445	1.07	ND	methanol				
				466,466	.98,1.0	ND	ethanol				
1-acetyl-2-hydroxy-3H-phenoxin-3-one	OH	H	1-Ac	421	1.55	ND	ethanol				
				440	1.74	ND	ethanol/HCl				
2-hydroxy-3H-phenoxazin-3-one	OH	H		404	1.48	ND	ethanol				
				398	1.14	ND	methanol/HCl				
	O^-	H		434	1.20	ND	ethanol/KOH				
				434	1.20	ND	methanol/KOH				
7-hydroxy-3H-phenoxazin-3-one	OH	H		476	1.00	ND	ethanol				
				410,674	.25,7.76	ND	methanol/base				
				606	4.37	ND	methanol/HCl				
1-methoxycarbonyl-2-hydroxy-3H-phenoxazin-3-one	OH	H	1-COOMe	405	1.91	ND	ethanol				
				446	1.95	ND	ethanol/HCl				
1,7,9-trihydroxy-3H-phenoxazin-3-one	H	OH	1,9-(OH)2	466	1.91	ND	ethanol				
				526	2.67	ND	ethanol (trianion)				
2,4-dimethyl-7,9-dihydroxy-3H-phenoxazin--3-one	Me	OH	4-Me	421	1.65	ND	ethanol				
			9-OH	440	1.74	ND	ethanol/HCl				
7-oxido-3H-phenoxazin-3-one	H	ONa		579	7.94	ND	ethanol				
7-ethoxy-3H-phenoxazin-3-one	H	OEt		550	1.00	ND	ethanol				
1,7-diacetoxy-3H-phenoxazin-3-one	H	OAc	1-OAc	442	1.31	ND	ethanol				
7,9-diacetoxy-3H-phenoxazin-3-one	H	OAc	9-OAc	460	1.23	ND	ethanol				

E3: Phenoxazin-3-one (continued)

Dye Name	Pos. 4-	Pos. 7-	other Pos.	Solubility H_2O	Solubility EtOH	Other Solvents	Other Information	Solubility H_2O	Solubility EtOH	Other Solvents	Other Information
2-amino-3H-phenoxazin-3-one	NH_2	H		464	2.00	ND	ethanol				
				422,434	2.19	ND	methanol				
				463	2.00	ND	methanol/HCl				
7-amino-3H-phenoxazin-3-one	H	NH_2		520,545	1.23,1.51	ND	ethanol				
				530,560	3.98	ND	methanol				
				610	3.98	ND	methanol/HCl				
2-phenylamino-3H-phenoxin-3-one	2-NHPh	H		448	2.82	ND	chloroform				
2-phenylamino-7-ethoxy-3H-phenoxin-3-one	2-NHPh	OEt		468	3.31	ND	ethanol				
1-methyl-7-dimethylamino-3H-phenoxazin--3-one	H	NMe_2	1-Me	560	4.90	ND	ethanol				
2-piperidino-3H-phenoxazin-3-one	$N(CH_2)_5$	H		443	1.66	ND	chloroform				
7-piperidino-3H-phenoxazin-3-one	H	$N(CH_2)_5$		580	10.0	ND	ethanol				
2-morphilino-3H-phenoxin-3-one	morpholino	H		440	1.66	ND	chloroform				
7-morphilino-3H-phenoxin-3-one	H	morpholino		568	4.46	ND	ethanol				
2-morphilino-7-hydroxy-3H-phenoxin-3-one	morpholino	OH		472	2.82	ND	ethanol				
	NHCOMe	H		400	2.45	ND	methanol				
	H	NHCOMe		471	2.20	ND	methanol				

E4: 5H-benzo[a]phenoxazin-5-one

Dye Name	Pos. 5-	Pos 9-	other Pos.	Absorbance nm	ε x 10^4 $l\,mol^{-1}cm^{-1}$	Fluorescence nm	Solvent	pKa	Solubility H_2O	EtOH	Other Solvents	Other Information
5H-benzo[a]phenoxazin-5-one	O	H		429	1.26	538	ethanol					
				427	1.26	ND	dioxane					
				432	1.36	ND	DMSO					
10-methyl-5H-benzo[a]phenoxazin-5-one	O	H	10-Me	440	1.35	ND	ethanol					
				456	1.45	ND	chloroform					
				HCl	1.48	ND	HCl					
9-amino-5H-benzo[a]phenoxazin-5-one	O	NH_2		496	ND	596	ethanol (solid)					
9-hydroxy-5H-benzo[a]phenoxazin-5-one	O	OH		463	2.29	ND	ethanol					
				561	2.09	ND	ethanol/HCl					
10-chloro-5H-benzo[a]phenoxazin-5-one	O	H	10-Cl	436	1.07	ND	ethanol					
				446	0.63	ND	HCl					
11-acetyl-5H-benzo[a]phenoxazin-5-one	O	H	11-Ac	440	1.29	ND	ethanol					
				460	1.12	ND	HCl					
9-dimethylamino-5H-benzo[a]phenoxazin-5-one	O	NMe_2		544	2.29	621	ethanol					
9-nitro-5H-benzo[a]phenoxazin-5-one	O	NO_2		430	1.10	ND	ethanol					
				446	1.02	ND	HCl					
5H-dibenzo[a,h]phenoxazin-5-one	O	benzo	8-benzo	481	1.48	ND	ethanol					
				472	2.09	ND	dioxane					
				481	1.45	ND	DMSO					
5H-dibenzo[a,j]phenoxazin-5-one	O	H	10,11-benzo	490	2.09	ND	ethanol					
				481	1.45	ND	DMSO					
11-methoxycarbonyl-5H-benzo[a]phenoxazin--5-one	O	H	11-COOMe	430	1.38	ND	ethanol					
				440	1.02	ND	HCl					
8,10-dichloro-5H-benzo[a]phenoxazin-5-one	O	H	8,10-Cl_2	430	0.89	ND	ethanol					
				440	1.02	ND	HCl					

