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The City University, London 1989

NOVEL LUMINESCENT COMPOUNDS FOR IMMUNOASSAY

by

STUART BLINCKO

A thesis submitted for the degree of PhD
in the Chemistry Department.

Supervisor: Professor R S Davidson

To Mum and Dad

with my love

And to Peter Rees

with much gratitude

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ACKNOWLEDGMENTS

So many people have made a contribution to this work that it is hard to list them all. I am very much in their debt either for help scientifically or for being good workmates or for hospitality and kindness. Hopefully the list below remembers most of them.

At City: Martin Walker, Sue Wilkinson, Fiona Henderson, Graham Yearwood, Brian Field, Tom Rose, Raj Bhardwaj, Alan Osborne, Chris Whitehead, Julie Pratt, Jane Baxter, Martin Hilchenbach and Stephen Collins.

My special thanks to Dave Abram for all his help in my first year and to Eva Radkowska for typing this work.

At Celltech: Richard Titmus, Alison Phipps and Andy Millican.

Thanks to Mike Eaton for all his help as my "industrial supervisor", to Celltech for sponsoring the work and for the three months I spent working with them.

At Boots-Celltech: Chris Bose, John Wright, Hishani Williams and Byron Boyce.

At Durham University: David Parker for his advice and help with the FAB mass spectra.

Thanks also to my longsuffering landlords and landladies:

Keith Bashford

Edith Roberts

Chris and Sue Dandridge

Monica Lamond

and David and Mel Toms.

Finally, I am very grateful to Prof Davidson for all his help, enthusiasm and patience with me. I count it a real privilege to have worked for him.

Foreword

This thesis describes the synthesis and application of luminescent compounds for immunoassay. The main body of the work describes the synthesis of luminescent transition metal complex compounds of ruthenium and chromium for antibody labelling. To set this work in context, the first four chapters are introductory.

Chapters 1 and 2 outline the theory of luminescent processes at a molecular level. Chapter 1 covers organic compounds and chapter 2 inorganic compounds. The main thrust of these two chapters is to provide a basis for the design of strongly luminescent compounds for antibody labelling. Chapter 3 describes antibodies with particular reference to their structure and chemistry. Chapter 4 discusses the ways in which immunoassays are carried out and sets the requirements for a successful luminescent transition metal-antibody conjugate for immunoassay development.

The next three chapters present my own work. Chapter 5 describes the work carried out to achieve these goals by synthesising ruthenium, chromium and aluminium complex compounds. Chapter 6 goes on to present the results of using the compounds made in chapter 5 as antibody labels, and discusses their properties. Chapter 7 details the experimental work that is discussed in the

previous two chapters.

Finally an epilogue, sets all the work in a much wider context, and is there for enjoyment and to stimulate further thought.

ABSTRACT

The thesis describes the synthesis of ruthenium bipyridyl and phenanthroline compounds that are luminescent. Several of the compounds were suitably derivatised for protein conjugation ie

$Rubipy_2bipy(CONCS)_2^{2+}.2PF_6^-$; $Rubipy_2bipy(CO_2H)_2^{2+}.2PF_6^-$;

$Rubipy_2bipy(COAnthr)_2^{2+}.2PF_6^-$

(Anthr = anthranilic acid);

$Ruphen_2phen(\phi SO_3H)_2^{2+}.2Cl^-$; $Ruphen_2phen(\phi SO_2Cl)_2^{2+}.2Cl^-$

and $Rudpphen_2phen(\phi SO_3H)_2^{2+}.2Cl^-$

(dpphen = 4,7-diphenylphenanthroline). The excitation and emission spectra were recorded for the above compounds and their luminescent lifetimes determined. Some of the above compounds were conjugated to antibody samples and their luminescent characteristics recorded. It was found that the complex-antibody conjugates had similar properties to their parent complexes possessing a large Stoke's Shift (about 130-140 nm) and a long luminescent lifetime (125-1200 ns). Hence these compounds may be of use as protein labels in time resolved immunoassays.

The thesis also describes the synthesis of some luminescent chromium and aluminium compounds ie

$Crphen_3^{3+}\cdot 3ClO_4^-$; $Crcyclam(CN)_2^+.ClO_4^-$

(cyclam = 1,4,8,11-tetraazacyclotetradecane); $Al(oxine)_3$

(oxine = 8-hydroxyquinoline) and Al(oxine-5-sulphonic acid)$_3$. Their luminescent characteristics were investigated. The preparation of several oxine derivatives was also attempted with varying success.

The thesis ends with an epilogue discussing science as a God-given activity.

Chapter 1

LUMINESCENT COMPOUNDS - PART 1

1.00 Introduction

This thesis involves the use of a large number of luminescent compounds with very different characteristics. Some, like fluorescein, have large quantum yields but short fluorescent lifetimes, whereas others like tris-(2,2'-bipyridine) ruthenous chloride have lower quantum yields than fluorescein but longer emission lifetimes. These next two chapters will review the properties and mechanisms involved in luminescence. A consideration will also be given to the best ways of optimising luminescent properties. This chapter will consider the main principles of luminescence and the theory involved in organic compounds.

1.01 Basic Principles [1,2,3,4,7,8].

A system that is luminescing is losing energy. This implies that a source of energy is required to produce the phenomena. Hence most kinds of luminescence are classified according to their sources of excitation eg. photoluminescence, chemiluminescence, electrochemically generated chemiluminescence and bioluminescence. In this thesis all the systems are photoluminescent (although many exhibit other forms of luminescence) that is to say that uv/visible light provides the excitation energy.

The equation $E = h\nu$ relates the energy of a photon to its corresponding frequency. The uv/visible frequencies are of interest as they are made up of photons which excite the outer electrons of molecules. It is these excitations involving bonding and antibonding orbitals that lead to photochemical and photoluminescent properties.

1.02 Organic Molecules

For an organic molecule it is possible to crudely represent the relative energies of the bonding and antibonding orbitals as follows:

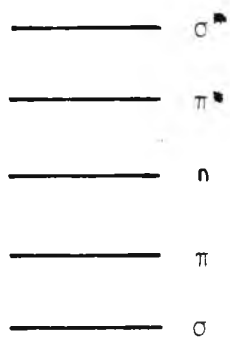


Fig 1

The σ, π and n orbitals may all be occupied by the electrons of a molecule in its ground state. A transition of an electron from one orbital to another results in a change of state. Saturated molecules contain bonding orbitals from which transitions may occur. However, the energy required for such

transitions is very high and they seldom lead to fluorescence. Unsaturated molecules involving π-π^* transitions will often fluoresce. These π-π^* transitions are usually strong, ε_{max}~10,000 - 100,000, unless they are symmetry forbioden. The n-π^* transitions are very weak by comparison, ε_{max}~ 100 due to symmetry factors. This means the populating of the excited state is inefficient and the emissive transition is unfavourable. Consequently, emission from these states is rare and when it does occur it is very weak.

These observations mean that when designing luminescent molecules, groups which give n-π^* transitions are to be avoided. However, that statement needs to be qualified somewhat. It must be remembered that charge transfer states may also lead to luminescence. A consideration of pyridine, pyrrole and aniline illustrates this point.

Pyridine has a lone pair of electrons that are not conjugated into the π system. The highest occupied molecular orbital is a non-bonding orbital and this leads to weak n-π^* transitions. Pyridine, consequently, is not luminescent. Pyrrole also has a pair of electrons, formally at nitrogen, but these are conjugated into the π system and so lead to π-π^* transitions. Pyrrole and many of its derivatives are

fluorescent. Aniline when it is planar has a lone pair of electrons conjugated into the system. However, charge transfer transitions are observed when the lone pair is out of plane with the phenyl ring. These charge transfer transitions lead to fluorescence in this compound.

1.03 The Jablonski Diagram

So far the types of excitation that may lead to luminescence have been reviewed. Now a more detailed consideration will be given to the excitation and emission processes. This will be based on the "Jablonski Diagram" as drawn below:

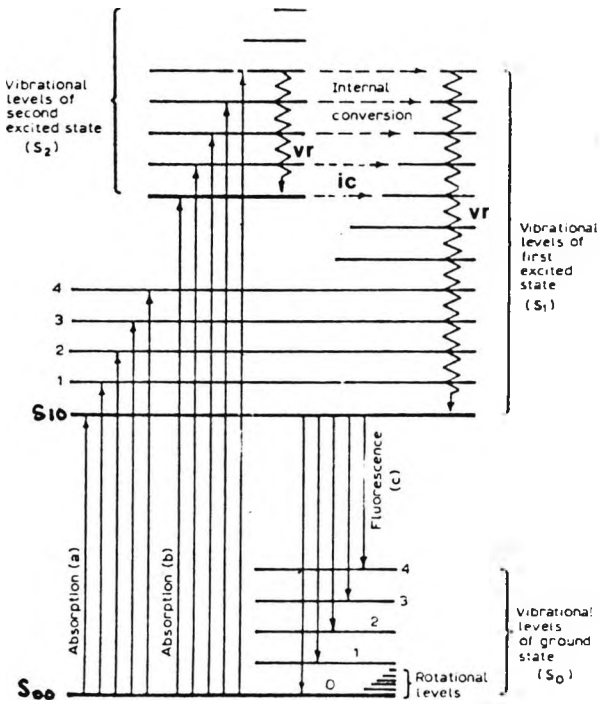


Fig 2

The absorption of a quantum of light by a molecule can only occur if the quantum of energy exactly corresponds to the difference in energy between two energy levels in the molecule. This may be simply illustrated as follows:

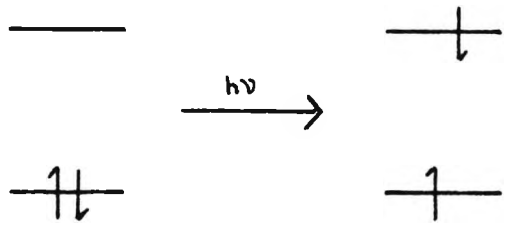


Fig 3

For a molecule the electronic energy levels are not the only important ones to consider. The vibrational and rotational energies are also quantised (during light absorption the translational energy will not change and so this is ignored). These energy levels are described by the Jablonski diagram.

At room temperature (where all the work for this thesis has been carried out) most molecules are in the lowest vibrational level of the electronic ground state ie. S_{oo}. (For all molecules in aqueous solution the rotational levels are so closely spaced that they are not spectroscopically discrete) Therefore transitions, due to an absorption of energy, occur from S_{oo} and a whole range of energy levels may be reached. In Fig 2, above, there are two main groups of energy levels shown.

These are the electronic excited states S_1 and S_2 with their associated vibrational levels.

When a molecule is raised to an upper vibrational level of any excited state it quickly loses its excess of vibrational energy by collisions with surrounding solvent molecules. This is known as vibrational relaxation (VR, Fig 2).

Another process operates called internal conversion (IC, Fig 2). This occurs when a molecule is raised to electronic states higher than the first. The molecule passes from a low level of an upper electronic state to a high vibrational level of a lower excited state. In doing so the molecule retains the same energy. This internal conversion is quicky followed by vibrational relaxation to S_{10}.

S_{10} is a singlet excited state and has a lifetime of about 10^{-9}s. It is from here that light emission may take place. Light emission occurs by the molecule returning to any of the vibrational levels of the ground electronic state. This process is called fluorescence as both electronic states have the same multiplicity.

1.04 Stokes' Shift

The Jablonski diagram shows that the energy of the

excitation process is greater than that of light emission. Consequently the emission wavelengths are longer than excitation wavelengths. This produces spectra of the type below in Fig 4.

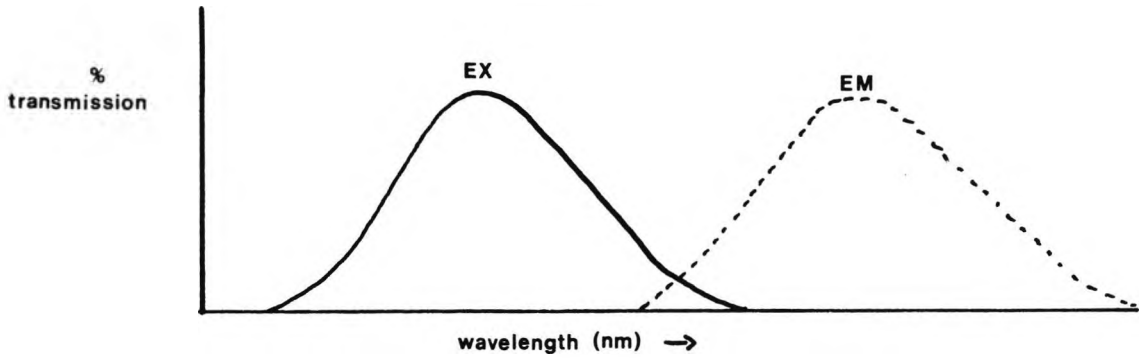


Fig 4

A law called "Stoke s' Law" is based on this observation. It states that the wavelength of florescence is always longer than the wavelength of the exciting light. The difference between the excitaton and emission maxima is called the Stoke s' Shift.

The Stoke s' Shift is determined by the difference in excitation and emission energies. These energies may be affected by different solvents. To understnad this it must be remembered that when a molecule is in its excited state the electronic distribution will be different to the ground state. This leads to a change in the dipolar characteristics and in some cases a change in the shape of the molecule. Excited ethylene is a simple example of this change in character (see Fig 5).

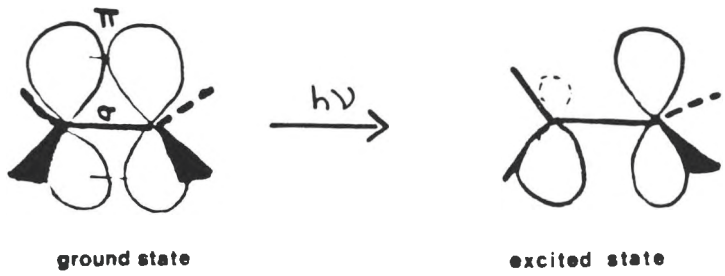


Fig 5

If either the dipole or shape of a molecule changes then so will its solvation characteristics. On excitation a molecule will undergo a re-organisation of its solvation sphere. This will affect the **Stokes'** Shift as follows:

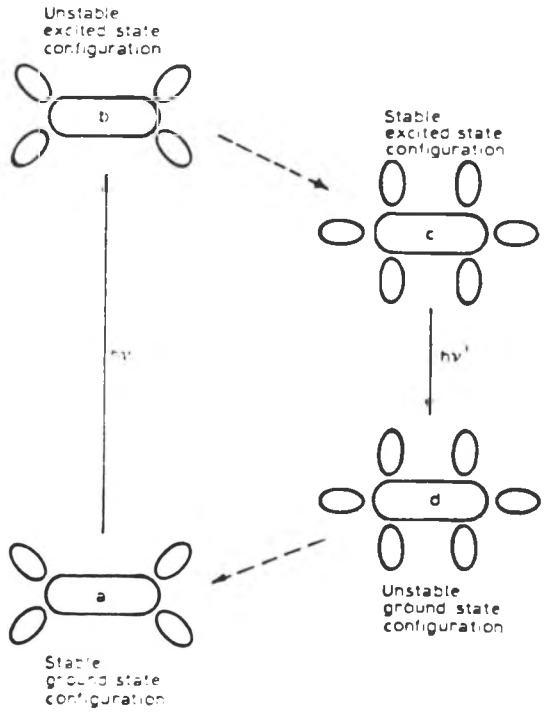


Fig 6

In fluid solution, at room temperature the solvent molecules will not have time to re-orientate themselves during the process of excitation (a)-(b). Hence the molecule is in the same state of solvation as it was in the ground state. This solution state is not stable and so the solvent rearranges to lower the energy of the excited state (b)-(c). It is from this new solvent configuration that the molecule emits light (c)-(d). On light emission a similar process occurs involving solvent re-organisation (d)-(a). The key fact to notice is that the solvent rearrangements lead to a marked difference in absorption and emission energies.

Hence some sense can be made of the statement that the Stokes' Shift is affected by different solvents. Different solvents will stabilise the excited state by different amounts relative to the ground state. More polar solvents tend to increase the Stoke's Shift by lowering the energy of the excited state.

1.05 The Efficiency of Light Emission

If all the molecules that originally absorbed light return to their ground state by light emission the system is said to fluoresce with a quantum yield of one. Normally only a fraction of the molecules excited fluoresce. This means fractional values, quoted decimally, are common. The reason not all the excited

states lead to light emission is that other competing pathways for energy loss operate. For example, molecules may lose energy from S_{10} by vibrational processes. Also the energy may be chanelled across to a triplet state (see 3.8) by intersystem crossing. Another way of losing energy is by chemical reaction. For this thesis the most important competing pathway is quenching by molecules in the solvent - in particular dissolved oxygen.

This effect is particularly marked if the excited state has a long lifetime - ie. more time for the oxygen quenching mechanism to compete. An equation relating quantum yield and lifetime is called the Stern-Volmer equation.

$$\frac{\phi_o}{\phi} = 1 + kq\,\tau\,[Q]$$

ϕ_o = quantum yield in the absence of a quenching species.

ϕ = quantum yield in the presence of a quenching species.

kq = rate constant for quenching.

τ = lifetime (in seconds).

[Q]= quenching species concentration.

The main observation is that quantum yield is increased by the absence of oxygen.

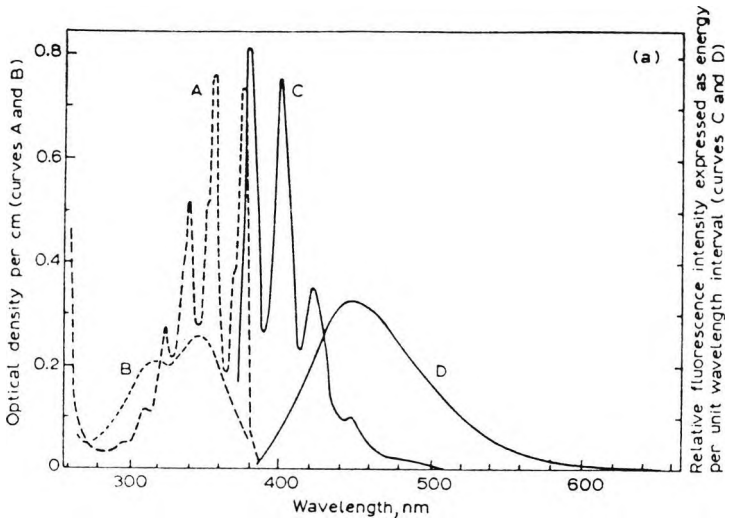
Another important consideration involves how to avoid a molecule losing energy by vibrational relaxation from S_{10}. The quantum yield is optimised by making the molecule as rigid as possible. This cuts down the vibrational pathways considerably. Hence the quantum yield of a compound may be improved by diminishing the effect of competing pathways to the light emission.

1.06 The "Shapes" of the Emission and Excitation Spectra

So far consideration has been given to the ideas of Stokes' Shift and Quantum Yield. However, it is notable that excitation and emission spectra have shape as well as frequency and yield. There are two main factors that govern the "shape" of these bands. These are, firstly, the distribution of vibrational levels within the electronic states and, secondly, the probabilities of transitions occuring between various states.

For simple molecules a vibrational fine structure may be observed (A and C in Fig 7). However, for more complex

molecules, the vibrational levels are so closely spaced that broad bands are observed (b and d in Fig 7).



Curves A and C from Anthracene

Curve B and D from Quinine bisuphate

Fig 7

The compounds handled in this thesis are of the more complex type, and so broad excitation and emission bands are observed.

The probability of transitions occuring is best understood by a description of the "Frank-Condon" principle. This principle is based on the fact that light absorption takes place in about 10^{-15} seconds and so is within the time period of vibration in the molecule. The conclusion from this fact is that the nuclear configuration of a molecule is the same before

and after light absorption. Consider the following diagram, Fig 8.

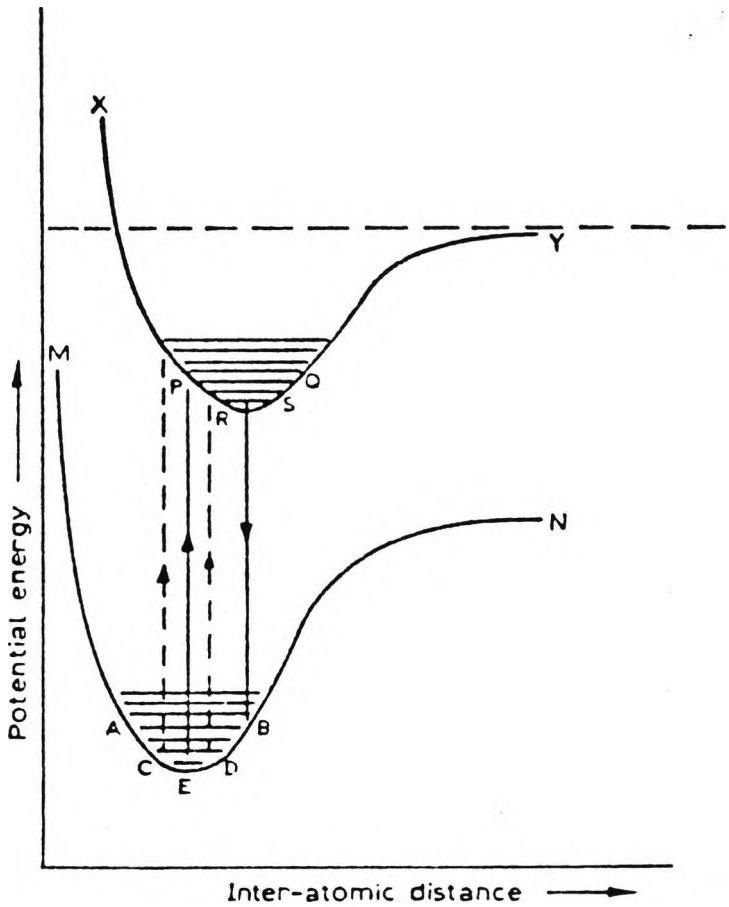


Fig 8

The above representation is two dimensional and so is only adequate for diatomic molecules. This plot shows how potential energy is related to interatomic distance in two electronic states.

The ground state (the lower set) contains a number of vibrational levels. Each of these levels has kinetic and potential energy components in varying proportions. The S_{oo} level has a most probable position (Fig 9) which is at the centre of the vibration.