C4: 5H-benzo[a]phenoxazin-5-one continued.

Dye Name	Pos. 6-	Pos 9-	other Pos.	Absorb-ance nm	ε x 10⁴ $M^{-1}cm^{-1}$	Fluor-escence nm	Solvent	pKa	Solubility H_2O	Solubility EtOH	Other Solvents	Other Information
Nile Red	O	NEt2		550	2.63	650	ethanol	1.0	0.02%	0.1%	s in E & Xyl	Purple red (570) in NaOH
Nile Blue A oxazone				643	4.00	641	methanol			red	s in most	Orange (491) in H_2SO_4, on dilution
Vile Blue				645		633	hexan-1-ol				organic	blue green (595,637)
CAS # 7385-67-3				605		533	hexane				solvents	Blue green (595,637) in HCl
				653		630	propan-1-ol				vs in DMSO	pHO3 blue with no fluorescence
				533		598	chloroform					(595,637), pHO3 red (570) with
				635		631	acetone					fluorescence at 652nm (50% methanol)
				643		632	pyridine					Dimer Abs @ 520nm & Fl. @ 650nm
				650		644	nitrobenzene					Molecular dimensions 3.9 x 9.4 x 16.8Å
				650		650	DMSO					τf = 3.70ns EtOH: τf = 2.86ns MeOH
				427/480	1.66	ND	H_2SO_4					τf = 1.89ns G: τf = 2.46ns EG
				528		585	ether					mp 203-205 °C
				635		640	acetonitrile					Temperature Dependence -0.08% per °C
				550		652	60% methanol					
				485		525	heptane					
				610		650	xylene					
				580		663	water					

E5: 9H-benzo[α]phenoxazin-9-one

Dye Name	Pos. 5-	Pos 9-	other Pos.	Absorb-ance nm	ε x 10^4 ($M^{-1}cm^{-1}$	Fluor-escence nm	Solvent	pKa	Solubility H_2O	Solubility EtOH	Solubility Other Solvents	Other Information
9H-benzo[α]phenoxazin-9-one	H	O		505	1.86	ND	ethanol					
				478	1.55	ND	dioxane					
				490	1.58	ND	DMSO					
				483	1.88	ND	ethanol					
8-methyl-9H-benzo[α]phenoxazin-9-one	NH_2	O		572	4.11	630	ethanol					
				595	6.12	630						
				615	8.33	630						
				522	3.24	615	ethanol/NaOH					
				510	3.47	570	cation					
				540,586	3.09,2.88	ND	dication					
5-hydroxy-9H-benzo[α]phenoxazin-9-one	OH	O		461	0.98	ND	ethanol					
				578	2.00	ND	ethanol (anion)					
10-morphilino-9H-benzo[α]phenoxazin-9-one	H	O	10-mor	491	2.40	ND	chloroform					
5-phenylamino-9H-benzo[α]phenoxazin-9-one	NHPh	O		455	1.05	ND	ethanol					
				625	1.09	ND						
				525	2.43	ND	anion					
				540	3.55	ND	cation					
				600	3.63	ND	dication					

E6: 10H-benzo[α]phenoxazin-10-one

Dye Name	Pos. 9-	Pos 10-	other Pos.	Absorb-ance nm	ε x 10^4 ($M^{-1}cm^{-1}$	Fluor-escence nm	Solvent	pKa	Solubility H_2O	Solubility EtOH	Solubility Other Solvents	Other Information
10H-benzo[a]phenoxazin-10-one	H	O		500	0.89	ND	ethanol					
				484	0.71	ND	dioxane					
				495	0.78	ND	DMSO					
9-piperidino-10H-benzo[α]phenoxazin--10-one	$N(CH_2)_5$	O		498	3.09	ND	chloroform					

§7: Thiazine

CI No	CAS #	Dye Name	Pos. 3-	Pos 7-	other Pos.	Absorb-ance nm	ε x 10^4 $l\,mol^{-1}cm^{-1}$	Fluor-escence nm	Solvent	pKa	er s.	Solubility H_2O	Solubility EtOH	Other Solvents	Other Information
52000	561-64-6	Thionin (Ehrlich) Lauth's Violet	NH_2	NH_2	-	598	6.01	616	water pH3	11.0 (11.2)		0.25% violet vs in hot water	0.25% violet	I in E, Xyl ss in Chl s in Ac & Gl	Molecular dimension 3.8 x 7.0 x 13.0 Å Brownish red ppt in NaOH. Yellowish-green in H_2SO_4, on dilution blue then violet. Blue in dilute HCl. Crimson in acetone, Mauve in pyridine. Saturated conc. 1.1 x 10^{-3}M (ethanol) Metachromic dye.
						597	6.31	622	methanol						
						597		ND	ethanol						
						600		628	acetone						
						600		629	nitrobenzene						
						603		ND	water						
						607		628	hexan-1-ol						
						612		632	pyridine						
						670		ND	20N H_2SO_4						
52002	531-57-7	Azur C	NH_2	NHMe	-	608		ND	water			2% blue	0.7% blue	I in D vss in Xyl ss in Chl & Ac	Pure blue in chloroform. 1% soln in deionised water previously adjusted to pH7 has a pH of 2.35
52005	531-53-3	Azur B, Azur I Methylene Azur	NHMe	NMe_2	-	650		670	water			s blue	ss blue	I in D, E & Xyl ss in Chl s in Py vs in Gl	Violet ppt with pink soln in NaOH Green in H_2SO_4, on dilution blue Blue in ethanol and water. 1% soln in deionised water previously adjusted to pH7 has pH of 7.0 Dimer Abs @ 600nm. Metachromic dye. mp mp 205°C (dec)
						638		665	methanol						
						643		667	ethanol						
52010	531-55-6	Azur A	NH_2	NMe_2	-	625		645	water			4.15% blue	0.65% blue	vss in Xyl, Chl & D I in E	Yellow (404,437) in fuming H_2SO_4 Green (404,437,705) in conc H_2SO_4 Blue (712) in conc HCl Dull pink ppt, pink soln (522) in NaOH Green in H_2SO_4. Blue in EtOH & water. Turquoise in ethylene glycol. 1% soln in deionised water previously adjusted to pH7 has a pH of 7.7 mp 290°C (dec)
						628		653	methanol						
52015	61-73-4	Methylene Blue CI Basic Blue 9 CI Solvent Blue 6	NMe_2	NMe_2	-	653	6.01	682	water			2.5% blue	2.0% blue	0.02% Chl I in E & Xyl ss in D & Chl. s in Ac, Gl & Py. vs in EG	mp = 100-10C (dec). Dimer Abs @ 570nm Dull pink ppt, violet soln in NaOH Yellow-green in H_2SO_4, on dil. blue. Reduced by Zn dust/dil H_2SO_4, colour restored slowly by air oxidation or anaerobically by light. Oxidation rate increased the higher the pH. Oxidises on standing and especially in soln to give demethylated forms. Reduced compound sticks to glass & is stable under N_2 and in the dark. Forms double salt with inorganic salts Saturated conc. 0.1M (ethanol) Excellent photostability.
						653		676	methanol						
						664	8.32	ND	pyridine						
						665	8.31	ND	HCl						
						668		683	0.01M NaOH						

67: Thiazines (continued)