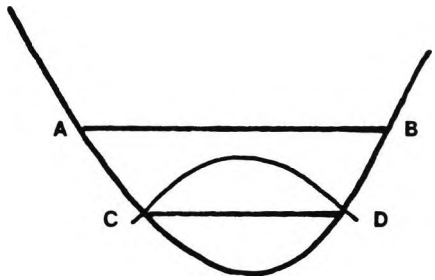


Fig 9

This centre position is where the kinetic energy is at a maximum. However, all the vibrational states above S_{oo} have a different probability profile. They have their most probable position at each end of the vibration where the potential energy is at a maximum - Fig 10.

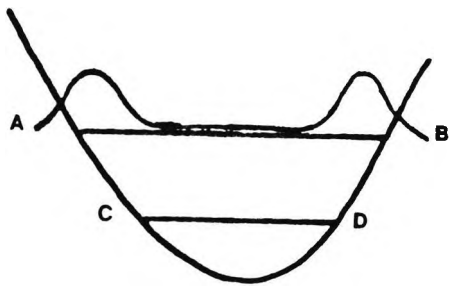


Fig 10

The relevance of this is seen when transitions occur. The "Franck-Condon" principle states that transitions from the ground state occur vertically. Hence some transitions are more probable than others - Fig 11.

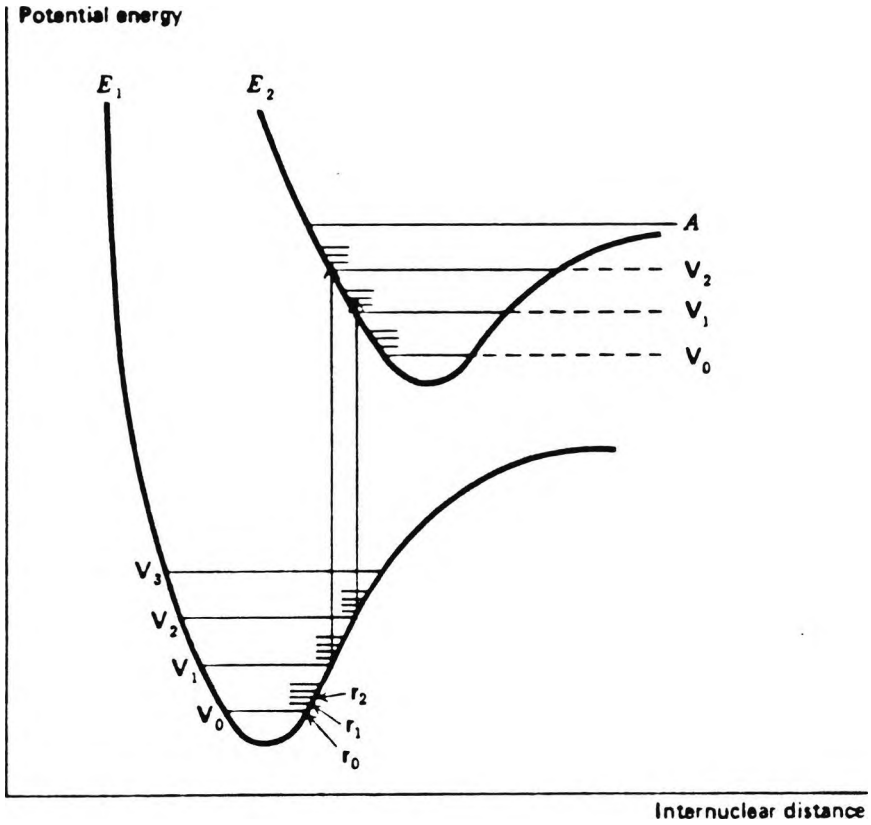


Fig 11

The probability of a transition will affect the intensity of the absorption of a particular wavelength. Therefore, the "shape" of the spectrum is determined by the probabilities of the various transitions. These same principles apply for emission spectra as well.

1.07 Phosphorescence

Fluorescence is not the only mechanism by which light may be emitted from an excited molecule. Another mechanism called phosphorescence is quite common. Phosphorescence is defined as a light emissive transition involving states of differing multiplicity. In practice this commonly occurs between a triplet excited state and a singlet ground state. Consider a more comprehensive Jablonski diagram, Fig 12.

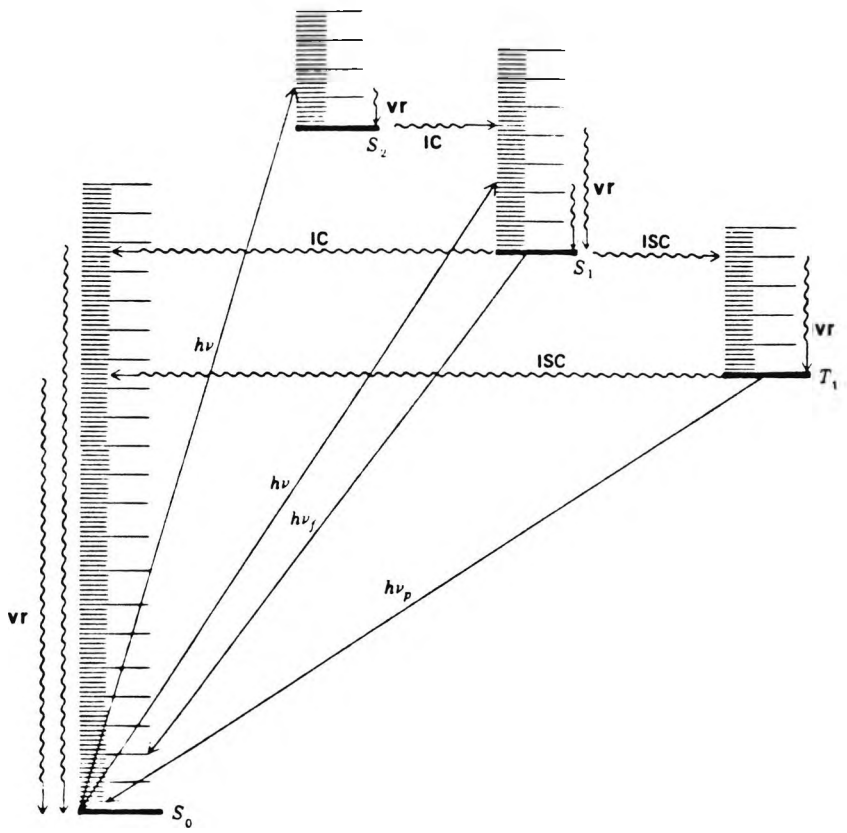


Fig 12

Triplet states are lower in energy than singlet states and their three levels are very close together. Theoretically, radiative transitions between states of differing multiplicity are forbidden. In practice, spin-orbit coupling makes these transitions possible but only with a low probability. This low probability has two consequences. Firstly, the absorption band $S \longrightarrow T$ is weak. Secondly the radiative lifetime of the emission will be long.

In general $S \longrightarrow T$ absorption is not the way the triplet state is populated. A mechanism known as intersystem crossing, (ISC Fig 12) where energy is transferred from the excited state to the triplet operates. The energy is transferred from S_{10} to an upper vibrational level of the triplet. This energy is quickly lost by vibrational relaxation until T_{10} is reached. In detail, this transfer may be understood by looking at the energy profiles of a two dimensional molecule, Fig 13.

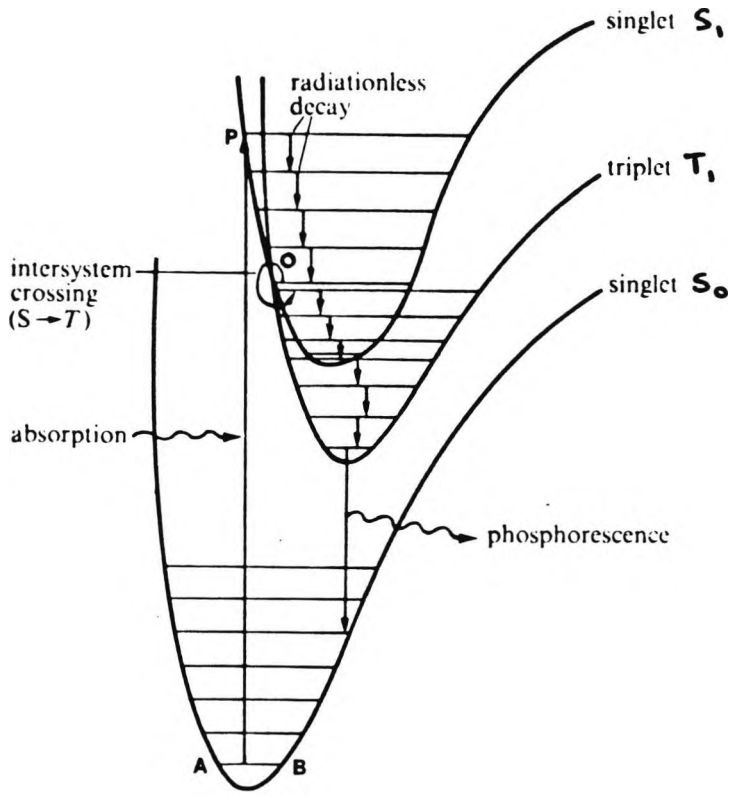


Fig 13

The lowest level of the T_1 curve is below that of S_1. The curves cross at a point O. Light absorption raises the molecule from S_o to the vibrational level P. Then the molecule loses its excess vibrational energy passing down the curve through point O. At this point the position and momenta of the atomic nuclei are identical for both singlet and triplet states. Hence the molecule may intersystem cross to the corresponding vibrational level of the triplet state. In more complex molecules a multi dimensional representation is required and so several points like O may exist.

It has alrady been noted that triplet states are lower in energy than singlet states. The consequence of this is that phosphorescence is observed at longer wavelengths than fluorescence. However, the signal is usually much weaker. This is because the longer lifetime of the phosphorescence allows competing radiationless pathways to have more opportunity. Another contributing factor to this weaker signal is that intersystem crossing is not often a very efficient process.

These factors mean that to observe phosphorescence the oxygen must be removed from a solution. Often rigid media at low temperature are used as well.

1.08 Lifetimes

The above section, 1.07, mentioned that phosphorescence has a longer lifetime than fluorescence. This section will quantify such a statement and lay some more groundwork for the properties outlined in the next chapter.

The radiative lifetime for fluorescence from a strongly "allowed" transition is of the order of 10^{-9} seconds.

These lifetimes are in marked contrast to phosphorescent lifetimes. These lifetimes are seldom less than 10^{-4} seconds and can even exceed one second.

1.09 Fluorescein [5,6]

Fluorescein is one of the most famous fluorescent compounds and is used a great deal for biological studies as its isothiocyanate derivative. The basic structure is as follows - Fig 14.

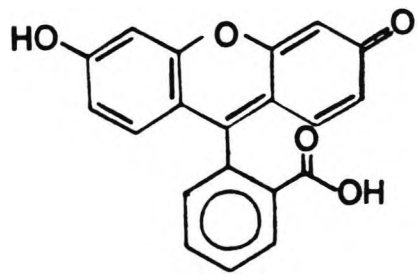


Fig 14

It is simply phenolphthalein (non-fluorescent) with an oxygen bridge. The increased rigidity is the major factor that enables fluorescein to fluoresce. The electronic transitions involved are efficient $\pi - \pi^*$ transitions localised in the xanthene section of the molecule. The $\pi - \pi^*$ transitions lead to a short fluorescent lifetime of about 4 ns and a high quantum yield of 0.96 (0.01 M KOH in 95% ethanol). The mechanism only involves S_0 and S_1 levels giving a small Stoke's Shift of about 30 nm. Both the excitation and emission bands are broad showing that a range of vibrational levels are involved in the mechanism.

Many derivatives of fluorescein have been made. Halogens, particularly bromine and iodine, promote intersystem crossing by spin orbit coupling. This leads to phosphorescent compounds.

This "heavy atom effect" reduces the quantum yield of the compounds. In the series fluorescein, tetrabromofluorescein and tetraiodofluorescein the quantum yields are 0.97, 0.67 and 0.08 respectively (0.01 M KOH in 95% ethanol) [6].

1.10 Proteins [5,9]

The work in this thesis all involves the application of luminescent compounds for biological studies. Proteins are themselves fluorescent. This fluorescence mainly comes from two amino acids, tryptophan and tyrosine. Phenylalanine also makes a less significant contribution.

The emission maxima of these three amino acids when isolated is as follows:

Tryptophan	348 nm
Tyrosine	303 nm
Phenylalanine	282 nm

When combined together with other amino acids in a protein the excitation maximum is at 280 nm and the emission maximum is between 330 and 340 nm. This leaves phenylalanine making a very small contribution to the emission. The emission of tryptophan, in particular, is very susceptible to solvent and other environmental changes. This is due to the indole part of the amino acid. Hence in a protein, where each amino acid has a unique environment, the emission is not merely the sum of tryptophan and tyrosine residues behaving in a similar way to their monomeric forms. The emission is made up of a combination of each tryptophan and tyrosine with its own fluorescent properties. For example,

bovine serum albumin shows a blue shift in its tryptophan emission compared to the free amino acid. This is explained as due to the tryptophan residue being buried inside the tertiary stucture of the protein and so not being exposed to water. In contrast glucagon, where the tryptophan is on the peripheral of the tertiary structure shows a red shift in its emission maximum.

Another marked difference between the protein and its free amino acids is that tyrosine gives a much smaller quantum yield in the protein. On denaturation this yield increases again. The explanation offered suggests an electron transfer from excited tyrosine to the tryptophan when in the protein. This would break down on denaturation. Certainly proteins are capable of electron transfer but it is not clear if this is the mechanism involved to rationalise these results.

More significantly the property of being a good electron transfer medium has a bearing on using fluorescent labels on proteins. It is expected that proteins will quench the fluorescence of the labels. This particularly concerned us as the metal complexes used in this thesis have long emission lifetimes and so are more susceptible to quenching [5].

For a typical protein the emission lifetime lies between 1 and 7 ns. The emission maximum for human IgG is at 333 nm. The quantum yields vary from protein to protein. The key fact that concerns this project is that proteins will always produce a signficant fluorescent signal that must be subtracted or screened from the signal of the labelling fluorochrome. In Ch. 4 this matter, along with other interfering signals, is discussed.

2.20 Summary and Conclusion

Organic molecules are able to emit light by two mechanisms called fluorescence and phosphorescence. These mechanisms are known collectively as luminescence. Fluorescence, generally, has a short lifetime and often a short Stokes' Shift. The quantum yield may be very high as in fluorescein. Phosphorescence, on the other hand, has a long lifetime and a larger Stoke's shift. The quantum yield may be high but is usually low at room temperature due to quenching effects.

The properties of organic luminescent molecules depend on many factors including energy levels, solvation effects, competing mechanisms, rigidity and functional groups.

Fluorescein, in particular, has found widespread use in protein and biological studies due to its high quantum yield. However, alternatives are being sought. This is due to the fact that proteins also fluoresce quite strongly creating a background signal. The next chapter examines some alternative compounds for protein labelling.

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Chapter 2

LUMINESCENT COMPOUNDS - PART 2

2.1 Introduction

This chapter will cover the properties of two other classes of luminescent compounds. These are the lanthanide chelates and the transition metal complexes. Initially both classes of compounds were investigated for a purely theoretical interest. However, more recently, their unusual properties have prompted research for their use in biological studies. These unusual properties are the subject of this chapter. Chapter 4 will then outline how they may be applied in immunoassay systems.

2.2 The Lanthanide Chelates

From the lanthanide metal ions there are several that form luminescent compounds when chelated to organic ligands. Of these, europium and terbium in their 3+ oxidation state are the most common eg. Europium diketonoate complexes [1,24].

The chelates are thought to luminesce by the following mechanism. The organic ligand is excited by uv light to a singlet state. Then the ligand enters its triplet state, by efficient intersystem crossing (the metal aids

this process by mixing the multiplicities of the excited states). The creation of the triplet state normally would lead to phosphorescence. In these complexes the triplet energy is transferred to the central metal ion causing "metal" electrons to be excited to higher orbitals. These excited electrons return to their ground state and in so doing they release energy as light. Fig 1 summarises these processes.

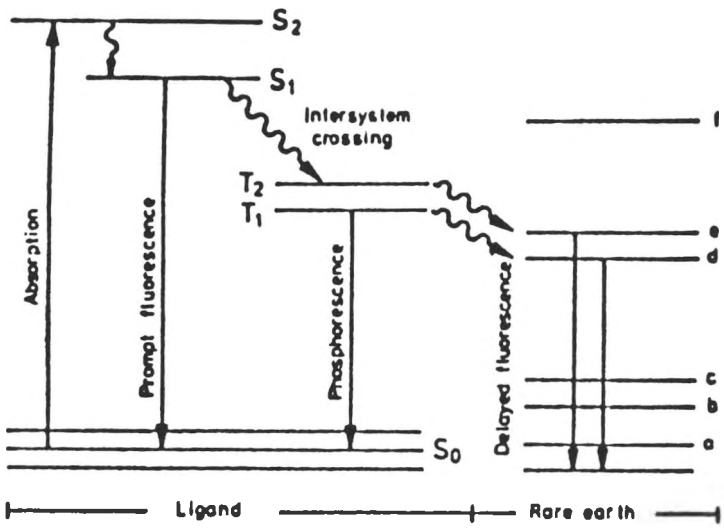

Fig 1

The spectral characteristics of the chelates are compatible with the above mechanism. The excitation band is broad and coincides with absorption band of the free ligand (250-360 nm). The Stoke's Shift is large due to the processes of excitation to a singlet followed by energy loss all the way to a metal-metal transition

for the emission. For these compounds the Stokes' Shift is normally greater than 250 nm. The emission band is sharp and characteristic of metal-metal transitions. The lifetime of the emission is very long,~100 - 1000 μs and this is due to the forbidden metal-metal transitions.

Chemically, the chelates are kinetically quite unstable compounds. This is true in aqueous solution where the ions are prone to filling their co-ordination sphere with nine water molecules in preference to the diketone ligands. Hence the triplet sensitisation of the ion by the excited ligand is prevented. To observe them most effectively they can be placed in micelles or encapsulated in latex beads. Chapter 4 will show how the properties of a large Stoke's Shift and a long emissive lifetime have been used to advantage in immunoassay systems. The table below summarises the properties of two lanthanide chelate compounds [1].

compound	λ_{ex} (nm)	λ_{em} (nm)	τ (ns)
$Eu(\beta\text{-NTA})_3$	340	613	500,000
Tb-EDTA-sulphosalicylic acid	300	490,545	150,000

3.3 Introduction to Transition Metal Complexes

Most of the work undertaken in this thesis involved the preparation of luminescent transition metal comlexes. The reason these were chosen was that they have competitive luminescent properties to the lanthanide chelates and in addition to this many are compounds that are kinetically very stable[25],In particular, complexes of ruthenium and chromium were of interest. The rest of this chapter wiil outline their physical properties enabling a comparison to be made with the lanthanide chelates and other luminescent compounds.

3.4 The Electronic Structure of Transition Metal Complexes

The most straight forward and, for this work, useful description of the electronic structure of transition metal complexes is given by crystal field theory [2,5]. For an octahedrally co-ordinated complex the d-orbitals of the metal ion are split in energy as follows (fig 2):

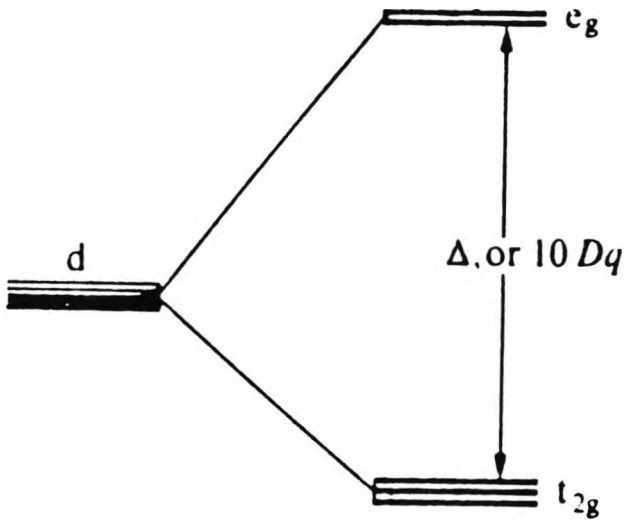

Fig 2

The d_z2 and d_x2-_y2 orbitals have lobes lying along the octahedral axes and so they increase in energy. The d_{xy} d_{xz} and d_{yz} orbitals correspondingly decrease in energy. The energy difference between the lower set of orbitals "t_{2g}" and the upper set "e_g" is labelled Δ.

The magnitude of this splitting energy is determined by three factors:

(i) The radius of the metal ion.

(ii) The charge on the metal ion.

(iii)The nature of the ligands.

For a given metal of specified charge the value of Δ can vary widely as the ligands are changed. The ligands may be arranged in the order of their power to split the d orbitals. This series is known as the spectrochemical series and here the series for nickel in its 2+ oxidation state is shown. I^-, Br^-, Cl^-, F^-, OH^-, $C_2O_4^{2-}$, H_2O, NCS, pyr, NH_3, en, bipy, phen, NO_2, CN^-. The order runs from the lowest to the highest in ability to split d orbitals. From this series it can be seen that a purely electrostatic model of ligand-metal bonding is inadequate. Some uncharged ligands are more powerful at splitting d orbitals than some charged species.

A more rigorous treatment by a molecular orbital approach may be undertaken to describe the electronic structure of complexes. However, it is convenient to divide the whole complex into two sets of orbitals - the metal set and the ligand set. The reason for this is mainly to ease describing metal to ligand charge transfer states (MLCT).

The metal orbitals (Fig 3) in Cr^{3+} and Ru^{2+}, in an octahedral complex are filled as follows [5].

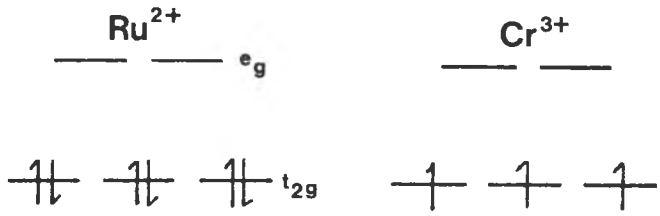

Fig 3

Ligands such as phenanthroline, bipyridyl, cyclam and cyanide ions are all high in the spectrochemical series. This leads to a large Δ and so the e_g orbitals remain unfilled. ($\Delta >$ Pr where Pr = pairing energy).