CI No	CAS #	Dye Name	Pos. 3-	Pos 7-	other Pos.	Absorb-ance nm	ε x 10^4 $M^{-1}cm^{-1}$	Fluor-escence nm	Solvent	Solubility H_2O	Solubility EtOH	Solubility Other Solvents	Other Information
52020	6722-15-6	Methylene Green CI Basic Green 5	NMe_2	NMe_2	4-NO_2	650 650 655	2.0	680 ND 678	methanol pH3 water	1.5%	.12%	I in Xyl vss in D s in Ac,Chl,Py	Violet black ppt, violet soln in NaOH Yellow-green H_2SO_4, on dil blue then violet. Greenish blue in water & EtOH
52025	6990-74-5	Thiazine Blue Thionin Blue GD New Methylene Blue H CI Basic Blue 25 Basic Blue GO	NMe_2	NEt_2	-	672		ND	water	I	0.03%		Violet with violet ppt in NaOH Yellowish-green in H_2SO_4, on dilution blue
52030	1934-16-3 6586-05-6	New Methylene Blue N CI Basic Blue 24	NHEt	NHEt	2,8-Me	636		ND	water	3.9%	1%		Chocolate brown ppt in NaOH Yellowish-green in H_2SO_4, on dil blue Violet blue in cold water, pale blue in hot water.
52035		Thiocarmine R	NEt-Be-SO3	NEt-Be-nSO3Na		ND		ND		s blue	ss		Grass green in H_2SO_4, on dilution bright blue.
52040	6586-04-5 92-31-9	Toluidine Blue O Tolonium Chloride CI Basic Blue 17 Methylene Blue T Methylene Blue T extra	NH_2	NMe_2	2-Me	629 629 640	0.3	650 655 666	ethanol methanol water	3.82% blue to violet	.57% blue	I in E vss in Chl ss in Xyl, Py, & D. s in Ac.	LD50 intro venus: mice 27.56 mg/kg, rats 28.93 mg/kg, rabbits 13.44 mg/kg Dark green (411,690) in H_2SO_4, on dilution blue green (590). Dull violet ppt in NaOH. pH 12 (530) Pink. 1% soln in deionised water previously adjusted to pH7 has a pH of 2.25 Dimer Abs @ 595nm
		Methylene Blue NNX New Methylene Blue NNX	NHMe	NHMe	2,8-Me	636		ND		13.3%	1.65%		
	92-84-2	Phenothiazine	H	H		ND		ND					mp 182C, bp 371C: Merck 11-7220

C8: Thiazin-3-one

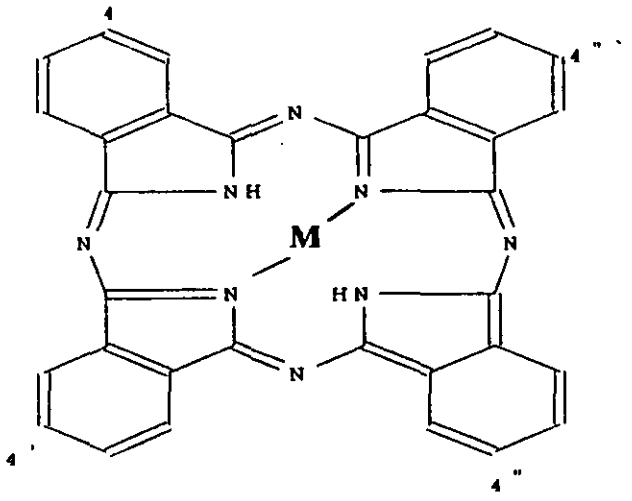
CI No	CAS #	Dye Name	Pos. 4-	Pos 7-	other Pos.	Absorb-ance nm	e x 10^4 $lM^{-1}cm^{-1}$	Fluor-escence nm	Solvent	pKa	Solubility H_2O	Solubility EtOH	Solubility Other Solvents	Other Information
		Phenothia-3-one	H	H		605		ND	methanol			s		
		Thionol	H	OH		618		ND	methanol	5.8		s	s in dil alkali	
						603		ND	methanol/HCl					
			H	O⁻		590	6.31	ND	ethanol/KOH					
						570		ND	methanol/NaOH					
52041	2516-05-4	Methylene Violet (Bernthsen)	H	NMe_2		610	17.78	ND	methanol		0.06%	0.4%	s in Xyl, Chl, & D. vs in Py.	Red in acetone. Metachromic dye. 1% soln in deionised water previously adjusted to pH7 has a pH of 9.25
						596		ND	water					
52045		Leucogallothianine DH	OH	NR_2	1-COR'	ND		ND			s in hot			R=Me or Et, R'=OH, OMe or NH_2 Pale violet blue in water Red in H_2SO_4, on dil. reddish violet Oxidised to bluish violet by NaOHaq.

C9: Benzo[α]phenothiazin-5-one

CI No	CAS #	Dye Name	Pos 7-	other Pos.	Absorb-ance nm	e x 10^4 $lM^{-1}cm^{-1}$	Fluor-escence nm	Solvent	pKa	Solubility H_2O	Solubility EtOH	Solubility Other Solvents	Other Information
52050		Indochrome S Indochromogen S	NEt_2	2-SO_3Na						s			Greenish Yellow in H_2SO_4, on dilution brownish yellow. Dull violet in NaOH, changing to blue on boiling. Reddish violet in water. Precursor, formed in situ.
52055	2861-02-7	Indochromine T Alizarin Brilliant Blue G, R CI Mordant Blue 61	NMe_2	2-SO_3Na					8.2	s in hot			Green in H_2SO_4 on dilution violet ppt Blue in water.
52060		Brilliant Alizarin Blue R	NEt-Be-nSO_3Na	6-OH	ND		ND			s blue			Green in H_2SO_4, on dil violet blue ppt Blue ppt in HClaq

Appendix F

PHTHALOCYANINES



F: Phthalocyanines

CI No	CAS #	Dye Name	Metal	4,4',4'',4'''- Position	Absorbance nm	ε x 10^4 $l\,mol^{-1}cm^{-1}$	Fluorescence nm	Solvent	Solubility H_2O	Solubility EtOH	Solubility Other Solvents	Other Information
74100	574-93-6	Phthalocyanine CI Pigment Blue 16	-		664,602,638, 665,700 406,772 340,622,686 332,384,610, 638,670,700	.97,2.69,4.17, 15.1,9.12	698 ND ND ND	chloronaphthalene solid state vapour phase dichloromethane	I	I	I in most solvents	ϕ_f=0.7 chloronaphthalene τ_p 0.28μs in 10% DMF @ 290K τ_p=1μs @296K, τ_p=150μs @77K. Olive solution, in H_2SO_4, on dilution blue suspension.
		Aluminium phthalocyanine chloride	Al		280,340,365, 696,633,662		ND	H_2SO_4			s in Chl & DCM	
74160:2	3317-67-7	Cobalt phthalocyanine	Co		212,296,368,738 657		ND ND	H_2SO_4 chloronaphthalene	s	ss	s in EG, Gl, Py ss in D, Chl, Ac I in E	Blue pigment, redder & duller than CuPc. Good light fastness. Stable to H_2SO_4. Olive when reduced. Destroyed by peroxide or hypochlorite
74160	147-14-8	Copper phthalocyanine CI Pigment Blue 15	Cu		620,650,670 312,325,657 446,779,821	3.98,3.98,10.0	ND ND ND	chloronaphthalene vapour phase H_2SO_4	I	I	I in most solvents s in 98% H_2SO_4	Ph 1062nm, $\phi_p=10^{-4}$ @ 300K Ph 1065nm, τ_p <3μs, $\phi_p=10^{-3}$ @77K Ph 1078nm, τ_p <10μs, $\phi_p=10^{-4}$ @2K in chloronaphthalene Olive solution in H_2SO_4, on dilution blue ppt. Merck 11-2515. Decomposed by hot HNO_3 or dil. acidic $KMnO_4$ to yield phthalimide. Stable to heat, alkalis or dilute acids.
		Chromium phthalocyanine	Cr		315,664		ND	vapour phase				Covalent dull green pigment. Poor lightfastness. Forms complexes with basic dyes.
	132-93-6	Iron phthalocyanine	Fe		676		ND	chloronaphthalene				
		Lead phthalocyanine	Pb		696		ND	vapour phase				
	1661-03-6	Magnesium phthalocyanine	Mg		568,667,610 332,666 666 ND	.39,.62,2,82	ND ND 683 680	pyridine vapour phase chloronaphthalene acetone	I		s in THF & DCM	τ_f=1ns, ϕ_f=.6, τ_p=1ms, Ph=1111nm τ_p=5x10^{-6} @77K in chloronaphthalene. Ph=1100nm @77K in acetone Covalent unstable blue pigment. Forms stable crystalline dihydrate & amine complexes. In organic solvents & peroxides gives red chemiluminescence.
	14325-24-7	Manganese phthalocyanine	Mn		718		ND	water				Covalent dull brownish green pigment. De-metallised by mineral acids.
74160:1	14055-02-8	Nickel phthalocyanine	Ni		327,676 738		ND ND	vapour phase H_2SO_4				Covalent greenish blue pigment. Outstanding lightfastness. Stable to H_2SO_4.

F: Phthalocyanines (continued)