The ligand orbitals are largely unaffected by complex formation. The important exception are the orbitals with electrons that are directly involved in bonding to the metal ion (eg. nitrogen lone pairs on bipyridyl).

The case of an N-heterocyclic ligand [5] is shown below (fig 4).

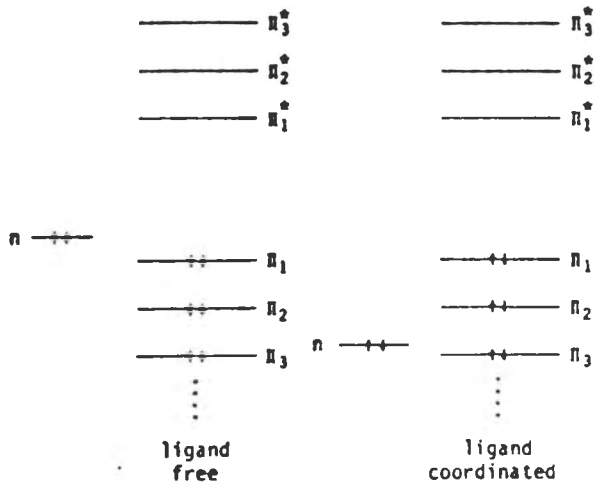

Fig 4

The diagram shows that on co-ordination the energy of the non-bonding electrons falls drastically.
However, the π orbitals remain at similar energies. The ligand orbitals have comparable energies to the metal orbitals and so a combined view of the metal and ligand orbitals may be described. The majority of luminescent transition metal complexes contain 6 d electrons and are based on Ru^{2+}, Os^{2+}, Re^{2+}, Rh^{3+} and Ir^{3+} ions [5]. For d^6 metal systems there are three possible ways in which the metal orbitals may combine with the ligand (fig 5). [27]

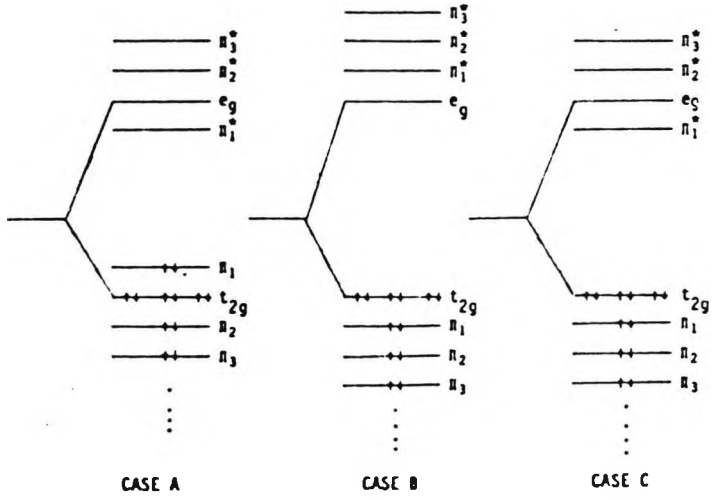

Fig 5

Case A

The π - π * excitation is the lowest in energy and so the metal orbitals play no role in the excitation process. The excitation and emission properties (if there are any) are characteristic of the ligand with its lone

pairs lowered in energy. $Rh(bipy)_3^{3+}$ is an example of such a compound.

Case B

The dd transitions are the lowest in energy. The energy of these transitions varies with the ligand's ability to split the d orbital energies. Often substitutional photochemistry results from these transitions due to the removal of an electron from the bonding orbitals. The following reaction is quite common.

$$Co(CN)_6^{3-}\ aq \xrightarrow[h\nu]{} Co(CN)_5H_2O^{2-}$$

Case C

Here the transition of lowest energy is the promotion of an electron from the t_{2g} set of metal orbitals to the π^* orbital of the ligand. For a species such as bipyridine, when alone in aqueous solution, the solution is colourless. On addition of Ru^{2+} ions a deep red colour forms. The absorption spectrum reveals that an entirely new set of electronic transitions have appeared. These are neither metal centred or ligand localised and are understood to be charge transfers from the metal's electrons to the ligand's antibonding orbitals. This process may be written as

$t_{2g}^{6} \longrightarrow t_{2g}^{5}\ \pi^{*1}$

eg $Ru(bipy)_3^{\ 2+} \longrightarrow Ru^{\ 3+}(bipy)_2(bipy^-)$

In the bipyridine complexes of ruthenium, osmium and iridium the charge transfer leads to excited states that lose their energy by light emission.

3.5 The Mechanism of the Luminescence in Ruthenium Polypyridyl complexes

The mechanism for the luminescence of the ruthenium bipyridyl complex $Ru(bipy)_3^{\ 2+}$ is not fully understood [3,4,6,8,17]. In outline an electron, metal localised, undergoes charge transfer to a ligand antiboding orbital (MLCT Fig 6). The energy for this comes from uv light, 450-500 nm in wavelength. By various relaxation and intersystem crossing processes (R_I R_{ISC} Fig 6) the complex arrives at an excited state from which emission may occur. This excited state is composed of a set of closely spaced electronic states in thermal equilibrium with one another. This set of states is called a "thexi state" (Thermally Equilibrated Excited State)[7].

Emission may occur from this excited state manifold to the ground state (E. Fig 6). However, it is the excited state configuration that is reached and so further relaxation (R_2 Fig 6) occurs to achieve the true ground state.

SIMPLIFIED MODEL OF THE PHOTOPHYSICAL PROCESSES INVOLVED IN $Ru(bipy)_3^{2+}$ LUMINESCENCE

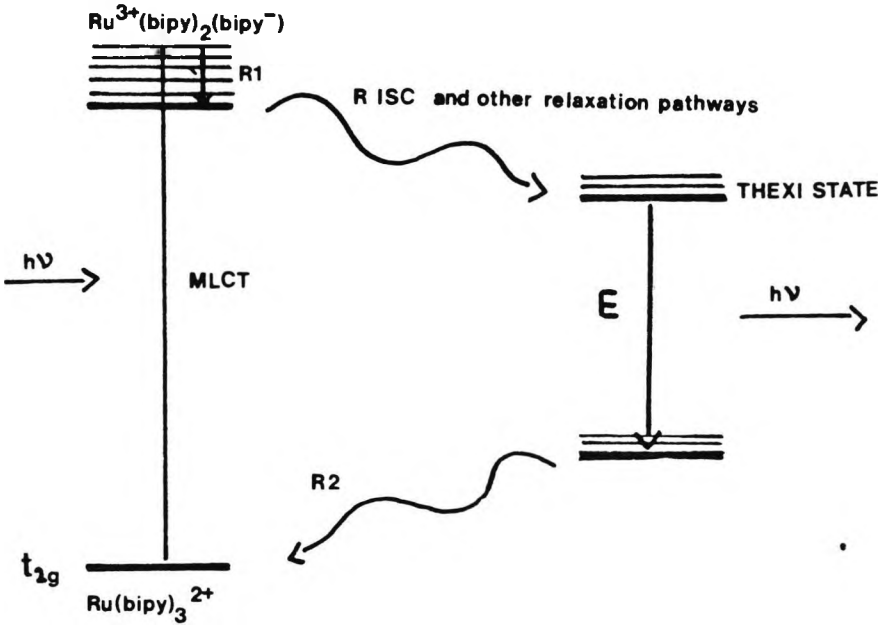

Fig 6

In detail the only clear part of the mechanism is that the excitation is a metal to ligand charge transfer. However, it is a subject of debate whether the charge is localised on one ligand or delocalised over all three. Earlier work, summarised by J Van Houten and R J Watts

[3], concluded that the charge was delocalised over all three ligands. This leads to a D_3 symmetry for the ground and excited states. More recently, Time Resolved Laser Raman Spectroscopy has given evidence that strongly suggests the charge is localised on one ligand only [4]. This gives a C_2 symmetry for the excited state.

The D_3 delocalised model is described as follows by Van Houten and Watts (Fig 7). "The low-lying CTTL (charge transfer to ligand) states of $Ru(bipy)_3^{2+}$ are believed to arise from promotion of a d electron of Ru(II) to a ligand π^* orbital of a_2 or e symmetry. Coupling of the promoted electron with the strongly spin-orbit coupled states of the d^5 core results in 16 levels which are comprised of 36 states. The lowest three levels of A_1, E and A_2 symmetry, which arise from coupling of a π^* (a_2) electron with the d^5 core are believed to be responsible for the luminescent properties. However, at ambient temperatures, as many as 36 spin-orbit coupled states of $d\pi^*$ parentage could lie within 3000 cm^{-1} of the lowest excited levels and could therefore contribute to the net decaying process [3]." The thexi state just described is thought to play a key part in giving a broad emission band (550 - 700 nm) in $Ru(bipy)_3^{2+}$ luminescence. This same paper also describes nine excited states of d-d parentage that may also contribute to the luminescent and photochemical properties of the

complex. These states are close in energy to the lowest excited state (with 3600 cm^{-1}) and probably lead to substitutional photochemistry in common with other d-d systems.

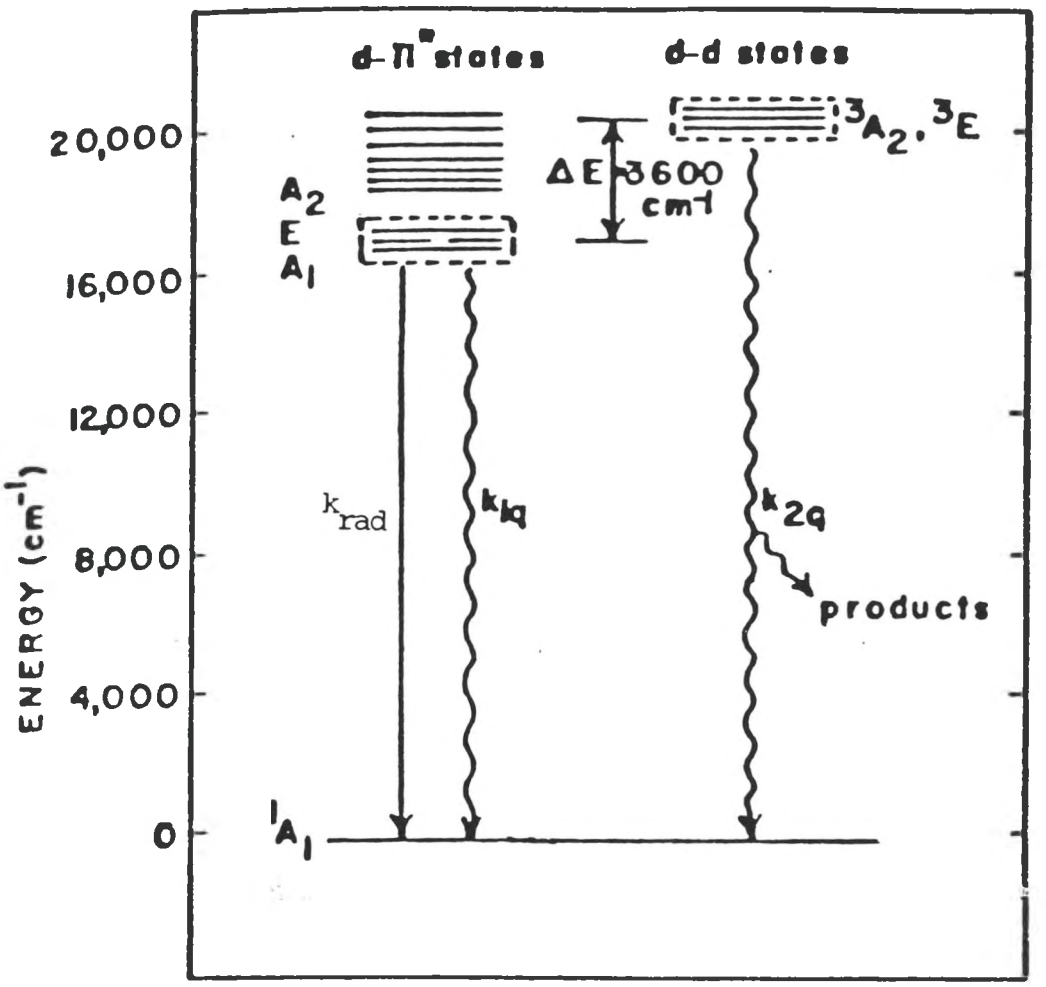

Fig 7

The C_{2v} model is described by Day and Sanders [18] and Ceulemans and Vanguickenborne [19]. Complete localisation, ie. the electron localised on one ligand throughout the lifetime of the emitting species, seems unlikely. This is because the emission spectrum and the

lifetime should exhibit dual behaviour in mixed ligand complexes as found for $Rh(bipy)_n(phen)_{3-n}^{3+}$ series of compounds [21]. This dual behaviour was not observed for ruthenium complexes [20]. The Time Resolved Laser Raman results are generally regarded as the most compelling evidence for charge localisation particularly during the first nanosecond of excitation [4]. Hence the best conclusion from the above models and results is to describe the excited state as follows:

The excited state electron is localised on one ligand but this interacts with the other ligands to a limited extent. Where a mixed ligand complex is made, the charge tends to localise on the ligand which is easiest to reduce [22]. I suspect that the ligand with the π^* orbital of lowest energy or a strong electron withdrawing group, such as CO_2H may be the place where electron localises.

What is clear from both theoretical and experimental work is that the excited state is made up of a set of levels that are closely spaced. It may also be inferred that some sets of these levels lead to photochemistry rather than to light emission. This may mean that by altering the excitation wavelength photofading of the complex may be reduced or enhanced.

Another property of these complexes that is clear from the above models is a large Stoke's Shift. Typically a

Stokes' Shift for ruthenium polypyridyl complexes is 150 nm. This is due to the processes that follow excitation. R_1 on Fig 6 shows vibrational relaxation (R_1) to an excited state from which intersystem crossing (R_{isc}) occurs to a second excited state (Thexi). In addition to this loss of energy there is an energy loss as the excited state adjusts its solvation sphere. The excited state molecule $Ru^{3+}(bipy)_2(bipy^-)$ is effectively an isomer of the ground state $Ru(bipy)_3{}^{2+}$. The dipole and solvation properties of the two isomers are different. Hence an adjustment of the solvation sphere will cause a further stabilisation and loss of energy of the excited state (see 2.5) [7].

Having seen that vibrational relaxation, intersystem crossing and solvent relaxation of the excited state contribute to a large Stoke's Shift there is an additional factor that can be observed to effect the Stoke's Shift. The Shift can be increased by increasing the conjugation on the ligand.

eg. Ru $bipy_3Cl_2.6H_2O$ SS = 135 nm

Ru $bipy_2bipy(CO_2H)_2.2PF_6$ SS = 160 nm

[10] and [17] p.152

Fig 8 shows how this can be drawn from the model of the ligand orbitals.

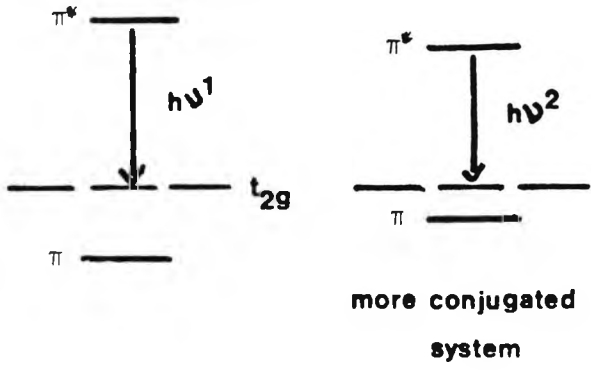

Fig 8

Increasing the conjugation results in a lowering of the π* energy. This means the excited state is at a lower energy than the unconjugated π* system.

The quantum yields of these compounds vary a great deal. The complexity of the mechanism means that there are plenty of competing processes to reduce the quantum yield of emission. Three factors are observed to increase the quantum yield when surveying the compound's luminescence. The first is expected - by increasing the rigidity of the molecules the quantum yield increases eg.Ru $bipy_3Cl_2.6H_2O$, ϕ = 0.042; Ru $phen_3Cl_2.7H_2O$, ϕ = 0.058. [9] Invariably, the phenanthroline series of complexes have higher quantum yields when compared to bipyridyl equivalents. The second factor was that by making phenanthroline or bipyridyl derivatives with phenyl groups gave an increase in quantum yield.

eg. $Ru(dp\ phen)_3^{2+}$	$\phi = 0.366$	MeOH/EtOH	
$Ru(phen)_3^{2+}$	$\phi = 0.019$	MeOH/EtOH	[28]

The reason for this is not clear but we suspect that the phenyl groups protect the excited state from solvent interactions. This would limit the loss of energy by vibrational process to the solvent molecules. However, if this is true it suggests that the solvent stabilisation of the excited state plays little or no role in the formation of a large Stoke's Shift. Finally, removal of oxygen from the solvent in which the compound is dissolved increases the quantum yield.
Eg. $Ru(bipy)_3Cl_2.6H_2O$ in air saturated water at 25°C, $\phi = 0.028$; $Ru(bipy)_3Cl_2.6H_2O$ in nitrogen saturated water at 25°C, $\phi = 0.0\ 42$ [9]. This effect is due to oxygen quenching of the excited state which always plays a part in compounds that have long lived excited states.

The emissive lifetime of ruthenium complexes varies from about 200-5500 ns. [9 ,17] These lifetimes are intermediate in length between those given by the lanthanide chelates and most organic molecules. The explanation given is that spin-orbit coupling causes the intersystem crossing involved in the mechanism to become more efficient. Second and third row transition elements cause a "mixing" of the multiplicities of excited states. For different metal ions this effect will vary. Hence a whole range of emissive lifetimes is

predicted from transition metal complexes and this is to some extent observed (eg. $Irbipy_3^{3+}$ T = 10 μs, $Rubipy_3^{2+}$ T = 0.6 μs [9,11]).

Lifetimes of emission were found to increase according to the same factors that increase quantum yield - namely increased rigidity, addition of phenyl groups and removal of oxygen from the solvent.

3.6 Conclusion to Luminescent Ruthenium Complexes

In general the luminescent properties of a large range of ruthenium complexes are known [17] and to a certain degree the mechanisms involved understood. The compounds have large Stoke's Shifts, intermediate emissive lifetimes and reasonable quantum yields. Enough is known from the large amount of compounds that have been made to design derivatives for protein labelling with the most favourable characteristics. A complex made up of a ruthenium 2+ ion and phenanthroline ligands with derivatised phenyl groups was thought likely to prove the best option.

3.7 The Mechanisms involved in Chromium Complex Luminescence

Luminescent chromium compounds have been studied for about 20 years but not as extensively as the ruthenium

compounds. For this work two chromium complexes in their +3 oxidation state were of interest. The first was a chromium phenanthroline complex $Cr(phen)_3.3ClO_4$. This was reported to have a luminescent lifetime of 270 μs and a quantum yield of about 1/20 of ruthenium tris bipyridyl. The Stokes' Shift was 300 nm with a broad excitation centred at 420 nm and a narrow emission at 728 nm. (All readings at room temperature) [12]. The other complex was trans-dicyano (1,4,8,11-tetra-azacyclotetradecane)-chromium III per chlorate. [13] This was reported to have a luminescent lifetime of 335 μs and a slightly higher quantum yield than the chromium phenanthroline complex. The Stokes' Shift was nearly 300 nm with the broad excitation peaking at 436 nm and the narrow emission at 720 nm. Both complexes gave increased emission lifetimes in D_2O. Also if the cyclam ligand was deuterated (replacing N-H with N-D) an emission lifetime of 1500 μs in water at 295 K was reported [14].

The mechanisms involved in the luminescence from these compounds are the subject of much debate but certain ideas dominate the literature. Unlike the ruthenium complexes these chromium systems are not excited via metal to ligand charge transfer. The ligand orbitals are energetically above and below the metal orbitals. This is illustrated earlier in section 3.4 "Case B".

Chromium in its 3+ oxidation state with octahedral microsymmetry has a t_{2g}^3 ground state of $^4A_2$ symmetry. For chromium phenanthroline complexes the transitions are mainly metal centred and can be illustrated as follows: [15]

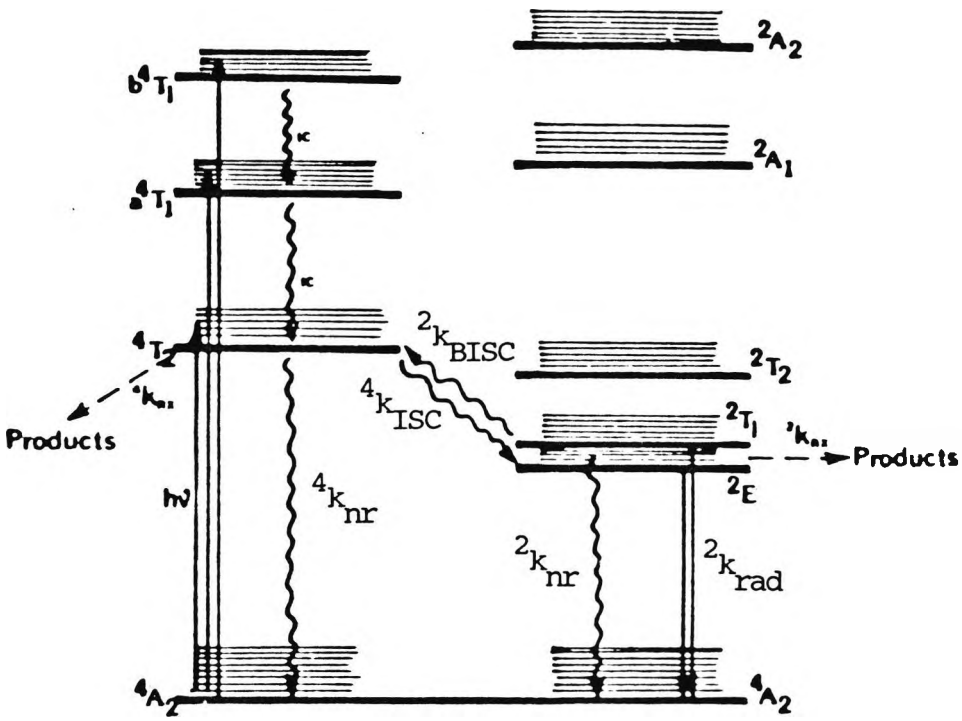

Fig 9

Several quartet and doublet states are present of which the lowest energy lead to luminescence. The lowest quartet state has the $t_{2g}^2\ e_g^1$ configuration.