CI No	CAS #	Dye Name	Metal	4,4',4",4"'- Position	Absorbance nm	ε x 10^4 ($l^{-1}cm^{-1}$)	Fluorescence nm	Solvent	Solubility H2O	Solubility EtOH	Solubility Other Solvents	Other Information
74220	36485-85-5	Copper tetrasulphonate phthalocyanine CI Acid Blue 249	Cu	$(SO_3Na)_4$	600,633, 655,695 602,633, 670,690 610(694)	3.98,6.31, 15.8,20.0 2.0,6.31, 15.8,9.77	 ND ND ND	 ethanol water water	0.7% blue	0.02%	ε in DMSO, DMF & MeOH 0.2% in EGME	τ_p=.02μs, $\phi_p=3.5x10^{-5}$ @298K EtOH $\phi_p=4x10^{-5}$ @77K ethanol $\phi_p=2.2x10^{-5}$ @298K water $\phi_p=5x10^{-5}$ @298K DMSO Yellow green in H_2SO_4. Green with ppt in conc. HCl. Decomposes in conc. HNO_3
	27835-99-0	Nickel tetrasulphonate phthalocyanine	Ni	$(SO_3Na)_4$	657		ND	water	0.2%	0.2%	0.05% in EGME	
74240	33864-99-2	Alcian Blue; CI Ingrain Blue 1 Alcian Blue 8GX	Cu	4 $MeSC(NMe_2)_3^+$	629 615		ND ND	water water	 0.3%	0.1%	1 in Xyl, Ac Chl & D s in Py vs in GI	Green solution in H_2SO_4, on dilution blue green solution. 1% soln. in deionised water previously adjusted to pH7 has a pH of 6.3 Unstable in alkali (pH>8) especially on warming.
		Cobolt tetramine phthalocyanine	Co	$(NH_2)_4$	320,570,622,666		ND	chloronaphthalene				$\phi_p=5x10^{-4}$ @298K, $\phi_p=1.6x10^{-3}$ in water
74120		Polychlorinated phthalocyanine	-	14-15 Cl	ND		ND					14-15 Cl atoms per molecule Olive solution in H_2SO_4, on dilution green ppt. Yellower green pigment than CI-74120.
74250		CI Pigment Blue 15 Monochloro copper phthalocyanine	Cu	Cl	ND		ND					Bright blue pigment. Olive solution in H_2SO_4, on dilution blue ppt.
74255		CI Pigment Green 37 Octachloro copper phthalocyanine	Cu	Cl8	ND		ND					Bluish green pigment. Olive solution in H_2SO_4, on dilution blue green ppt.
74260	1328-53-6	CI Pigment Green 7 Phthalocyanine Green	Cu	14-16 Cl	ND		ND					
74265	14302-13-7	CI Pigment Green 36 Copper polybromochloro-phthalocyanine	Cu	12-4 Cl 4-12 Br	ND		ND					
		Alcec Blue	Cu	~3 $SO_2NHPrN^+Me_3$	611		ND	pH5.7 acetate	0.3%	0.6%	0.1% in EGME	Stable to at least pH12.
		Cupromernic Blue	Cu	?	ss				ss			Basic phthalocyanine with quaternary ammonium groups. Used at 0.5% in pH5.7 0.025M sodium acetate buffer.

F: Phthalocyanines (continued)

CI No	CAS #	Dye Name	Metal	4,4',4",4"'- Position	Absorbance nm	ε x 10^4 $l\,mol^{-1}cm^{-1}$	Fluorescence nm	Solvent	Solubility H2O	Solubility EtOH	Solubility Other Solvents	Other Information
	14320-04-8	Zinc phthalocyanine	Zn		326,661 667 696,783 ND		ND ND ND 683	vapour phase DMF H_2SO_4 chloronaphthalene				ϕ_f=3x10^{-3} @300K; τ_f=100ns, ϕ_f=0.3, τ_p=1.1ms, ϕ_p=10^{-4}, Phw1093nm @77K τ_p=1.1ms, ϕ_p=10^{-4}, Phw1092nm @2K In chloronaphthalene Covalent greenish blue pigment. Unstable to light. Forms stable acid salts which can be isolated.
		Tetraphenylphthalocyanine	-	(Ph)4	615,649, 679,713	3.31,4.79, 12.9,13.5	ND	chlorobenzene				
		Cobolt tetraphenylphthalocyanine	Co	(Ph)4	620,690	2.19,10.5	ND	chlorobenzene				
74280		Copper tetraphenylphthalocyanine Ingrain Green 3	Cu	(Ph)4	ND		ND					Green solution in H_2SO_4, on dilution green suspension Green pigment.
		Lead tetraphenylphthalocyanine	Pb	(Ph)4	655,725	2.04,12.6	ND	chlorobenzene				
		Manganese tetraphenylphthalocyanine	Mn	(Ph)4	630,670,745	.72,.83,4.67	ND	trichlorobenzene				
		Palladium tetraphenylphthalocyanine	Pd	(Ph)4	616,676	8.51,7.76	ND	chlorobenzene				
		Tin tetraphenylphthalocyanine	Sn	(Ph)4	645,720	4.67,26,3	ND	chlorobenzene				
		Tetrakis-t-butylphthalocyanine	Pb	(t-C4H9)4	420,550,582, 630,639,654, 696	.61,.33,2.69 3.71,6.75,11.6 17.4	ND	heptane				
		Aluminium tetrasulphonate phthalocyanine	Al	$(SO_3Na)_4$	ND		680	chloronaphthalene				τ_f=5ns, ϕ_f=0.6, τ_p=330µs @298K pH3-12
74140		Cobolt phthalocyanine CI Vat Blue 29 Indanthrene Brilliant Blue 4G	Co	SO_3Na	617	ND	ND	water	s	ss	vss in Xyl,Chl, D & Ac s in Gl, Py & EG	Partly sulphonated, bright blue pigment
74180	1330-38-7	CI Direct Blue 86 Durazol Blue 6G	Cu	$(SO_3Na)_2$	617(670)		ND	water	s	vss	i in Xyl, Ac & Chl. vss in Py & D s in Gl	Yellowish green in H_2SO_4, on dilution greenish blue with bluish green ppt. Bright greenish blue pigment. Not influenced by pH. No colour changes in either dil. HCl or NaOH.
74200	1330-39-8	CI Direct Blue 87; CI Pigment Blue 17	Cu	$(SO_3Na)_3$	ND		ND					Green in H_2SO_4, on dilution greenish blue with olive green ppt. Bright greenish blue pigment.
		Chromium tetrasulphonate phthalocyanine	Cr	$(SO_3Na)_4$	344,477,602, 655,695	.86,3.96,- -,14.8	ND	methanol				

G Phthalocyanines Trade Names and Manufacturers

Acid phthalocyanine dyes			
	CI Acid Blue 185	Cibacrolan Blue 8G	Ciba-Geigy AG
		Pergacid Turquoise Blue RAL	Ciba-Geigy AG
		Eriosin Fast Blue 8G	Ciba-Geigy AG
		Coomassie Turquoise Blue 3G	ICI, Manchester
	CI Acid Blue 197	Oxanal Fast Turquoise Blue FGLL	ICI, Manchester
	CI Acid Blue 242	Acid Brilliant Blue DH	Sandoz AG
	CI Acid Blue 243	Aluminium Turquoise PLW	Sandoz AG
	CI Acid Blue 254	Aluminium Blue RL	Sandoz AG
	CI Basic Blue 33	Methic Fast Paper Blue 6G	ICI, Manchester
Direct phthalocyaine dyes			
74180	CI Direct Blue 86 (Cu Pc disulphonate disodium salt)	Chlorantine Fast Turquoise GLL, VLL	Ciba-Geigy (UK)
		Pergasol Turquoise Blue GAL	Ciba-Geigy (UK)
		Solophenyl Turquoise Blue GL, GTL,	Ciba-Geigy AG
		Durazol Blue 8G	ICI, Manchester
		Cuproxil Printing Blue Green B	Sandoz AG
		Dermafix Blue GLL	Sandoz AG
		Finisol Blue Green G	Sandoz AG
		Solar Turquoise Blue GLL	Sandoz AG
	CI Direct Blue 199 (Simillar to CI 74180	Chlorantine Fast Turquoise Blue BRLL Solophenyl	Ciba-Geigy AG
		Turquoise Blue FL	Ciba-Geigy AG
		Durazol Turquoise Blue FBS	ICI, Manchester
		Nylomine Acid Turquoise P-B	ICI, Manchester
		Carta Turquoise GL	Sandoz AG
		Cuproxil Blue Green FBL	Sandoz AG
		Cuproxil Turquoise Blue FBL	Sandoz AG
		Solar Turquoise Blue FBL	Sandoz AG
74200	CI Direct Blue 87 (Sodium salt of Cu Pc trisulphonic acid)	Durazol Paper Blue 10G	
	CI Direct Blue 189	Solophenyl Turquoise Blue GRL	Ciba-Geigy AG
		Durazol Turquoise Blue GR	ICI, Manchester
		Pyrazol Fast Turquoise FBLN	Sandoz AG
	CI Direct Blue 262	Cartosol Turquoise B-GL	Sandoz AG
Ingrain phthalocyanine dyes			
74240	CI Ingrain Blue 1 (Cu Pc with 4 omium groups)	Alcian Blue 8GX	ICI, Manchester
	CI Ingrain Blue 3	Alcian Blue 7GX	ICI, Manchester
	CI Ingrain Blue 4	Alcian Blue 2GX	ICI, Manchester
	CI Ingrain Blue 8	Alcian Blue 5GX	ICI, Manchester
Mordant phthalocyanine dyes			
	Mordant Blue 58	Panduran Blue Turquoise	Sandoz AG
	Mordant Blue 77	Panduran Blue B	Sandoz AG
	Mordant Green 54	Panduran Green G	Sandoz AG