This is particularly reactive photochemically as e_g orbitals are mainly σ antibonding in character. This leads to a weakening of the metal-ligand bonds and so substitution by other species (notably solvent molecules) may occur. The vacant t_{2g} orbital also promotes the formation of 5 and 7 co-ordinate transition states with competing ligands. In the chromium phenanthroline complex it is thought that excitation follows allowed quartet - quartet transitions. The excess vibrational energy is lost until the $^4T_2$ state is reached. From there, several competing processes operate. These include photochemistry and non-radiative relaxation to the ground state. A low efficiency intersystem cross also occurs to the $^2T_1$ and ^2E states It is from these states in thermal equilibrium that the emission is thought to occur. Hence the emission is known as phosphorescence. The doublet states must be very close together for this theory to hold as the emission is a sharp band. These states are able to lose energy by means other than light emission - photochemistry, non-radiative and even back intersystem crossing (very unlikely) is thought to occur. [15]

The intersystem crossing aided by the presence of a metal ion is compatible with the large Stoke's Shift and long emissive lifetime of these systems. The low quantum yields of emission are expected with all the competing processes in operation. For Cr $bipy_3^{3+}$ the

quantum yield of substitutionary photochemistry is 0.15 whereas for $Cr(phen)_3^{3+}$ is is 0.047. Hence as expected $Cr(phen)_3^{3+}$ has a higher quantum yield of emission (twice) and a longer luminescent lifetime than $Cr\ bipy_3^{3+}$.

The chromium cyclam complex shows similar properties to the above. There are two isomers of $Cr(cyclam)CN_2.ClO_4$. The CN^- groups may be cis or trans to one another and it is only the trans form that is luminescent. A complex with two axial ligands that are higher in the spectrochemical series than the the four equatorial ligands gives a distorted octahedral field. This may be shown diagramatically as follows:

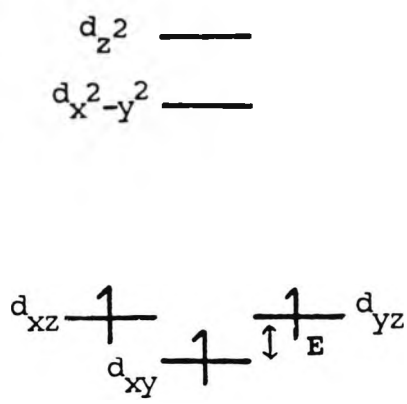

Fig 10

The perturbation of the regular octahedral field makes the assignment of the excited states not as clear as for $Cr\ phen_3^{3+}$. For a d^3 configuration the ground state is a quartet as the pairing energy is greater than E (Fig 10). On excitation excited quartet and doublet

states may be formed in a similar way to $Cr(phen)_3^{3+}$. The lifetime and quantum yield data (similar to $Cr(phen)_3^{3+}$) makes it likely that intersystem crossing to a doublet state is involved in the mechanism. The picture as far as it is understood for chromium - amine complexes bears some similarity to the chromium-polypyridyl one. For complexes such as $Cr(NH_3)_6^{3+}$ photochemistry is the dominant result from irradiation (ϕ = 0.51). By contrast $Cr(en)_2(SCN)_2.ClO_4$ (trans) shows substitutional photochemistry (ϕ = 0.18) with weak luminescence [12,16]. By linking the en ligands to make a cyclam lignd the substitutional photochemistry becomes nearly impossible ($\phi = 3x10^{-4}$) and so the luminescence quantum yield increases [16].

3.8 Conclusion to the Luminescent Chromium Complexes

A range of chromium complexes have been made that exhibit luminescence. The lifetimes suggest a partially improved efficiency phosphorescence. The complexes with the best characteristics are those that have the least ability to undergo substitutional photochemisty. Of these $Cr(phen)_3^{3+}$ and trans-Cr cyclam $(CN)_2^{+}$ compounds have the strongest emissions. Suitable derivatives of these complexes may be found but their low quantum yield may preclude their usefulness.

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Chapter 3

ANTIBODIES

3.1 The Structure and Function of Antibodies [1,2,3]

Antibodies are an integral part of the defence mechanism of many organisms. This chapter will describe their structure and function. To begin with, the structure of antibodies, paying particular attention to their binding sites, will be described. For this thesis the interest in antibodies comes from their ability to bind specific targets (such as haptens, enzymes and cell walls) and to ignore other substances.

The antibody fraction of serum is made up predominantly of a group of proteins with a molecular weight of around 150,000. The major component is IgG (Immunoglobulin G) with another component, IgM, of molecular weight 900,000. Hence the first and most important fact is that antibodies are proteins. The chemistry carried out in this thesis reflects this fact.

The structure of antibodies was elucidated as follows: IgG antibodies were split into three fragments by a papain digest. Two of these fragments were identical and bound antigen (known as Fab - Fraction antigen binding). Another enzyme, pepsin, produced two

fragments from IgG of which only one bound antigen. This fragment was divalent in its binding properties. The antibody was also reduced and broken down into its constituent peptide chains. Two types of peptide were found. One, the larger, is known as the "heavy chain" and the other is known as the "light chain".

On the basis of these experiments Porter and Edelman proposed the following structure for IgG.

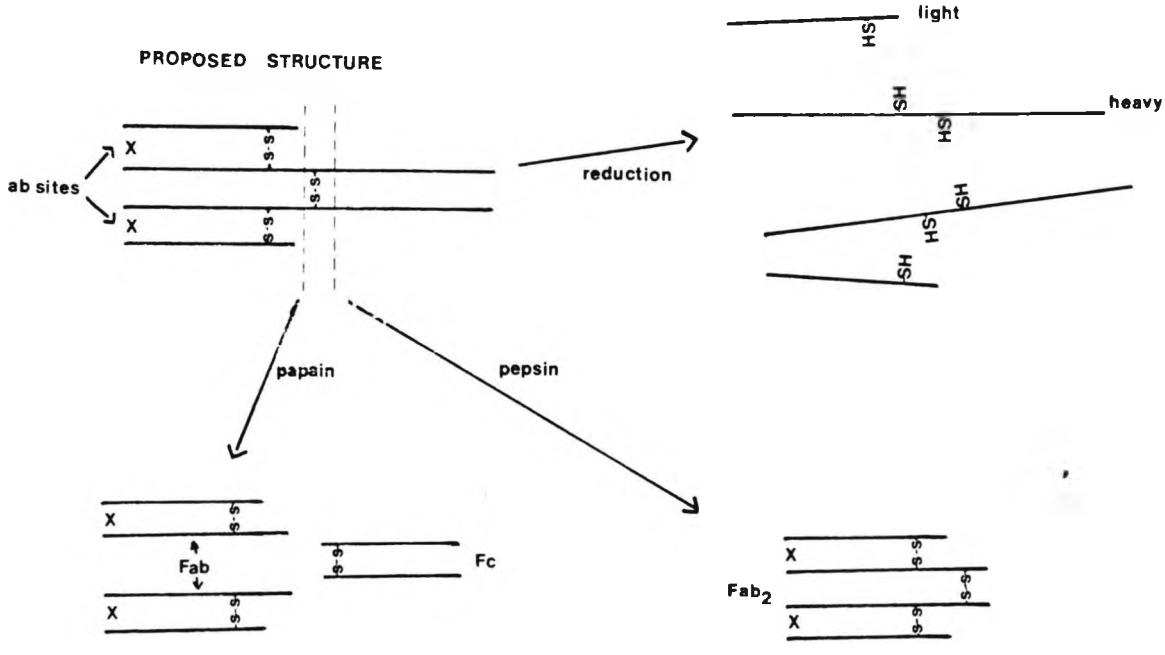


Fig. 1

Abbreviations: Fab - Fraction antigen binding

ab - antigen binding

Fc - Fraction crystalisable

[1]

The antibody is thought to be a protein that has a symmetrical four peptide structure with two "heavy" and two "light" chains. The diagrams above show this schematically and also show the rationale behind the enzyme digests.

Studies by electron microscopy have shown antibodies to be "Y" shaped molecules with "arms" that swing to angles up to 180°. The amino acid sequence of the "hinge" region (Fig. 1) reveals a large number of proline residues. This sequence prevents α-Helix formation and so enables the peptide to be flexible at this point. Another confirmation that this shape is correct has come from divalent hapten studies. Haptens are synthetic antigens and in an experiment antibodies were raised against dinitrophenylene (DNP). These antibodies were then mixed with bis-N-dinitrophenyl octamethylenediamine (Fig. 2).

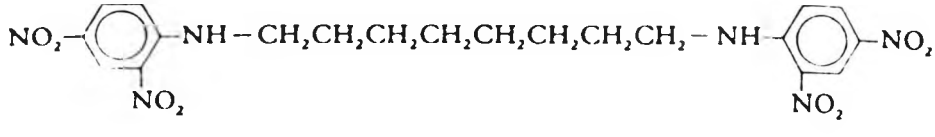


Fig. 2

The two "haptenic" DNP groups can each bind antibody molecules. Hence polymeric structures were possible. Electron micrographs showed dimers, trimers, tetramers and pentamers. The illustration below (Fig. 3) shows how a trimer may be formed.

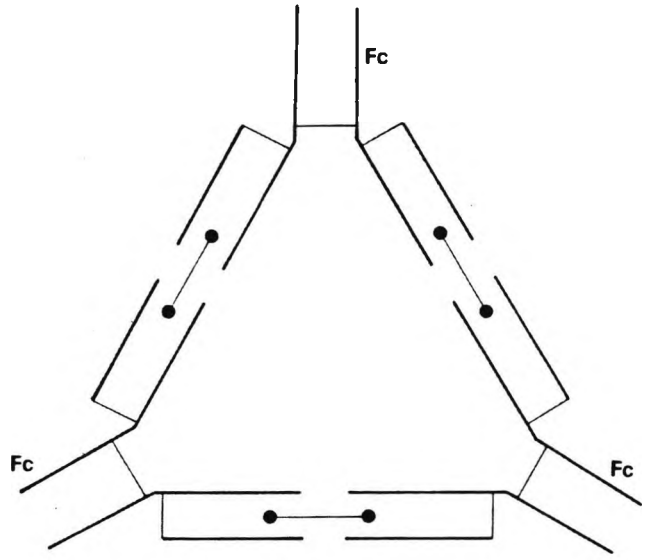


Fig. 3

The overall shape of the antibody seems to be clear and also there is strong evidence to suggest that the antibody binds antigen at sites near the N terminal residues of the peptides.

For some time it was not possible to isolate a specific antibody for a particular antigen. This problem was solved by studying myeloma proteins. In "multiple myeloma" one cell, a lymphocyte making one particular, specific antibody divides uncontrollably in a cancerous manner. The patient is then flooded by one type of antibody. These antibodies were isolated and sequenced to discover their primary structure. This has revealed, for a number of myeloma proteins, that within a major immunoglobulin class, such as IgG, some useful facts. The N-terminal portions of both "heavy" and "light" chains show considerable variations whereas the remaining parts of the chains are quite constant in

sequnce. Each "variable" region has itself a basic overall sequence common to antibodies with different specificities. However, within this sequence there are regions which vary a lot. These region are known as hypervariable regions. Fig. 4 shows the sequence data on a number of myeloma proteins. [1]

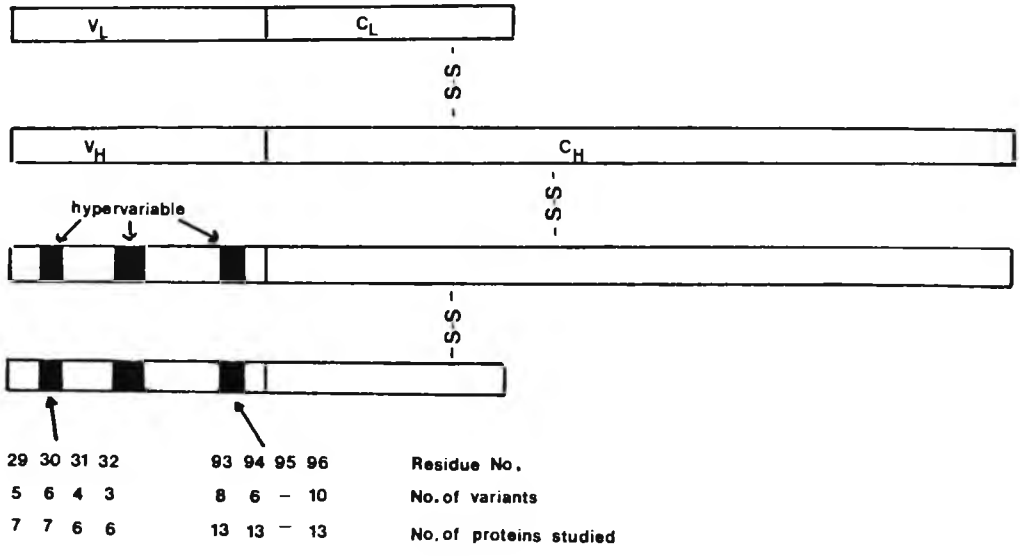


Fig. 4

The amino acid sequence only gives data on the primary structure. Proteins are three dimensional in structure and their properties depend on this fact. The data from the hypervariable regions suggest that the specificity of an antibody is determined by a change in sequence at particular points in the peptide chains. Schematically the regions can be seen to work together as follows.

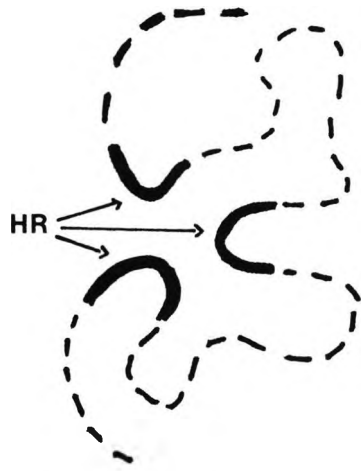


Fig. 5

HR = Hypervariable regions

Another line of evidence that suggests that the hypervariable regions work together comes from the fact that antigen binding is considerably reduced on separating "heavy" and "light" chains.

Experiments so far, suggest that the structure of the antibody's binding site is a three dimensional contribution from the hypervariable regions of the protein. Variation of the residues in the binding area will alter the shape, electrostatic, hydrogen bonding, hydrophobic and Van der Waal's properties of the site. Hence by a change in amino acid sequence a vast range of specificities may be achieved.

These properties will now be examined in turn. Electrostatic forces are due to the attraction of oppositely charged ionic groups (eg. NH_3^+ lysine and CO_2^- aspartate). The force of attraction is inversely proportional to the square of the distance between the charges. Dipoles, also will interact so as to align positive with negative. Charge transfer interactions may also cause electrostatic attraction (eg. tryptophan - e^- donating to dinitrophenyl - e^- accepting).

Hydrogen bonding is a relatively weak force in comparison to electrostatic forces but in nature it is vitally important. This bonding occurs between hydrophilic groups such as OH, NH_2 and CO_2H. For hydrogen bonding to occur these groups have to come close to one another.

Hydrophobic forces are also important. They are estimated to make up to 50% of the total antigen - antibody binding strength. These forces are most readily observed when non-polar oil droplets merge together in water. In the same way non-polar side chains of valine, levcine and phenyl alanine tend to associate in aqueous environments. The driving force for hydrophobic bonding is derived from the fact that water has its highest entropy when it is able to hydrogen bond in all directions. This entropy is decreased as the area of contact between water and

hydrophobic surfaces increases. Hence, if two hydrophobic surfaces cover each other the overall energy of the system decreases. For a protein in an aqueous environment the covering of one hydrophobic region by another lowers the total energy of the system.

Van der Waals forces are induced dipolar forces that occur by the interaction of external "electron clouds". In non-classical terms the interaction results from a temporary perturbation of the electron density of a molecule which creates a temporary dipole. This dipole may induce a temporary dipole in neighbouring molecules and so a weak attractive force is set up. The force of attraction is inversely proportional to the seventh power of the distance.

The four types of force have one common requirement for them to be active. They all depend on a close interaction between species. Hence the shape of the binding site in three dimensions must produce a "snug" fit. This sequence of amino acids is a "wonderfully made" [6] molecular fragment.

The rest of the antibody is made up of the "constant" regions. Fig. 6 shows a fuller picture of IgG.

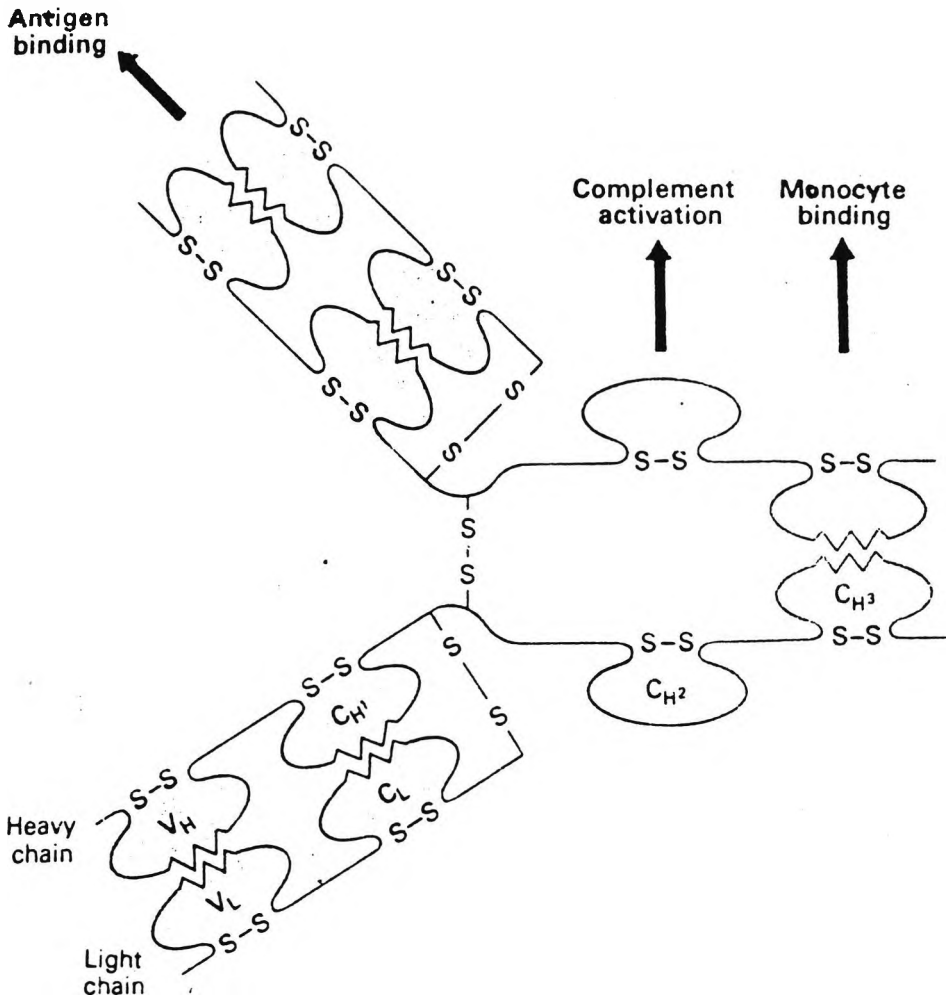


Fig. 6

The antibody is part of a defence network in the body and the constant regions possess properties that link the antibody into the defence network. The antibody may be divided into domains. These domains are the result of grouping together parts of the peptide chain and is achieved by intramolecular disulphide bonds. The constant domains C_H1 and C_H2 are involved in complement activation. The C_H3 domain is able to bind other proteins such as "J" chains.

The constant regions of the antibody are only constant within one major class of antibody eg. IgG. There are five main classes of antibody in the human body. The shape and properties of their constant regions determine their biological properties. IgA, for instance, is able to pass into the tears, sweat and saliva so it is able to defend the external surfaces of the body. It is able to do this by binding to a protein of a molecular weight of 60,000 known as the secretory component.

3.2 The Production of Monoclonal Antibodies [1,2]

Undoubtedly the greatest revolution in antibody technology has been the production of monoclonal antibodies in the laboratory. A monoclonal antibody sample contains antibodies that are all of the same Ig class with the same variable regions, structure and specificity for a given antigen. What is more, this specificity is directed to one part of the antigen only. Consider the following antigen:

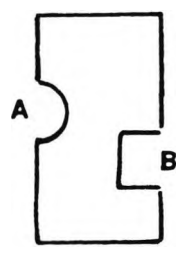


Fig. 7

Antibodies may be raised against that antigen but they will be of two types. One will be specific for site A and the other will be specific for site B. A monoclonal antibody sample will be specific for one site only. This property has proved to be of great use (see chapter 4).

The production of monoclonal antibodies may be briefly outlined as follows[7]. A mouse is injected with an antigen. After a period of time the mouse starts to produce antibodies in its spleen cells. The spleen is then removed from the mouse and individual spleen cells are fused in polyethylene glycol with B tumour cells. The cells are placed in wells at a concentration of less than one cell per well. The tumour cell's characteristic means that each cell will rapidly divide and so create a large sample of cells of identical properties producing identical antibodies. Different wells will produce antibodies specific for the same antigen but, possibly, specific for different sites on that antigen. Hence the samples in each well are kept separate. The antibodies are then removed from the cell culture supernatant for use. The cell line may be stored frozen and grown up when required.

3.3 Polyclonal Antibodies

Antibodies may also be raised against a particular hapten or protein by immunising animals and collecting the antisera. Proteins (with molecular weight greater than 5000) are injected into the animal (often inter-peritineal) in an adjuvant. Antibodies are produced by the animal and will be specific for the various antigenic sites on the protein. Hence they are called polyclonal.

Haptens (eg. drug derivatives) are not immunogenic by themselves as they are too small. The body does not normally raise antibodies to drugs. To make an animal produce an immune response the haptens have to be bonded to the surface of a large protein.

Then antibodies may be raised successfully. The antibodies are polyclonal as some populations of the samples are more specific for the drug and others are more specific for the protein. Affinity Chromatography enables the best populations to be isolated.

3.4 The Chemistry of Antibodies [4,5]

As is already clear antibodies are proteins that contain a large variety of amino acids. This means that it is

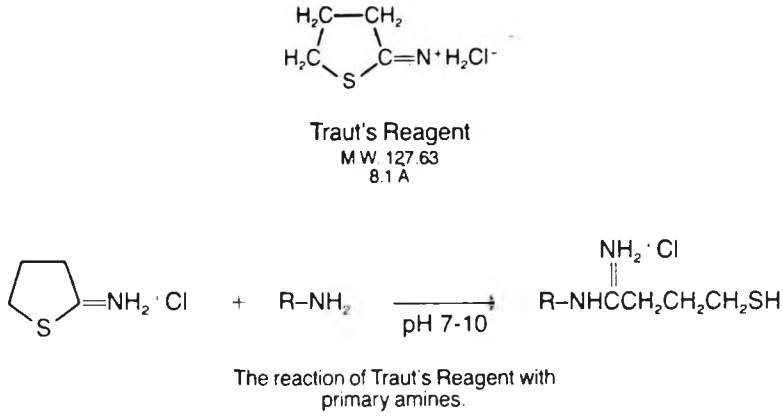
possible, using suitable reagents, to attach molecules to them. The most common functional groups available for chemistry are as follows:

group	amino acid
$-CO_2H$	Asp Glu C terminal
-phenol	Tyr
-OH (aliphatic)	Ser Thr
-SH	Cys(or Traut's reagent)
$-NH_2$	Lys N terminal
-NH-	His

Several classes of reactive groups have been utilised to derivatise fluorescent molecules so they can be attached to antibodies. The most important are listed as follows:

reactive group	target group on protein
-N=C=S	$-NH_2$ (-SH)
$-SO_2Cl$	$-NH_2$ (-SH)
malemide	-SH
active esters	$-NH_2$ (-NH-) (-SH)

Normally an antibody has no free -SH groups. However, specific reduction to form "Fab" fragments or the use of Traut's reagent (fig. 8) gives free -SH groups.



The reaction of Traut's Reagent with primary amines.

Fig. 8

To carry out a labelling reaction targeting for amino groups it is vital to keep the pH basic. This avoids $-NH_3^+$ formation. However, by varying the pH between 7.0 and 9.5 it is possible to vary the labelling levels and position of labelling. The pKa's of the various groups on a protein are different. Hence for a high level of indiscriminate labelling a high pH is used. The other ways of controlling labelling levels involve varying concentration and the time for reaction. By varying these parameters it is possible to label antibodies without swamping their biological characteristics.

1.5 Conclusion

Antibodies are remarkable proteins capable of binding a specific site on an antigen. It is now possible to produce polyclonal and monoclonal antibodies for use in the laboratory. This breakthrough has led to the desire to attach (amongst other things) fluorescent compounds to the antibodies to carry out the diagnostic tests and other biological studies.

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Chapter 4

IMMUNOASSAYS AND LUMINESCENCE

4.1 Immunoassays [1,2,3]

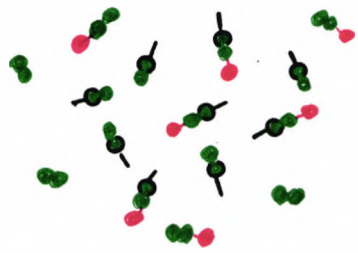
Immunoassay is an analytical technique, typically used to measure hormones, proteins and other biological substrates. The technique depends on the use of antibodies that are specific for a particular substrate. Where monoclonal antibodies are used the specificity will be for a particular antigenic characteristic of the substrate. (See Chapter 1)

There are several methods of immunoassay and this section will describe some of them.

A simple radioimmunoassay RIA may be carried out as follows: This system is described as "competitive".

1. The substrate (unknown concentration) is mixed with ^{125}I labelled substrate (of known concentration).

2. This is mixed with antibody specific for the substrate to give the following:



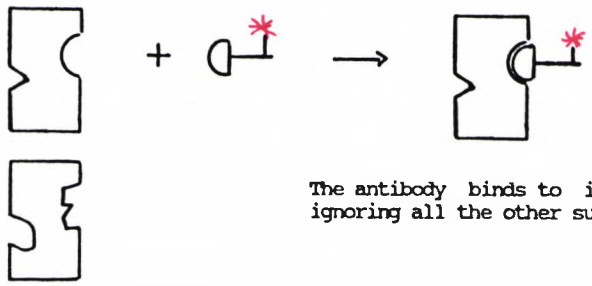
3. The antibodies are precipitated from solution by adding ammonium suphate, sodium sulphate or polyethylene glycol and the supernatant removed after centrifugation.

4. The ^{125}I signal is recorded for each tube of precipitate.

If the substrate concentration is high then the ^{125}I reading will be low. If the substrate concentration is low then the ^{125}I reading will be high. Known amounts of substrate are measured every time the assay is performed and a standard curve plotted. From this curve the unknown concentration is determined.

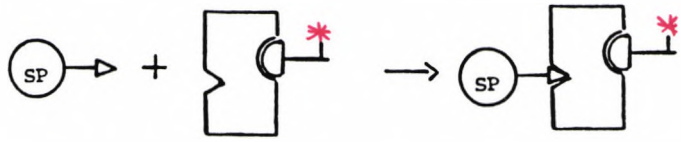
More advanced immunoassays with ^{125}I using monoclonal antibodies have since been developed [6].

1. Serum containing a particular substrate is added to a labelled monoclonal antibody specific for that substrate.



The antibody binds to its target ignoring all the other substances.

2. The above solution is added to a solid phase (beads "SP") that has been coated with another specific antibody for the substrate.



3. The solid phase is separated from the solution and washed leaving only the labelled substrate bound to the solid phase.

4. The label is counted which leads to an assay of the substrate.

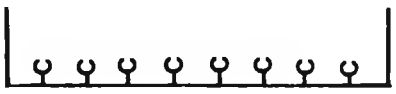
= substrate with two different sites that are bound by different monoclonal antidies.

= labelled antibody (^{125}I).

Hence by using two monoclonal antibodies one labelled and one secured to a solid phase an assay of a particular substrate may be achieved. Systems based on the above principles using ^{125}I are widely marketed. However, the need for a replacement for radioactive labelling is increasing. This is because radioactive substances are potentially hazardous, they have a short shelf life and are incresingly subject to government legislation. In particular, the short shelf life of the reagents (about 8 weeks at most) makes them expensive as staff are required to make new batches of labelled material.

The alternatives to radioactive labelling include luminescent, chemiluminescent and enzyme labels. A typical enzyme immunoassay (IEMA) can be done as follows [7].

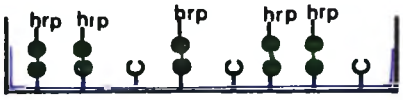
1. Coat microtitre wells with an antibody specific for the substrate to be measured.



2. "Block" the well with a small protein to prevent the substrate binding non-specifically to the plate.

3. Add substrate and leave to incubate with plate bound antibodies.

4. Wash and add horseradish peroxidase HRP labelled antibody specific for the substrate.



5. Wash and add hydrogen peroxide and ortho-phenylene diamine.

6. This produces a colour change which is read spectraphotometrically. Plates of 96 microtitre wells are often used enabling standards and unknowns to be read together by an automated plate reader. The colour change should increase with substrate concentration.

Other enzymes are also used apart from HRP such as urease and alkaline phosphatase. Perhaps the greatest advantage of using enzymes that turn over a colourimetric substrate is that for every molecule of enzyme many molecules of substrate are changed. This means the signal is amplified many times over. A further increase in sensitivity may be gained by using a substrate that releases a fluorescent product.

4.2 Luminescent Labels for Immunoassay

The luminescent compounds used at present in immunoassay are of two main classes. They are the organic and lanthanide chelate classes [2,3].

Typically organic labels fluoresce with a short emission lifetime of a few nanoseconds (see chapter 2). Fluorescein isothiocyanate is the most commonly used organic protein label and is has the following properties:

	FITC
excitation wavelength λ_{ex}	492 nm
emission wavelength λ_{em}	520 nm
Stoke s' Shift $\lambda_{em} - \lambda_{ex}$	28 nm
quantum yield	0.85
emission lifetime	4.5 ns

The main advantage with fluoresceinisothiocyanate is its large quantum yield which aids its detection. However, at low concentration in a protein containing environment, some problems occur that limit its detection severely. Firstly the quantum yield of fluorescein isothiocyanate when conjugated to protein, drops considerably (about ten times). This may occur due to the change of environment on conjugating fluorescein to the protein [5]. Another problem occurs because

samples of biological interest are often turbid. This is no problem to radioactive detection but for luminescent measurements it can create strong interference. When light travels through a transparent medium such as distilled water a fraction of it is scattered. The following spectrum of distilled water was recorded on a Perkin Elmer MPF 44A Spectrofluorimeter. The excitation wavelength was 350 nm with the excitation and emission slits at 5 nm [8].

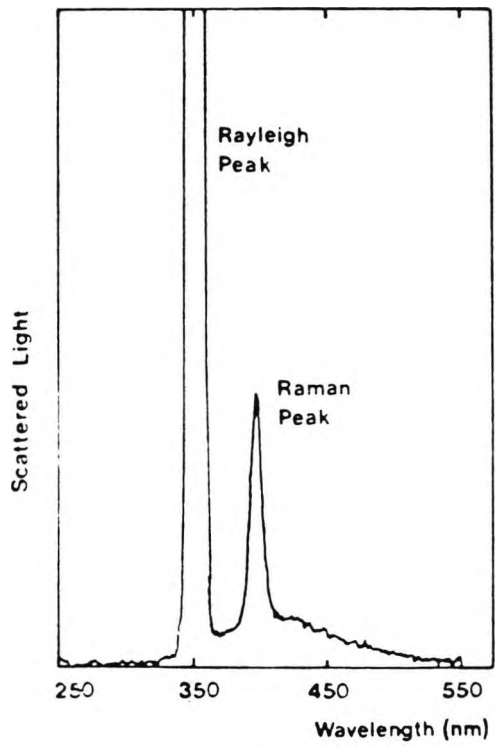


Fig 2

The spectrum shows Rayleigh and Raman scattering. Rayleigh scattering is caused by particles of a size very much smaller than the wavelength of the exciting light. This gives rise to a single peak centred at the excitation wavelength. Raman scattering is caused by scattered light which has lost (or gained) energy by the

transfer of one or more vibrational quanta to the scattering centre. In practice most of the scattering molecules are in their vibrational ground states and the energetics of the transfer process favour the transfer of a single vibrational quantum. This means that only the first low energy (longer wavelength) Raman peak is of appreciable intensity. The spectrum of distilled water (Fig 2) is changed considerably on addition of proteinacious or cellular material. The Rayleigh peak broadens and covers any Raman effects. Turbid samples in particular, broaden this central scattering peak. This is due to Tyndall and "large particle" scattering. Tyndall scattering is caused by colloidal particles of about 0.05-0.20 μm in size. "Large particle" scattering is caused by particles of a greater size [4,8].

These effects will compete with the emission signal of a compound such as fluorescein and at low concentration may become prohibitive. Fluoresceinisothiocyanate has a small Stoke s' Shift. This means the emission signal is near to the scattering effects. As the concentration of the fluorochrome drops so the competition from the scattered light becomes significant.

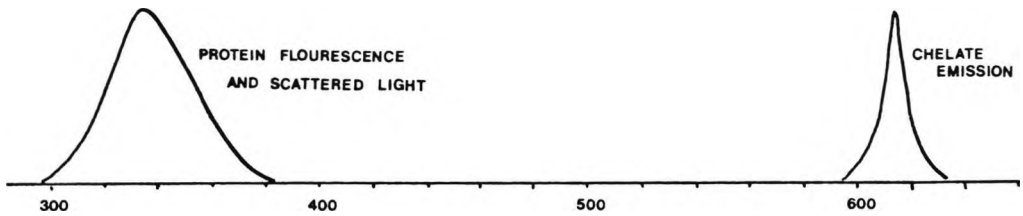
For fluoresceinisothiocyanate and other organic compounds of similar properties scattering is not the only problem. Proteins themselves fluoresce, mainly due

to tryptophan and tyrosine residues. This fluorescence, peaking in intensity at 333 nm, causes a background signal. Once again it is as the fluorochrome concentration becomes low that this background signal becomes significant.

The combined effects of scattering and protein fluorescence mean that the detection limit for fluoresceinisothiocyanate in serum is 1000 ng/ml. This compares with a 30 ng/ml detection limit in phosphate buffer [3].

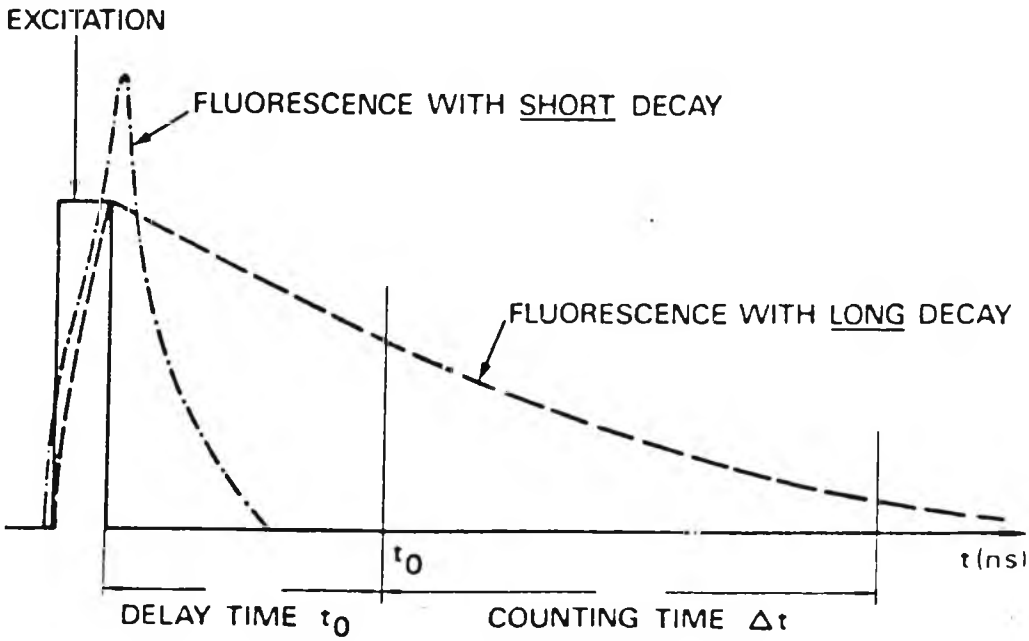
Some of the background may be removed by filters. The low wavelength protein fluorescence (333 nm) is far enough away from the fluorochrome's emission (520 nm) for this to be separated. However, the short Stokes' Shift of Fluorescein and many other organic fluorochromes means that the scattering effects cannot be removed by filters.

The lanthanide chelate class of protein labels was introduced to enable the background signals to be removed. This was achieved by using their properties of a long emission lifetime and a large Stokes' Shift. The technique is called Time Resolved Fluorimetry. The large Stoke s' Shift of the compounds meant that filters could be used to exclude both the protein and scattering signals. (Both signals peak around 340 nm).



The long lifetime enables the fluorochrome, typically a Europium-β- diketonoate complex to be analysed as follows:

Fig 3



The chelate is excited by a pulsed light source (Stage 1). The protein fluorescence and scattering effects are all short lived ($\sim$10 ns) and so the detector is kept shut for a few hundred nanoseconds. Then the detector is opened. All that will be seen is the long lived emission of the lanthanide chelate (lasting $\sim$1 ms). This sequence when combined with a good filter system cuts out the background effects almost entirely.

Immunoassay systems have been marketed around this technology by Pharmacia-Wallac. They claim to have a sensitivity that is competitive with ^{125}I labelling. [3] A possible drawback is that many of the chelates are unstable in an aqueous environment. Europium, for example, has a strong affinity for co-ordinating nine water molecules in place of the diketone ligands [3]. This problem is overcome by adding a detergent to form a micelle during the assay [11]. These compounds have been used in extremely sensitive assays. The most recent development has been in the use of a europium chelate in a non-separation immunoassay [15]. This enables the assay for estrone-3-glucuronide to be extremely simple. The sensitivity, precision and speed make this assay a strong improvement over the comparable RIA.

Conclusion

The best fluorophores for immunoassay that are currently available are the Lanthanide Chelates. In particular, the availability of stable compounds has greatly increased their usefulness. This thesis investigates whether Luminescent Transition Compounds have the properties to make them suitable as alternatives to the Lanthanide Chelates.

4.3 The Use of Transition Metal Complexes in Time Resolved Analysis

This thesis describes the synthesis and conjugation to antibody of a new class of protein labels, the Transition Metal Luminescent Complexes. The work would build on the ability of time resolution and a large Stokes' Shift to remove background effects in photoluminescent analysis.

Their luminescent properties are typified by the following examples:

Compound	Solvent	excitation λ_{ex} nm	emmission λ_{em} nm	lifetime τ ns.	quantum yield
$Ru(bipy)_3^{2+}$	H_2O	453	608	650	0.042
$Ru(phen)_3^{2+}$	H_2O	450	610	1000	0.058
$Ru(dpphen)_3^{2+}$	EtOH:MeOH	464	618	4680	0.36

Table 1

These three complexes are representative of a large family of ruthenium polypyridyl derivatives [9].

The Stoke s' Shift of these compounds is large (150 - 160 nm) and their luminescent lifetime is longer than scattered light and protein emission. The main difference between these ruthenium complexes and the

lanthanide chelates is in the order of magnitude of the emission lifetime. Typically a ruthenium polypyridyl complex gives a luminscent lifetime of 1.0 μs (10^{-6}S). On the other hand the europium-β-diketonoate complexes give emissive lifetimes of about 1.0 ms (10^{-3}S).

This means that, to observe the emission in a time resolved manner of the ruthenium complexes, the equipment will have to be changed.

The lanthanide chelates are excited by a pulsed Xenon flash lamp. This produces a bright pulse of light for about 100 μs at 340 nm. This pulse of light is not suitable for most transition metal complexes as it is longer lived than the luminescent lifetime. Two possible solutions can be made to this problem. Firstly and ideally the excitation source could be a laser. An argon ion laser would be ideal. This could be pulsed to a few nanoseconds and would provide a powerful burst of excitation energy at 480 nm.

At the time of working on this thesis argon ion lasers were thought to be too expensive to use for an analytical instrument of this type. The competitive instruments based on xenon flashlamps and radioactive counting would not be displaced by a more expensive instrument. However, as time goes on the price and reliability of argon ion lasers is expected to improve

so laser excitation may become commercially viable.

The second solution to observing the metal complexes involves the use of "phased fluorimetry" [10]. This technique excites the sample with an oscilating light source. The exciting light follows a sinusoidal pattern and the emission is seen as a delayed wave.

Conventional lamps may be used. However, phased systems are relatively inefficient and would be probably unsuitable for use with these compounds. Of the two methods the pulsed laser excitation which is so powerful is the preferred method for the analysis of complexes in immunoassays.

This combination of an appropriate labelling compound with a laser source of excitation could prove to be a very sensitive technique. An additional benefit of using these compounds is in their stability. They may even be boiled in acidic or basic solutions without substitution [12].

4.4 Chemiluminescent labels for immunoassay

Chemiluminescence may be defined as the production of light by a chemical reaction which produces an electronically - excited state in one of the reactants

or products. As the excited state loses energy this may be accompanied by the emission of light. The significant example of this phenomenon is the emission from isoluminol with hydrogen peroxide and microperoxidase [20]. Derivatives of this and several other organic chemiluminescent molecules eg aminonaphthal hydrazides, acridinium esters and active oxalates have been made for immunoassay work [11]. The great advantage of chemiluminescent emission is that no excitation light source is required and so the background noise is very low (cf the scattering effects described earlier). However, chemiluminescent reactions are extremely sensitive to interference from compounds found in biological media [11]. This means that their use in analytical techniques such as an immunoassay can be unreliable. Another factor that affects chemiluminescent processes is temperature. This once again tends to indicate that they are not always suitable for robust assays.

4.5 The Use of Transition Metal Complexes in Chemiluminescent Analysis

The chemiluminescent emission of Ruthenium tris bipyridyl complexes has been well investigated [9,14,17]. There are two ways to generate the excited state $*Ru(bipy)_3^{2+}$ by chemical reaction. One way is to oxidise $Ru(bipy)_3^{+}$ with a species X having an oxidising

potential greater than 0.84V (equ 1). The other way is to reduce $Ru(bipy)_3^{3+}$ with an agent Y with a reduction potential greater than -0.86V (equ 2) [9].

(1) $Ru(bipy)_3^{+} + X \longrightarrow {}^{*}Ru(bipy)_3^{2+} + X^{-}$

(2) $Ru(bipy)_3^{3+} + Y^{-} \longrightarrow {}^{*}Ru(bipy)_3^{2+} + Y$

$${}^{*}Ru(bipy)_3^{2+} \longrightarrow Ru(bipy)_3^{2+} + h\nu$$

Oxidants such as $S_2O_8^{2-}$ [16] and reductants such as e^{-}_{aq}, H^{-}, N_2H_4, oxalate and OH^{-} [9,17] have been used. The emission is bright and peaks at about 610 nm. For our purposes both the creation of the species to be oxidised or reduced and the oxidant/reductant are important. For example the use of $NaBH_4$ (H^{-}) on $Ru(bipy)_3^{3+}$ requires the prior oxidation of $Ru(bipy)_3^{2+}$. This was achieved by mixing a solution of $Ru(bipy)_3^{2+}$ with solid lead oxide [17]. Hence for complex-antibody conjugates two reactions would be required to produce light emission in an immunoassay.

For an analytical technique the main disadvantage in using the chemiluminescent method is that $Ru(bipy)_3^{+}$ or $Ru(bipy)_3^{3+}$ have to be made in situ and are not stable over a length of time. This when combined with the problems encountered in chemiluminescent assays (as stated earlier) probably would make this an unsuccessful

method for use in immunoassays.