Pigment phthalocyanine dyes			
74100	CI Pigment Blue 16	Irgalite Blue 3GT	Ciba-Geigy AG
	(Pc)	Irgazine Blue 3GT	Ciba-Geigy AG
		Monastral Fast Blue G	ICI, Manchester
		Polymon Blue G	ICI, Manchester
		Vulcafor Fast Blue G	ICI, Manchester
74160	CI Pigment Blue 15	Irgalite Blue BCX, BGL, BL, BLP, BNL	Ciba-Geigy AG
	(Cu Pc: unstable	Irgalite Blue BNS, BNX Paste,SPV1	Ciba-Geigy AG
	form)	Irgalit Paper Blue BNL	Ciba-Geigy AG
		Tinofil Blue BL	Ciba-Geigy AG
		Tinolite Brilliant Blue MRL	Ciba-Geigy AG
		Daltolite Fast Blue B	ICI, Manchester
		Monastral Fast Paper Blue B	ICI, Manchester
		Monastral Fast Blue B, BNV, BV, BX, FB	ICI, Manchester
		Vulcafor Fast Blue BN	ICI, Manchester
		Vulcatex Fast Blue BS	ICI, Manchester
		Vynamon Fast Blue BLBA	ICI, Manchester
	CI Pigment Blue 15:1	Irgafin Blue S2	Ciba-Geigy AG
	(α form, non-	Irgafiner Blue E2	Ciba-Geigy AG
	crystallising)	Irgalite Blue BCA, BCS, B3NF, RPB	Ciba-Geigy AG
		Sandorin Blue BNF	Sandoz AG
	CI Pigment Blue 15:2	Graphtol Blue BLF	Sandoz AG
	(α form, flocculation		
	resistant)		
	CI Pigment Blue 15:3	Irgafin Blue S1	Ciba-Geigy AG
	(β form, solvent	Irgafiner Blue E1	Ciba-Geigy AG
	stable, greener	Irgalite Blue CPV2, GFR, GLA, GLV, GLSM	Ciba-Geigy AG
	than the α form)	Irgalite Blue GST, LGLD, PD55, PR7, PR3N	Ciba-Geigy AG
		Monastral Fast Blue BG, LB, LBC, LBX	ICI, Manchester
		Graphtol Blue 2GLS	Sandoz AG
74180:1	CI Pigment Blue 17:1	Monosol Fast Blue 2G, 2GP	ICI, Manchester
	(Ba salt of Cu Pc		
	di/trisulphonic acids)		
74260	CI Pigment Green 7	Chromphthal Green GF	Ciba-Geigy AG
	(Cu Pc with 15-16	Irgafin Green S1	Ciba-Geigy AG
	chlorine atoms per	Irgalite Green CPV4, GLN, GLNP	Ciba-Geigy AG
	molecule)	Irgalite Paper Green 3GL	Ciba-Geigy AG
		Tinofil Green GLN	Ciba-Geigy AG
		Tinolite Green MB	Ciba-Geigy AG
		Daltolite Fast Green GN	ICI, Manchester
		Monastral Fast Green GD, GN, GTP, GTV	ICI, Manchester
		Polymon Green G, 6G, GN	ICI, Manchester
		Vulcatex Fast Green GS	ICI, Manchester
		Vynamon Green BE	ICI, Manchester
		Graphtol Green 2GLS	Sandoz AG
		Sandorin Green GLS	Sandoz AG
	CI Pigment Green 36	Monastral Fast Green 3YA, 6Y	ICI, Manchester
	(polybromo/chloro Pc	Vynamon Green 6Y	ICI, Manchester
	Yellower shades have		
	higher no. of Br atoms)		

Reactive phthalocyanine dyes.

74460	CI Reactive Blue 7 (Cu Pc tetra-. sulphonic acid, with 1 amide & 1 sulphonyl amide group)	Cibacron Turquoise G-E	Ciba-Geigy Ag
		Procion Turquoise H-G	ICI, Manchester
	CI Reactive Blue 14	Cibracron Brilliant Blue FC4G-P	Ciba-Geigy AG
	CI Reactive Blue 15	Cibracron Turquoise FGF-P	Ciba-Geigy AG
		Procion Turquoise H-GF	ICI, Manchester
	CI Reactive Blue 18	Reactone Turquoise Blue FGL	Ciba-Geigy AG
		Drimarne Turquoise X-G, X2G, Z-G	Sandoz AG
	CI Reactive Blue 25	Procion Brilliant Blue H-5G	ICI, Manchester
	CI Reactive Blue 41	Cibacron Turquoise 2G-E	Ciba-Geigy AG
		Procion Turquoise H-2G	ICI, Manchester
	CI Reactive Blue 63	Procion Supra Turquoise H-2GP	ICI, Manchester
	CI Reactive Blue 71	Procion Turquoise H-A	ICI, Manchester
	CI Reactive Blue 72	Cibacron Turquoise Blue GR-D	Ciba-Geigy AG
	CI Reactive Blue 85	Reactofil Turquoise Blue GL	Ciba-Geigy AG
	CI Reactive Green 5	Cibacron Brilliant Green C4GA	Ciba-Geigy AG
		Pergasol Brilliant Green 3GAL	Ciba-Geigy AG
		Procion Brilliant Green H-4G	ICI, Manchester
	CI Reactive Green	Drimarene Brilliant Green X-3G, Z-3G	Sandoz AG

Solvent phthalocyanine dyes

	CI Solvent Blue 46	Acetosol Blue GLS	Sandoz AG
		Telasol Blue GLS	Sandoz AG
	CI Solvent Blue 52	Orasol Brilliant Blue G	Sandoz AG
	CI Solvent Blue 67	Orasol Brilliant Blue GN	Sandoz AG

Ciba-Geigy AG, Basel, Switzerland.
Ciba-Geigy (UK), Manchester, England.
ICI Ltd, Manchester, England.
Sandoz AG, Basel, Switzerland.