4.6 Electrochemically Generated Chemiluminescence

This method generates the excited state of $Ru(bipy)_3^{2+}$ by electrochemical means. Here the problems of instability of the species $Ru(bipy)_3^{3+}$ and $Ru(bipy)_3^{+}$ is overcome by their either continuous generation by electrode or by them being used instantaneously in the light emission process eg electrochemical reduction of $Ru(bipy)_3^{2+}$ in the presence of a strong oxidant [9]. In this case the luminescence was obtained upon continuous reduction of $Ru(bipy)_3^{2+}$ at a working electrode in the presence of $S_2O_8^{2-}$ [16].

The first applications of similar methods for an immunoassay have been reported for pyrene [18] and $Ru(bipy)_3^{2+}$ [19]. This method may have a great potential as most of the problems encountered in chemiluminescent systems are overcome by this "steady" way of generating the excited state. The complexes described in this thesis would probably be suitable for this type of analysis.

4.7 Conclusion

Tris bipyridyl and phenanthroline complexes of ruthenium can be analysed by photo-, chemi- and electrochemi-

luminescent techniques. The photoluminescent and electrochemically generated luminescent methods are probably the most suitable for immunoassay. Both methods would require new assay equipment to be made (which is to their disadvantage). However, they have the potential to be incorporated into very sensitive assays. None of the derivatives that have been reported in the literature are suitable for conjugation to protein. Therefore, there is the need to develop the chemistry to introduce functional groups onto the complexes that will enable them to be covalently linked to protein. This is the aim of the thesis.

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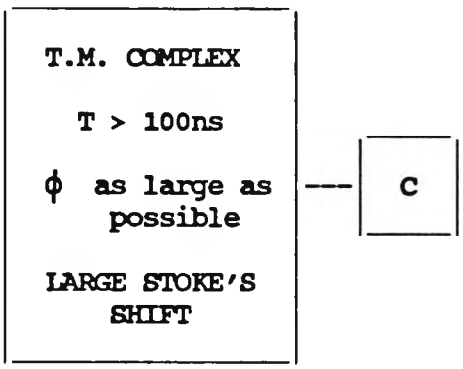
Chapter 5

THE SYNTHESIS OF METAL COMPLEX COMPOUNDS FOR ANTIBODY LABELLING.

5.00 Introduction

This chapter describes the work carried out to make metal complexes suitable for use in an immunoassay. The aim was to synthesise compounds that would covalently bind to antibodies and have the required luminescent characteristics when bound. This may be summarised as follows:



where group C is able to form a covalent link with amine groups on the antibodies.

Several compounds of the ruthenium bipyridyl and phenanthroline "families" were successfully prepared. These were linked to antibodies and some possessed the required properties. Complexes of chromium and aluminium were also prepared but none were successfully linked to protein. This project may be understood best

as a reconnaissance into the suitability of luminescent metal complexes for protein labelling. The purpose was to cover as many avenues of approach as possible with patent justification in mind.

THE SYNTHESIS OF LUMINESCENT RUTHENIUM COMPOUNDS FOR ANTIBODY LABELLING

5.10 Synthetic Strategies for Ruthenium Compounds

Chapter 3 reviewed the properties of ruthenium bipyridyl and phenanthroline complexes. A huge number of derivatives of the simple Ru $bipy_3^{2+}$ and Ru $phen_3^{2+}$ complexes have been made [1-4,22]. However, none of the derivatives were suitable for forming covalent bonds with proteins directly.

Ruthenium complexes with three bipyridyl or phenanthroline ligands may be made by two main approaches [24].

1 $RuCl_3.3H_2O \longrightarrow Ru\,bipy_3^{2+}X_2^-$

2 $RuCl_3.3H_2O \longrightarrow Ru\,bipy_2Cl_2$

$\longrightarrow Ru\,bipy_2\,bipyD^{2+}X_2^-$

"bipyD" may be bipyridine or any derivative of bipyridine, eg. 4,4'-Dimethyl-2,2'-bipyridyl.

X^- is Cl^- or PF_6^-.

Most derivatives of bipyridyl and phenanthroline tend to be "bis".

eg. 4,7-diphenyl-1,10-phenanthroline

or 2,2'-bipyridyl-4,4'-dicarboxylic acid. Hence reaction 1 would give six functional groups on each complex if a derivatised bipyridyl was used. Even the use of mono-substituted bipyridyls would give complexes with three functional groups. In this work the use of reactive functional groups was required but a large number on each complex could cause crosslinking of the antibodies. Crosslinking is known to impair the ability of antibodies to bind antigens and so this is undesirable. Approach 2 offers a strategy in which each complex would have one or two reactive groups. This strategy was applied to most of the ruthenium compounds made. For addition of a third ligand to Ru $bipy_2Cl_2$ the use of protic solvents like water, methanol and ethanol is preferred.

The reaction proceeds as follows [28]:

$$Ru(bipy)_2Cl_2 + MeOH \longrightarrow [Ru(bipy)_2Cl\ MeOH]^+$$
$$\longrightarrow [Ru(bipy)_2bipyX]^{2+}$$

DMF and DMSO also may be used [29] where $[Ru(bipy)_2ClDMSO]^+$ may be isolated as the intermediate. A common solvent mixture for the addition of a third ligand to Ru $bipy_2Cl_2$ is DMF:Ethanol, 1:1.

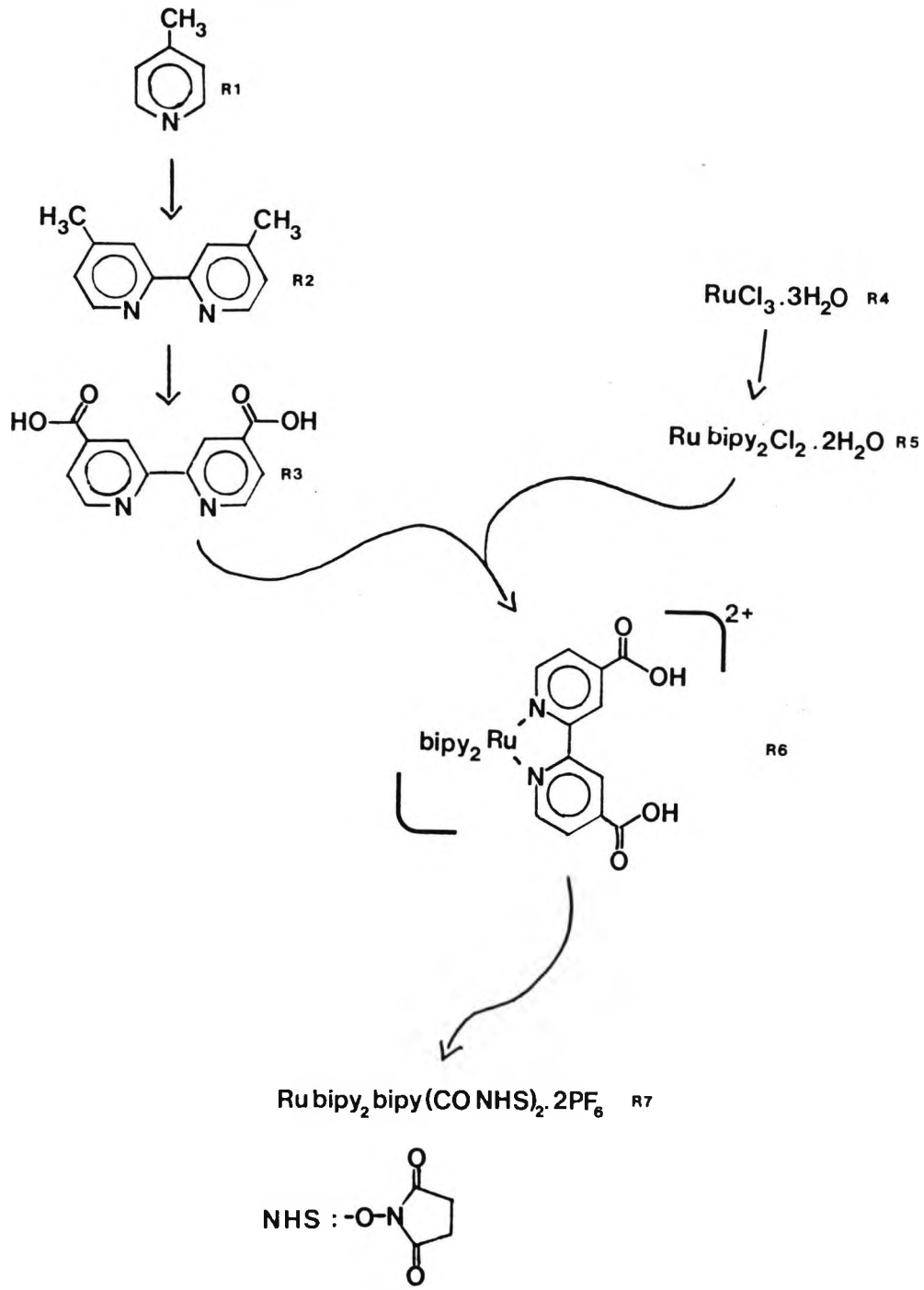
Isolation of the compounds was usually achieved by precipitation. Addition of a large counter-ion such as hexafluorophosphate or perchlorate to an aqueous solution of a complex often leads to precipitation of the complex eg. tris-bipyridyl ruthenous chloride (Ru $bipy_3Cl_2.6H_2O$) in aqueous solution forms a red precipitate on the addition of ammonium hexafluorophosphate. This precipitate is tris-bipyridyl ruthenous hexa-fluorophosphate (Ru $bipy_3.2PF_6$). In some cases this reaction is nearly quantitative leaving an almost colourless solution of ammonium chloride. The precipitation is thought to occur because large ions have low hydration energies giving a more thermodynamically stable product on forming the solid [30].

$$\text{eg. } \underset{-66}{Cs^+_{(aq)}} + \underset{-70}{I^-_{(aq)}} \longrightarrow \underset{-141 \text{ (lattice energy)}}{CsI_{(s)}} \qquad \Delta H^o = -5 \text{ kcal/mol}$$

For ruthenium complexes there was an exception to this rule when the ligands have phenyl groups attached. The complexes tend to be insoluble in water without the addition of hexafluorophosphate.

Purification of complexes was achieved by recrystallisation where possible. Products with unsatisfactory CHN microanalysis were sometimes used crude to label protein. This was possible as the purification of the complex-antibody conjugate by gel filtration (G25 Sephadex) would remove impurities. Gel filtration of products using LH20 sephadex (not suitable for complex-antibody conjugates) with various organic eluents was attempted for a few impure complexes. The sephadex captures small molecules in its pores and allows larger species to run down the column faster. However, Ru $bipy_2$ $bipy(CO_2H)_2$ $^{2+}$ showed no improvement in microanalytical data after passing down a 20 cm LH20 column eluting with methanol. The rest of this section outlines the synthetic strategies adopted to produce antibody labels.

5.11 Strategy 1



This was the first strategy attempted. The dicarboxylic acid complex R6 had been made by Whitten et al [1] for esterification with long chain alcohols. These were used to make surfactant layers for the photo-cleavage of water. For purpose of antibody labelling a carboxylic

acid converted to an active ester seemed a good approach. There are a large number of methods for activating carboxylic acids to nucleophilic attack. In particular peptide synthesis involves such chemistry [5]. The formation of amide bonds with proteins is very attractive for this work due to their stability in biological conditions and buffers.

A widely used method for activating carboxylic acids involves converting them to an N-hydroxysuccinimide active ester [6]. Preparation of the bipyridyl-diacid R3 followed the method of Whitten et al [1] and was performed routinely. 4-Methylpyridine, R1, was added to 10% palladium on carbon and the mixture refluxed for 5 days. The palladium on carbon was removed by filtration and the liquid reduced in volume. The remaining solution was allowed to stand at room temperature until a good crop of crystals formed. These were recovered and recrystallised from ethylacetate. This pure product: 4,4'-dimethyl-2,2'-bipyridyl, R2, was mixed with potassium permanganate in water. The solution was refluxed to give a brown precipitate. The precipitate (MnO_2) was removed and the aqueous solution washed with ether to remove any unreacted 4,4'-dimethyl-2,2'-bipyridyl. The acid, R3, was precipitated from the aqueous phase using hydrochloric acid. The preparation of Ru $bipy_2Cl_2.2H_2O$ was done by adding $RuCl_3.3H_2O$ to a two fold molar excess of bipyridine in DMF. The mixture

was refluxed for 5 hours, reduced in volume and quenched with a large excess of acetone. Black crystals formed after 24 hours at $0^{o}C$. This was only successful on addition of lithium chloride to the reaction mixture at the start.

The diacidic complex R6 formed readily in solution on mixing the bipyridyl-diacid, R3 and Ru $bipy_2$ Cl_2 in methanol and aqueous sodium bicarbonate. The mixture was refluxed for 2 hours to give a bright orange/red solution. However, isolation of the complex proved very difficult. The method of Whitten et al [1] was attempted several times without any success in obtaining a precipitate. The method states that adding ammonium hexafluorophosphate to the mixture followed by refrigeration overnight should give a red precipitate. We found a precipitate only formed after neutralisation of the solution with hydrochloric acid followed by addition of ammonium hexafluorophosphate. This is understandable on consideration of the complex in acid and basic solutions.

Ru $bipy_2$ bipy $(CO_2^-)_2$ $^{2+}$ basic solution.

This complex is neutral over all and so will not combine with an anion such as hexafluorophosphate.

Ru $bipy_2$ bipy $(CO_2H)_2$ $^{2+}$ acidic solution.
The complex is now positively charged and will combine and precipitate with hexafluorophosphate as observed.

The product R6, although not pure according to its microanalysis, gave a satisfactory FAB Mass Spectrum, 'Hnmr and UV spectra. In particular the UV in acidic and basic solutions matched the literature spectra [7].

The formation of the N-hydroxysuccinimide active ester of R6 was carried out by mixing N-hydroxysuccinimide and DCCI with R6 in dry DMF for 2 hours at room temperature. (The way carbodiimides are thought to work is detailed later in the section). Isolation of this complex was not attempted as removing the DMF (heat required) and adding aqueous ammonium hexafluorophosphate would hydrolyse the ester. Instead the complex was added to a solution of antibody in carbonate buffer (pH 9.5). The DMF solution was kept at a 1:10 ratio to the aqueous solution to avoid denaturing the protein. The mixture was left overnight at $0^{o}C$ and then passed through a 20 cm G25 sephadex column eluting with PBS. The antibody fraction was coloured red and ran on the solvent front. The unreacted complex ran more slowly and was discarded. Fluorescence spectra of the antibody fraction showed a fluorescent compound to be present with similar characteristics to other ruthenium bipyridyl complexes.

λ_{ex} = 450 nm

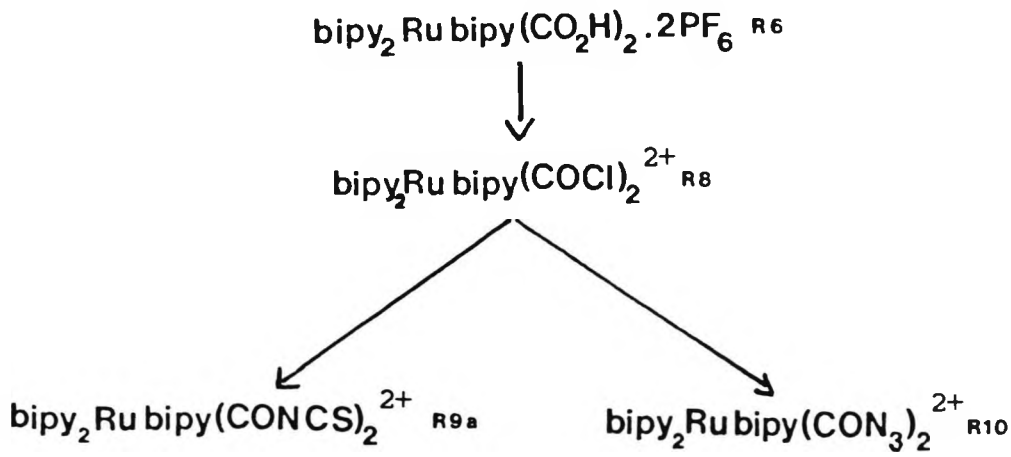
λ_{em} = 630 nm

luminescent lifetime 410 ns (aerated solution).

Further discussion of labelling, spectra and luminescent lifetimes will be given in the next chapter. The method, in outline, for labelling antibodies is as shown above with detail and variations described in Chapter 6 (6.00 and 6.10).

This methodology, although somewhat unreliable, prompted further work as the complex-antibody conjugate showed the target properties stated at the beginning of this chapter.

5.12 Strategy 2



There are other ways apart from active esters to derivatise carboxylic acids. This strategy attempted the preparation of acyl isothiocyanates and acyl azides.

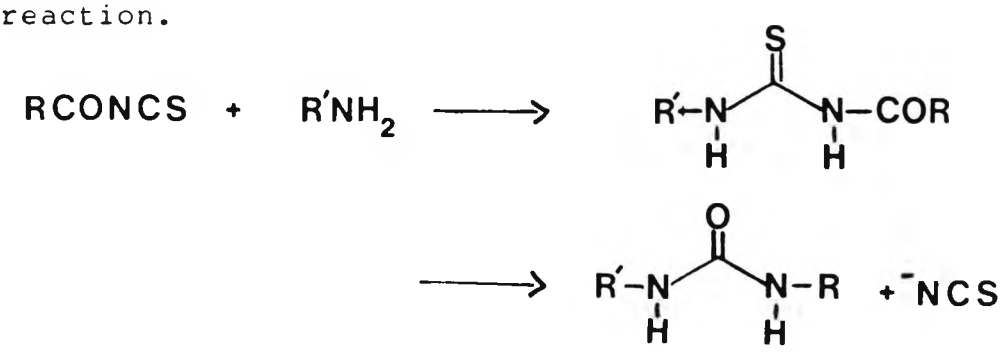
The acid chloride complex R8 was prepared using an excess of thionyl chloride following Whitten et al [1]. However, the product was not pure. It was suspected that not all the thionyl chloride was removed by vacuum. The preparation of R9a was attempted by adding potassium thiocyanate to the acid chloride dissolved in acetone. A white precipitate formed (KCl) and was removed by filtration and the acetone was removed to give a brick red solid. Thiocyanate ions have "hard" and "soft" ends:

$$^{-}N{=}C{=}S \longleftrightarrow N{\equiv}C{-}S^{-}$$

The negative charge localises itself more towards the nitrogen atom (more electronegative) and so it is this end of the ion that is the dominant nucleophile. In practice this gives a mixture of products with the isothiocyanate isomer the dominant product.

$$RCOCl + KNCS \longrightarrow RCONCS + RCOSCN + KCl$$

The IR spectrum of the product showed the main product was RCONCS (2050 cm^{-1} st) with little evidence of RCOSCN formation. Only RCONCS is reactive towards nucleophiles itself. There are two possible mechanisms for the reaction.



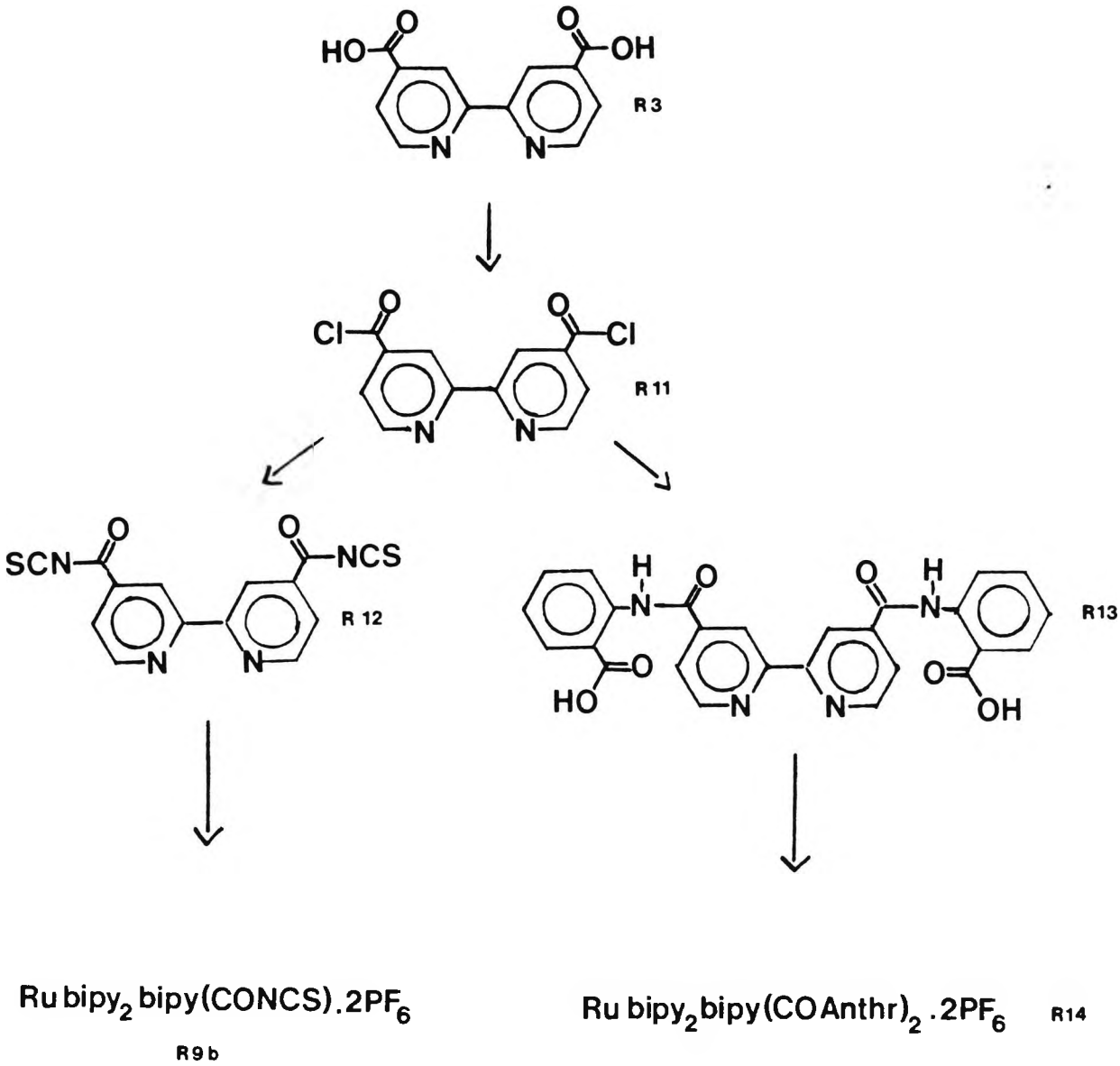
Evidence from studies on mixing m-toluyl isothiocyanate with aniline suggests a thiourea linkage is the product [23]. Also consideration of the effect of a carbonyl group on the isothiocyanate would suggest that NCS carbon is more susceptible to nucleophilic attack ie. electrons are pulled away from this carbon by both the nitrogen and carbonyl groups.

R9a in its crude form was used in a labelling experiment on polyclonal and monoclonal antibodies. It appeared to bind to both (G25 separation) but electrophoresis of the monoclonal sample removed the luminescent complex from the antibody (Electrophoresis carried out at Boots-Celltech Diagnostics Ltd). This suggested that a non-covalent bond was formed with the antibody.

The synthesis of the aryl azide complex, R10 was unsuccessful. It was attempted by adding sodium azide in water to diacid chloride, R8, in acetone. A pink solid was isolated but did not show any evidence that an acyl azide had formed.

The lack of success with this strategy probably stemmed from R8 being impure. Hence an alternative approach was adopted avoiding Whitten's synthetic methods.

5.13 Strategy 3



This approach avoids making a complex with 2,2'-bipyridyl-4,4'-dicarboxylic acid. Instead more chemistry is carried out on the ligand before complex

formation. This seemed a wise approach as organic reactions are often altered by the inductive effects of transition metal ions. 2,2'-Bipyridyl-4,4'-dicarboxylic acid, R3, was converted to its acid chloride derivative, R11, using thionyl chloride in toluene. R3, the acid, was only slightly soluble in this mixture but the acid chloride product, R11, was readily soluble. Hence the reaction was complete when all the solid diacid had disappeared. The work up was more thorough than for R8 and followed the method of Valenti et al. [9] The thionyl chloride was removed by twice fractionally distilling the mixture, followed by rotary evaporation and vacuum removal of any remaining solvent (1 mm Hg). The product was a grey/yellow solid and was very unstable to moisture. It began to decompose in a vacuum desicator after a few days. The TLC of the product showed there were two compounds present (one major, one minor). The instability of the acid chloride meant it had to be used quickly to make R12 and R13.

The acyl isothiocyanate dervative, R12, was made using a 6:1 molar excess of potassium isothiocyanate in acetone. Solid potassium chloride and some decomposed acid chloride (to the parent acid?) were recovered by filtration. TLC of the acetone solution showed two products were present. Mass spectral analysis did not give a molecular ion but did give bipy(CONCS)CO^+: (m/z 268). Infrared analysis gave a clear NCS stretch at 2064 cm^{-1}.

This lies within the R-NCS range 2050-2150 cm^{-1} but not in the RSCN range of 2135 - 2170 cm^{-1} [8]. This crude material was taken and mixed with $Rubipy_2Cl_2.2H_2O$ to make R9b. To make this complex successfully the isothiocyanate groups would have to be kept intact. Hence contact with nucleophilic species was kept to the minimum. In section 5.10 it was mentioned that the addition of a third ligand to a ruthenium complex requires a protic solvent such as ethanol, methanol or water. These solvents if used at reflux temperatures would have destroyed the isothiocyanate. DMF or DMSO would have been suitable except that they would require too much heat for their removal. Hence acetonitrile was used as it was strongly polar without being nucleophilic. There was no precedent for using it but the reaction proceeded successfully. After 2 days of refluxing the mixture in dry acetonitrile under nitrogen a strong red colour had formed. The acetonitrile was easily removed with gentle heating by rotary evaporation until 3 mls of the solution remained. An excess of water was added and the mixture filtered. This removed unreacted $Rubipy_2Cl_2.2H_2O$ and unreacted bipyridyl species (all these are insoluble in distilled water). To the resulting clear red solution an excess of ammonium hexafluorophosphate was immediately added. The precipitate was quickly filtered and dried to give a red solid. Infrared analysis showed the isothiocyanate

group had survived the reaction (-NCS stretch at 2103 cm^{-1} str). The compound gave a luminescent lifetime of 275 ns in acetonitrile (aerated). The compound successfully labelled a polyclonal antibody sample (human IgG) giving the following fluorescence spectrum and luminescent lifetime.

λ_{ex} (max) = 470 cm

λ_{em} (max) = 620 nm

lifetime = 125 ns (PBS aerated)

It is possible that the $Ru(bipy)_2(CH_3CN)Cl^+$ complex forms as an intermediate during this preparation. $[Ru(bipy)_2(CH_3CN)Cl]Cl$ has been isolated from a similar reaction mixture [27].

The anthranilic acid derivative, R13, was prepared so as to give a carboxylic acid functional group some distance from the "luminescent part" of the molecule. p-Aminobenzoic acid was the preferred reactant to anthranilic acid on paper. However, anthranilic acid was far more soluble in acetonitrile so enabling the concentration of reactants to be kept high. The acid chloride and anthranilic acid were both dissolved in separate quantities of acetonitrile. On mixing at room temperature with vigorous stirring (acid dripped into acid chloride) a yellow precipitate formed. This was recovered and dried etc. 'Hnmr (360 MH_z) showed the desired product had formed with some unreacted anthranilic acid as an impurity. Mass spectral analysis did not give a molecular ion but gave $M^+ - 2H_2O$ (446, 7.2%) and $M^+ - H_2O$ (464). Anthranilic acid would not interfere with the next reaction and would not be precipitated by ammonium hexafluorophosphate hence the

impure material was used.

$Rubipy_2Cl_2.2H_2O$ was mixed with the above derivative (a 1:0.9 excess of ligand was used) in DMF. After one hour of refluxing the mixture, no colour change was observed. Some ethanol was then added and after one hour the solution began to turn red. After 24 hours of refluxing the mixture the solution was bright red. The solvent was reduced to 5 mls in volume and a large excess of water was added. This was filtered to remove any impurities and then an excess of ammonium hexafluorophosphate was added. The resulting precipitate was collected. FAB Mass Spectroscopy showed that the desired product had formed (m/z 895 at centre of typical Ru isotope pattern). The fluorescence spectrum showed:

λ_{ex} = 492 nm broad

λ_{em} = 660 nm broad

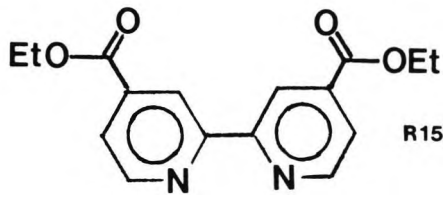
solvent CH_3CN

lifetime of emission = 653 ns (aerated)

This complex was mixed with a water soluble carbodiimide (2:1 excess of carbodiimide) in acetonitrile and stirred. The mixture was evaporated to dryness and the solid was added to antibody. This successfully bound to the antibody and will be discussed in more detail in the next chapter. The antibody - complex conjugate gave a similar fluorescence spectrum to the parent complex and

had an emissive lifetime of 625 ns in PBS (aerated). The carbodiimide was 1-Cyclohexyl-3-(2-morpholino-ethyl) carbodiimide metho-p-toluenesulphonate.

5.14 Strategy 4



R15

↓

Ru bipy$_2$ bipy(CO$_2$Et)$_2$.2PF$_6$ R16

↓

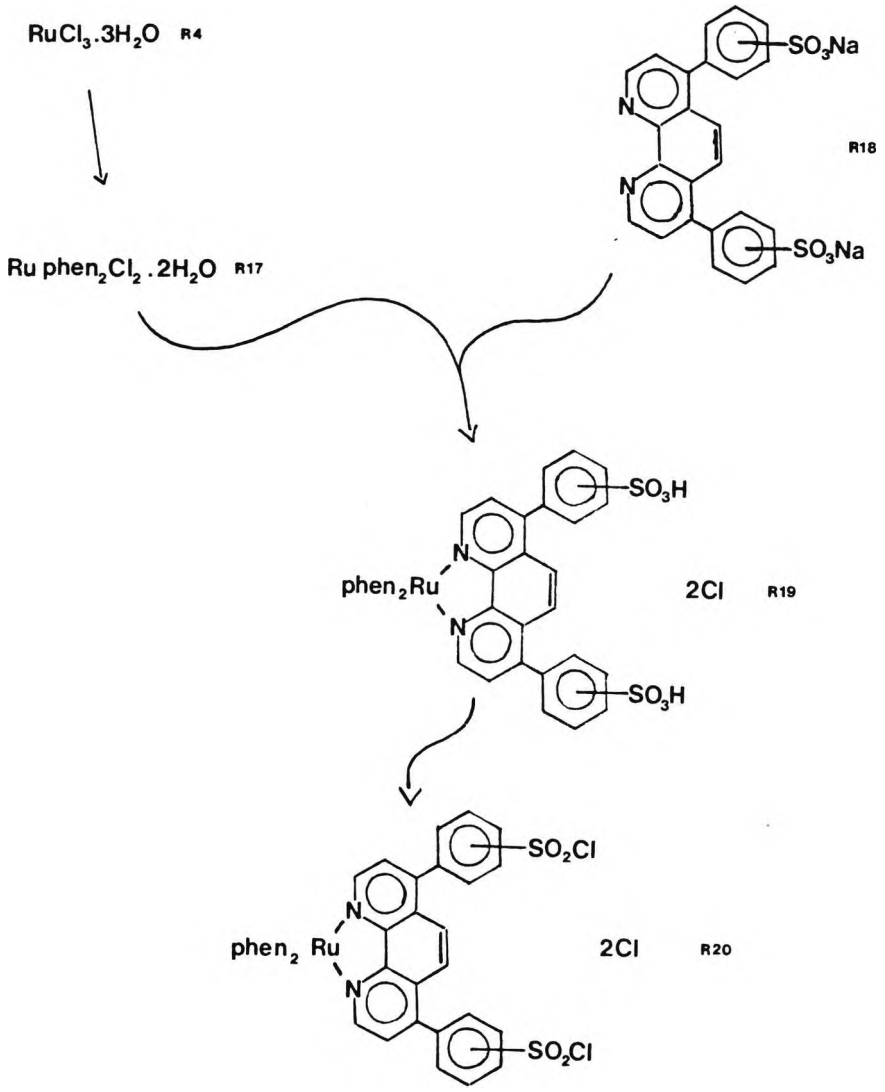
Ru bipy$_2$ bipy(CO$_2$H)$_2$ .2PF$_6$ R6

This strategy was begun as the synthesis of the diacid complex, R6 was proving difficult (see 5.11). When the problems had been overcome this method became superfluous. Fiona Henderson made the diethyl ester derivative R15, and this was mixed with Rubipy$_2$Cl$_2$.2H$_2$O in ethanol. After 2 days of refluxing the mixture, most of the ethanol was removed. An excess of water followed by excess ammonium hexafluorophosphate was added giving a red precipitate. The precipitate was recovered and dried. FAB Mass Spectroscopy showed the desired product

was present (m/z 714 = M^+). The strategy was not pursued further as R6 was reliably prepared using strategy 1. The conversion of R16 to the diacid R6 involves boiling a solution of R16 in aqueous base.

5.15 Strategy 5

From the literature [1-4,22] it was clear that phenanthroline complexes of Ru^{2+} have higher quantum yields and longer emissive lifetimes than their bipyridyl analogues. This prompted the development of a strategy involving phenanthroline ligands.



The disulphonic acid derivative of 4,7-diphenyl-1,10-phenathroline R18 is available from Aldrich. Sulphonic acids are commonly converted to sulphonyl chlorides for protein labelling (dansyl chloride is probably the most well known example). $Ruphen_2Cl_2.2H_2O$ was made by the same procedure as for $Rubipy_2Cl_2.2H_2O$. This complex was then added to the disulphonic acid R18 in a water/ methanol mixture. The mixture was refluxed for 2 hours and a bright orange/red precipitate formed. The precipitate was recovered and recrystallised from water. FAB mass spectroscopy and microanalysis showed the product to be $Ruphen_2phen(\phi SO_3H)_2.2CL^-.3H_2O$ (R19). M^+ at m/z 952 was observed in the centre of a characteristic Ru isotope pattern. The fluorescence spectrum gave λ_{ex} = 480 nm broad

λ_{em} = 615 nm broad

Luminescent lifetime was 1.20μs in $H_2O:CH_3CN$, 1:1 (aerated).

Sulphonic acids may be converted to sulphonyl chlorides by three main methods:

(i) addition of chlorosulphonic acid

(ii) addition of phosphorus pentachloride

(iii) addition of phosphorous oxychloride

Method (i) was adopted first. R19 was dissolved in fresh chlorosulphonic acid and the mixture was heated to 120°C for 2 hours. The solution turned green

indicating that the compound was oxidised from Ru^{2+} to Ru^{3+} (Ru^{3+} is green in solution) cf $Rubipy_3{}^{2+}$ which forms green $Rubipy_3{}^{3+}$ in concentrated sulphuric acid.

After cooling, the above mixture was carefully tipped into ice, drop by drop. The solution turned to a red colour again (Ru^{2+}) and the sulphonyl chloride derivative was extracted into dichloromethane from the aqueous phase. The extract was dried with calcium chloride, filtered and evaported to dryness. This gave a red hydroscopic solid in variable yields of 2-30%. The infrared spectrum showed clear RSO_2Cl stretches at 1380 cm^{-1} and 1180 cm^{-1}. These peaks were not present in the parent acid. The fluorescence spectrum gave typical characteristics:

λ_{ex} = 475 nm broad

λ_{em} = 620 nm broad

Luminescent lifetime = 250 ns in CH_3CN (aerated).
The drop in lifetime to 250 ns from the parent acid (1200 ns) was consistent with the covalent addition of chlorine atoms which quench luminescent emission [25] possibly, in this case, by dissociation of the SO_2Cl group [26].

This compound although not pure by microanalysis was used to label antibody successfully. The complex - antibody conjugate gave a luminescent lifetime between 1000 and 1200 ns (aerated PBS). This was again

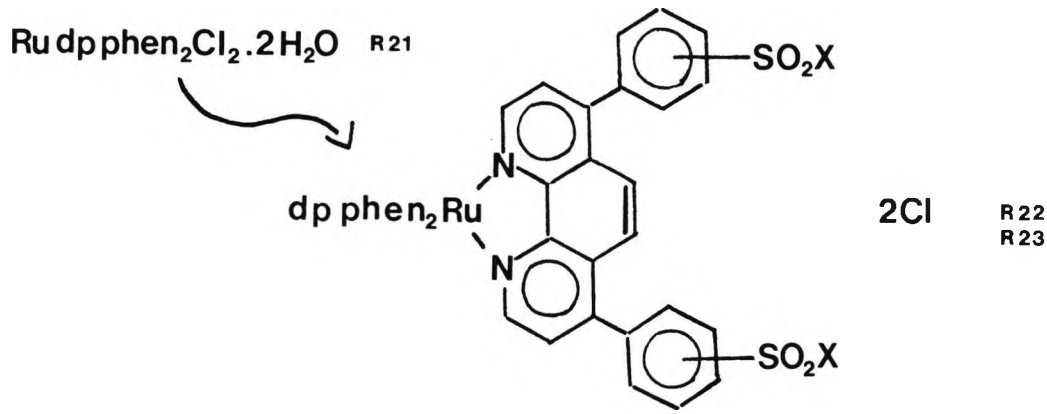
consistent with the loss of chlorine atoms from the molecule. Clearly this compound was very useful for antibody labelling work. It was linked to a monoclonal antibody sample at Boots-Celltech Ltd and was not separated from the antibody by electrophoresis. However, the yields from the above reaction were often poor and so other ways of making the complex were pursued.

Firstly the sulphonic acid complex R19 was mulled together with PCl_5. Unfortunately no effervescence occurred and no complex was recovered that was soluble in dichloromethane. (This method was practiced with 5-dimethyl aminonaphthalene-1-sulphonic acid to make dansyl chloride 7.13). Since completing my practical work Graham Yearwood was produced an excellent product using a $POCl_3/PCl_5$ mixture.

The disulphonic acid compound R19 was also linked to antibody in a similar way to the anthranilic acid compound R14. The compound was mixed with a 2:1 excess of a water soluble carbodiimide (1-Cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluenesulphonate) in acetonitrile. After stirring for 2 hours at room temperature the mixture was evaporated to dryness and a small amount added to antibody. G25 sephadex separation gave a red-coloured antibody fraction with a typical luminescent spectrum. This is discussed further in Chapter 6.

5.16 Strategy 6

The 4,7-diphenyl-1,10-phenanthroline complex of ruthenium 2+, $Ru(dpphen)_3^{2+}$ is one of the best luminescent compounds of this type [22]. This prompted the idea of replacing the two phenanthroline ligands in Strategy 5 with two 4,7-diphenylphenanthroline ligands (dpphen). Schematically the strategy is identical to strategy 5 with the products as follows:



X = OH R22
X = Cl R23

The synthetic work proceeded smoothly with $Rudpphen_2Cl_22H_2O$, R21, being produced in the same way as R5 and R17. The method adopted to make the disulphonic acid complex, R22, was from the previous strategy. Hence R21 and R18 were dissolved in methanol and water. However, after refluxing the mixture for 16 hours, no colour change from the purple colour was observed. Some ethanol was added and within 10 minutes a red precipitate began to form. It is possible that

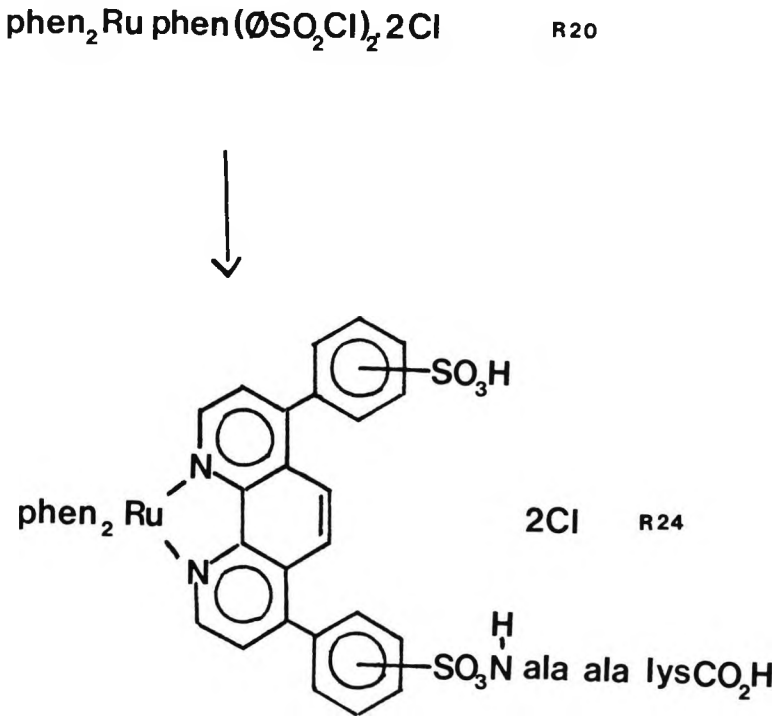
$Rudpphen_2Cl_2.2H_2O$ was insoluble in the solvent mixture of methanol and water (4:1) thus preventing reaction. The addition of ethanol may have changed that.

The precipitate was recovered, washed and dried. Microanalysis for Ru $dpphen_2$ $phen(\phi SO_3H)_2.2Cl.2H_2O$ (R22) showed a pure product with the reaction yield of 85%.

Preparation of the sulphonyl chloride, R23, was attempted using both chlorosulphonic acid and PCl_5. Neither reaction gave an identifiable product nor would either label antibody successfully. Instead of a fraction containing an antibody - complex conjugate separating on the G25 sephadex column there was a red "streaking" of colour down the whole column. To check why this might be happening the parent acid was mixed with antibody (same conditions etc) and was run down a G25 sepahdex column eluting with PBS. The same "streaking" occurred that was observed before. It may be that non-specific, non-covalent binding occurred between the phenyl groups on the complex and the hydrophobic regions of the protein. Such bonds are well known in protein studies. Another more unlikely cause

may involve the interchelation of the complex into the three-dimensional structure of the protein. By comparison Ru dpphen$_3$ $^{2+}$ has been interchelated into DNA helices [10].

5.17 Strategy 7



This strategy was followed successfully and led to the development of a method that would enable a wide range of functional groups to be attached to a protein label. The idea was to build amino-acid "spacer groups" between the labelling complex and the antibody. These might help to reduce quenching of the luminescent properties of the complex and also enable a range of functional groups to be used for protein binding.

eg. ala ala lys gives a $(CH_2)_4NH_2$

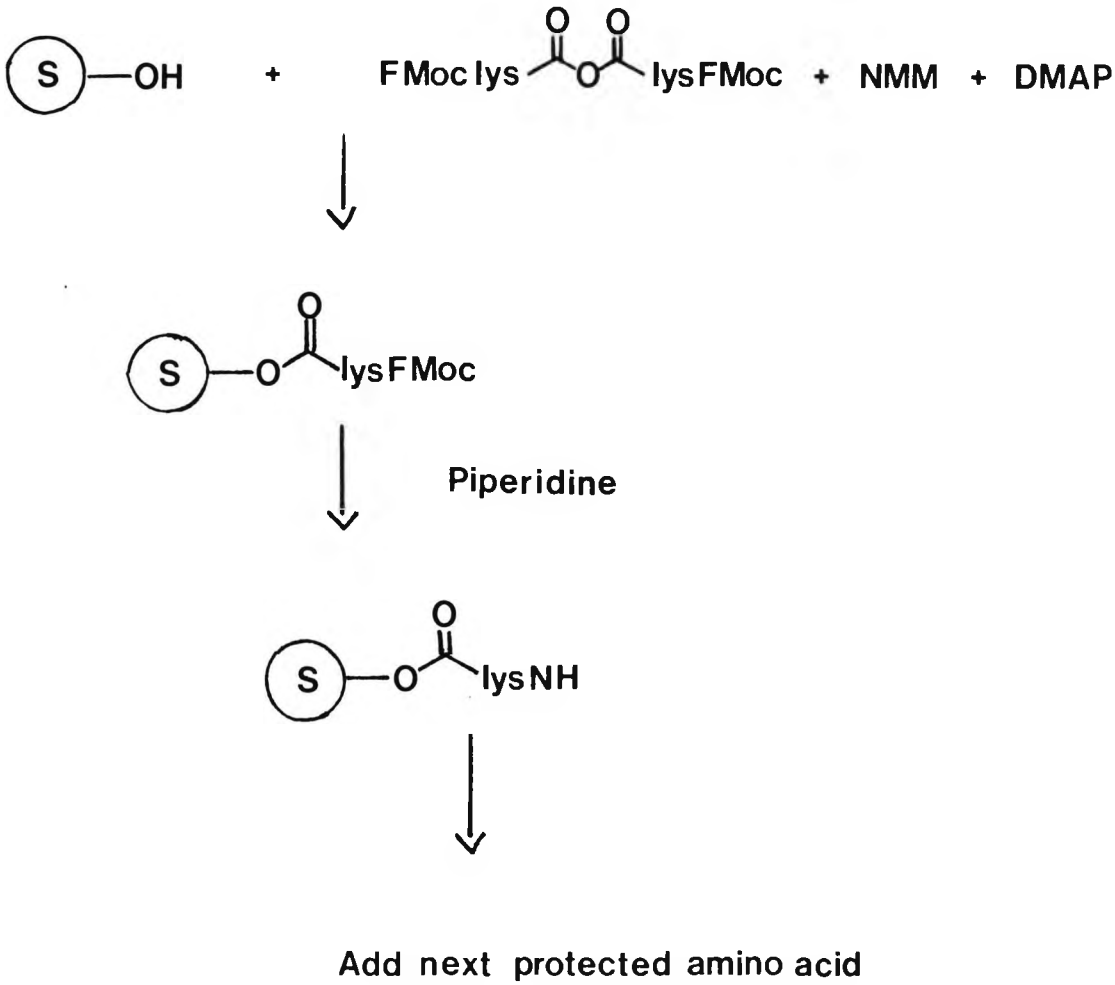
ala ala ala gives a CH_2CO_2H

Hence by varying the number and type of amino acids different functional groups can be attached to a labelling molecule. Also the character of the chain can vary from hydrophobic to hydrophilic.

eg. ala leu leu ala hydrophobic

ala arg arg ala hydrophilic

The strategy used solid phase peptide synthetic methods following the "FMoc" approach [11].



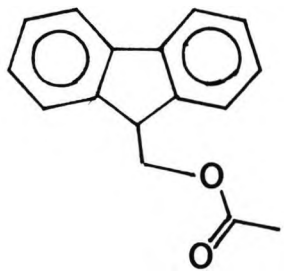
Each amino-acid was protected by the "FMoc" group. The anhydrides were made by mixing a twofold molar excess of

the protected amino-acid with DCCI in dichloromethane. The side chain of lysine $-(CH_2)_4NH_2$ was protected by a Boc group which is acid labile. Hence the lysine amine would not interfere with the synthesis which is carried out in basic conditions.

Each symmetrical anhydride was added to the solid phase in turn until the tripeptide was built up. The first amino-acid (the C terminus of the tripeptide) was added to the solid phase using N-methylmorpholine as base and dimethylaminopyridine as catalyst. This was to ensure that as many OH groups became O^- and to make "in situ" active esters of the anhydride. Also the first amino-acid is added twice to use as many OH groups as possible.

The tripeptide was submitted for amino-acid analysis and gave a 2.16:1 ratio of alanine:lysine.

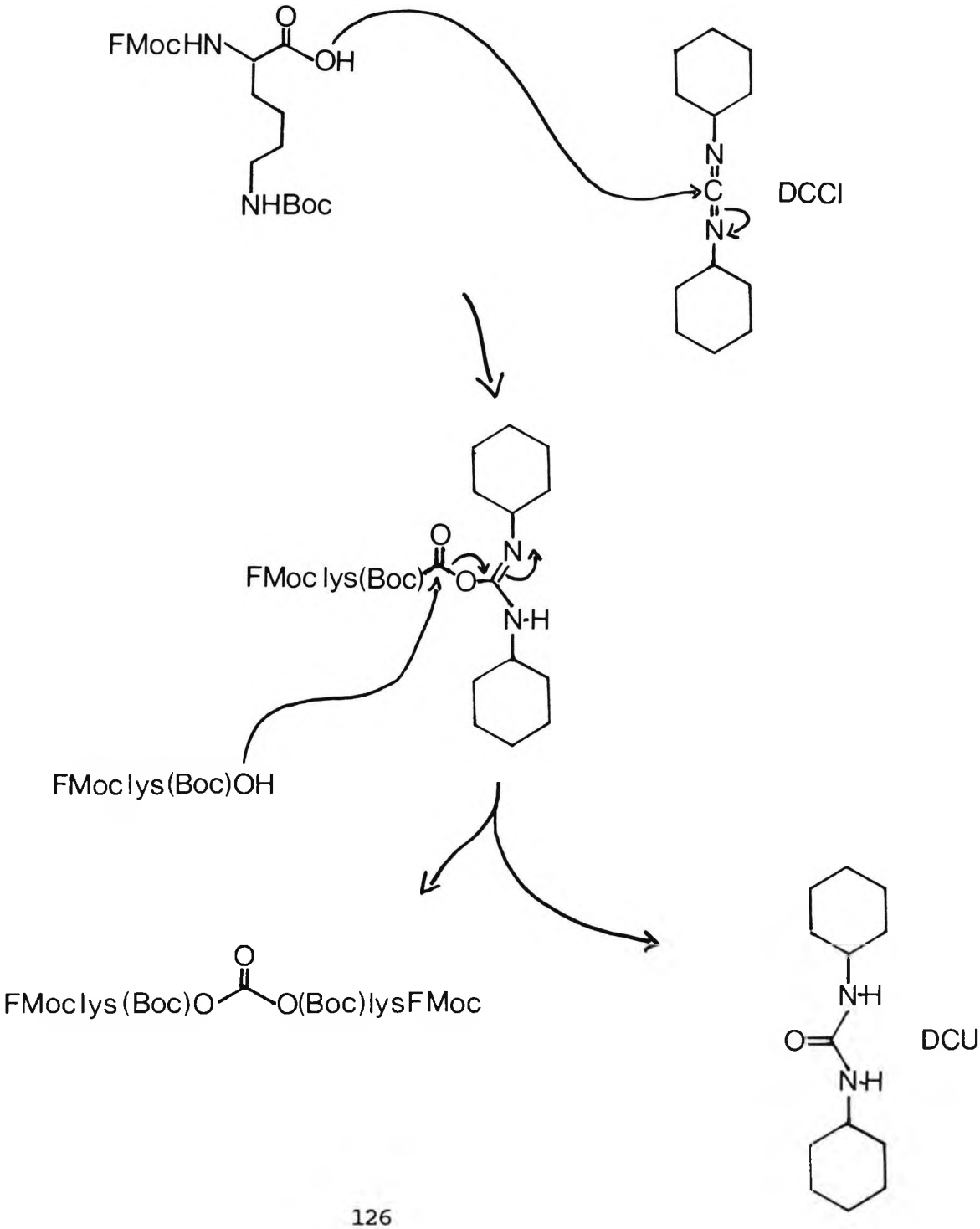
The reactions are now described in more detail:

FMoc = 

= Boc

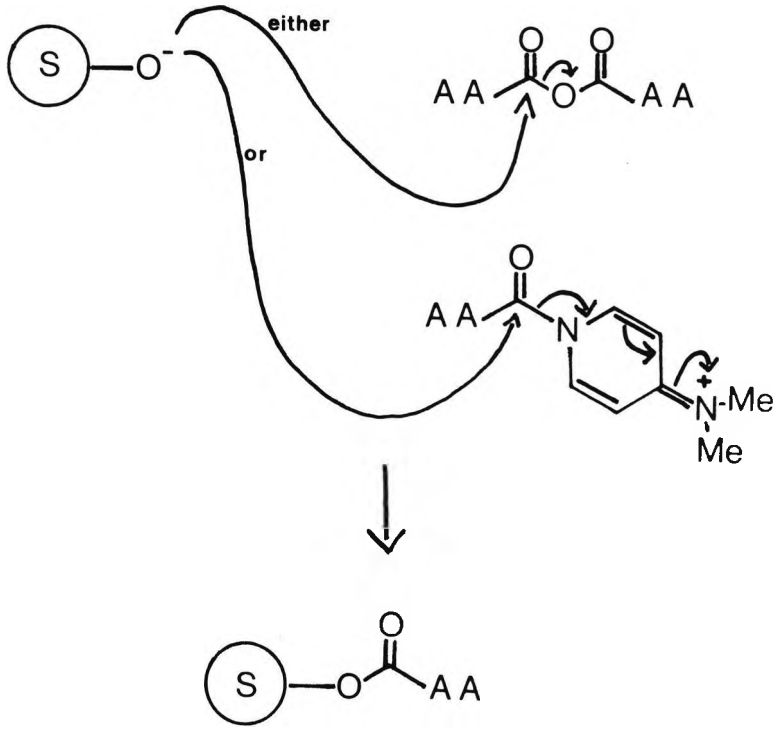
(i) Symmetrical Anydride Formation

The protected amino acids were mixed with DCCI (2:1 molar ratio AA:DCCI). DCU precipitated as the anhydride formed and this was removed. The solution was added to the solid phase as described later.

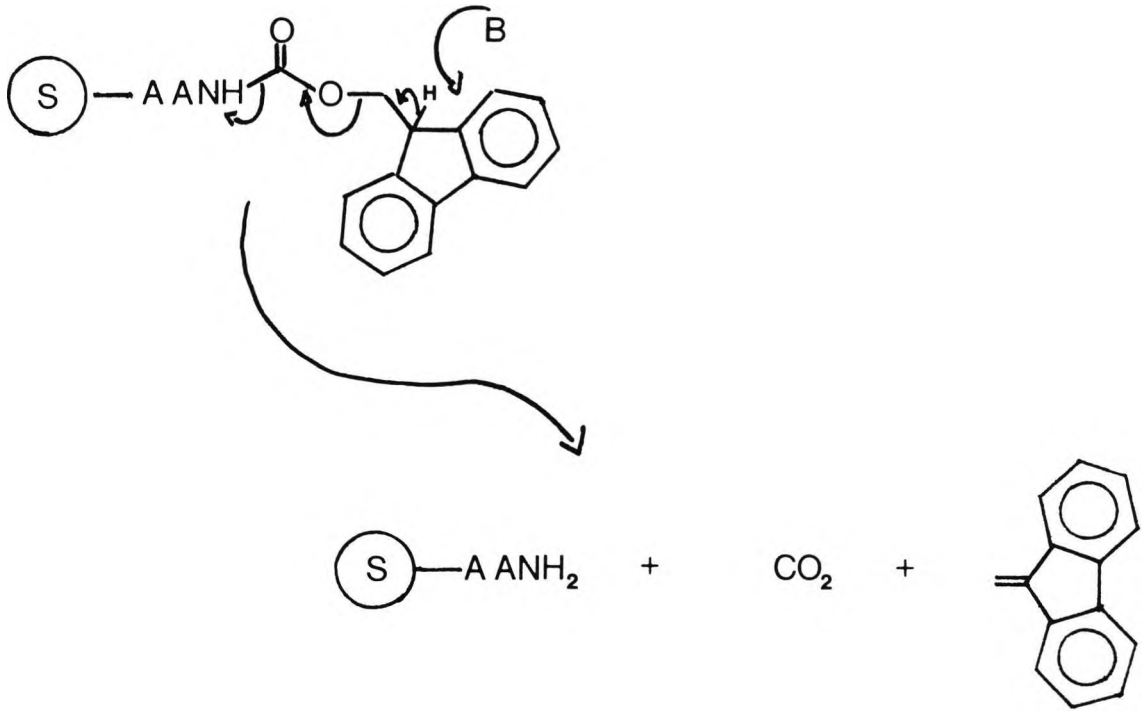


(ii) Reaction between Celite resin with hydroxyl functionality and the anhydride.

The use of N-methylmorpholine converts the solid phase OH groups to O^-. The use of dimethylaminopyridine converts the anhydrides to active esters. The two reactions lead to the amino acid "AA" being covalently linked to the solid phase.



(iii) Deprotecting the amino group with piperidine (B). This followed a β-elimination mechanism.



(iv) Continuing the synthesis

The next FMoc protected amino-acid (ala) was converted to its symmetrical anhydride and added to the deprotected amino-acid bound to the solid phase. Each stage of the synthesis involved excess reagents which helped to keep the yield at a maximum.

(v) Reacting the complex with tripeptide: The sulphonyl chloride derivative **R20** was added to the tripeptide which had a free amino function on alanine. This formed a sulphonamide link and after washing the unreacted complex away the peptide was removed from the solid phase using trifluoroacetic acid. This removed the red colour from the solid phase into solution. The trifluoroacetic acid was removed and the tripeptide-complex compound was passed through an ion exchange

(HPLC) column to attempt to purify it. (Reverse phase chromatography was also tried but gave too many peaks). The ion-exchange method gave three main peaks at 1.3, 2.9 and 13.0 mins (see Ch 8 Experimental for details). The peak at 13.0 minutes was coloured red (luminescent) and was collected. This solution was passed down a G10 sephadex column to remove the salt used in the ion-exchange run. Unfortunately the product absorbed onto the column and was lost. Further samples from ion-exchange were kept but no way of removing the salt could be found. This synthesis which had clearly worked had one main problem in that it had to be performed on a small scale. This did not enable enough work to be done on the purification steps. However, the success of using a solid phase peptide synthesis to put functional groups on a labelling compound was most encouraging and hopefully could lead to a whole range of derivatives. If R24 had been purified the free lysine amino group would have been reacted with thiophosgene to make an isothiocyanate or succinic anhydride to make a hemisuccinate.

5.18 Conclusion

The aim of making luminescent transition metal complex - antibody conjugates with the properties outlined at the start was fulfilled in using the following compounds:

Ru $bipy_2bipy(CO_2NHS)_2$ $^{2+}$	R7
Ru $bipy_2bipy(CONCS)_2$ $^{2+}$	R9b
Ru $bipy_2bipy(CO\ Anthr)_2$ $^{2+}$	R14 + carbodiimide
Ru $phen_2phen(\phi SO_2Cl)_2$ $^{2+}$	R20
Ru $phen_2phen(\phi SO_3H)_2$ $^{2+}$	R19 + carbodiimide

Of these Ru $phen_2phen(\phi SO_2Cl)_2$ $^{2+}$ made the best complex-antibody conjugate and was used for some equipment development at Applied Photophysics.

The main criticism of this work is that it failed to produce a synthesis for R20 that was reliable and of sufficiently high yield. This aim has now been achieved by Graham Yearwood at the University. The next chapter will tabulate the labelling results and discuss the characteristics of the complex-antibody conjugates in more detail.

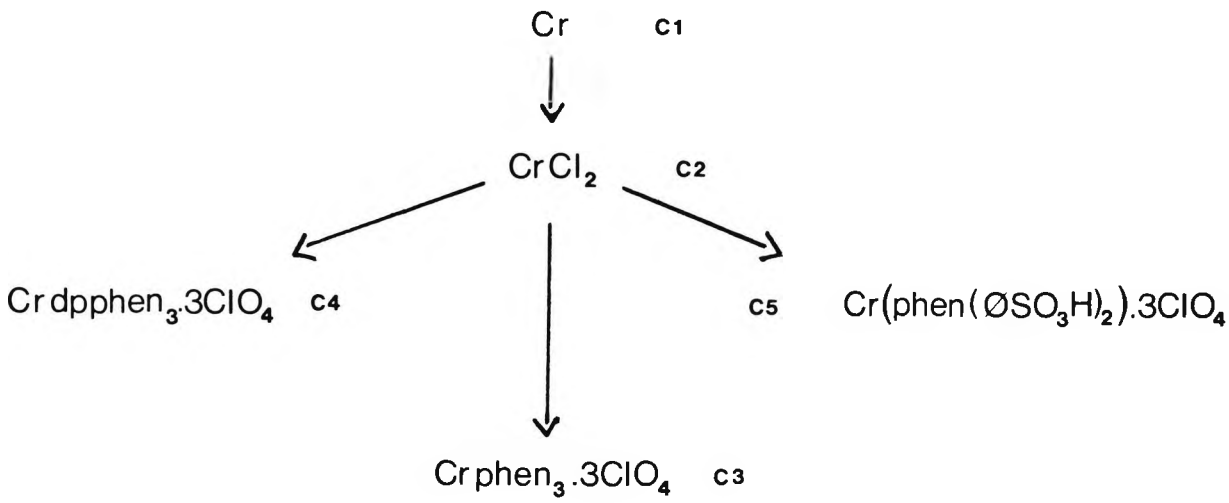
5.20 Introduction to Chromium Complex Chemistry

Chromium bipyridyl, phenanthroline and cyclam (1,4,8,11-tetraazacyclotetradecane) complexes are reported to exhibit luminescence [12-15]. As was reviewed in chapter 3 the luminescence is long lived (several hundred microseconds) and the Stoke's Shift large ($\sim$ 300 nm). Unfortunately, the quantum yields are low (about 1/20 of $Rubipy_3$ $^{2+}$) but the other properties were sufficiently attractive to warrant some work being done on these compounds. Also the patent being filed

required work to be done on other metals apart from ruthenium. Boots-Celltech advanced some work on Iridium - phenanthroline complexes while the group at City University worked on chromium complexes.

It was decided to make, for this project, Chromium Phenanthroline and cyclam complexes to investigate their properties and to, hopefully, link them to antibodies.

5.21 Strategy 1



Cr $phen_3$ $^{3+}$, C3, was the first compound made. This was done to build up experience in making this type of complex and to enable us to study its photophysical properties. Cr $phen_3.3ClO_4$ was made by adding a 3:1 excess of phenanthroline to freshly made $CrCl_2$, C2. Commercially available $CrCl_2$ was not pure enough for this task (tried twice). Instead chromium metal was dissolved in 5M hydrochloric acid under nitrogen.

3 hours of sonication was required to dissolve all the chromium. On adding an excess of water a blue/green solution formed to which the phenanthroline (in ethanol) was added. The resultant mixture was cooled and then sodium perchlorate was added forming a green precipitate. This solid was $Crphen_3.2ClO_4$ and could have been isolated if we had desired. Instead the compound was oxidised by addition of perchloric acid and bubbling oxygen through the suspension for 24 hours. The green solid turned yellow ($Crphen_3.3ClO_4$) and was filtered through a G4 sinter. This left a pink solid on the disc and a fine yellow precipitate in the solution. The yellow precipitate was then carefully filtered (by gravity) and dried . Microanalysis and the luminescence spectrum confirmed the product to be $Crphen_3.3ClO_4$ (C3). However, the lifetime of the emission at 728 nm was only 130 ns in acetonitrile or 1M sulphuric acid (purged with argon for 15 minutes). This was in contradiction to other studies [12] and will be discussed in the next chapter. Attempts to add 4,7-diphenyl-1,10-phenanthroline and bathophenanthroline disulphonic acid to $CrCl_2$ by similar methods both failed (C4 and C5). The green colour (presumbaly CrL_3^{2+}) formed but no precipitate of these compounds could be recovered by addition of perchlorate, extraction or removal of solvents.

5.22 Strategy 2

$CrCl_3.3THF$ C6

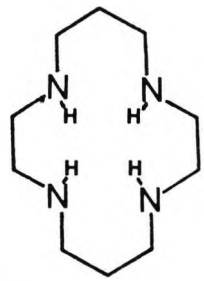
↓

Cr cyclam $Cl_2.ClO_4$ C7

↓

Cr cyclam $(CN)_2.ClO_4$ C8

Cyclam = 1,4,8,11-tetraazacyclotetradecane



This strategy was carried out in parallel to the chromium phenanthroline work so that a comparison could be made between the two types of compound. The preparations followed some work by Kane-Maguire et al [16]. The chromium chloride tetrahydrofuran complex C6 was purchased from Aldrich and was always handled under a nitrogen atmosphere. This compound was dissolved in hot DMF and an excess of cyclam added. The mixture was refluxed for 2 hours and then filtered to give a mixture of solids. Washing with hot DMF removed the excess cyclam to leave a grey solid. This was washed with acetone and dried. The solid was then dissolved in 100 mls of water and heated to 50°C. An excess of

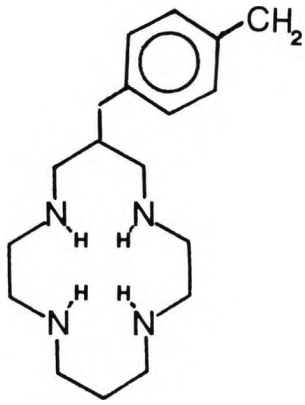
perchlorate was added to give a pink-mauve precipitate Cr cyclam $Cl_2.ClO_4$. This compound is a cis/trans mixture.

The separation of the isomers is important as only the trans form gives the desired photophysical properties [16]. The problem was overcome by Kane-Maguire et al[16] by synthesising Cr cyclam $(CN)_2.ClO_4$ which is completely trans.

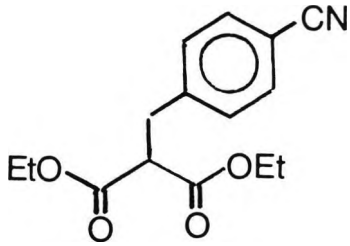
Cr cyclam $(CN)_2ClO_4$ was prepared by dissolving Cr cyclam $Cl_2.ClO_4$ in DMSO at 62°C and adding an excess of finely ground sodium cyanide. The mixture was stirred at 62°C for 2 hours and then was allowed to cool. A yellow solid was recovered, washed, redissolved in water and filtered. To this solution excess perchlorate was added to give a fine yellow solid. Microanalysis and the luminescence spectrum confirmed the compound to be trans-Cr cyclam $(CN)_2.ClO_4$. The Stoke's Shift was 299 nm with an emission maximum at 715 nm. However, the emisson quantum yield is very close to $Crphen_3^{3+}$ measured at 715 nm.

It was decided to stop pursuing work in these systems as only the Stoke's Shift of about 300 nm was reliably

found. The low intensity emission signals observed on our apparatus showed that for both phenanthroline and cyclam complexes of chromium there would be no potential for them as luminescent antibody labels. Hence the plan to make:



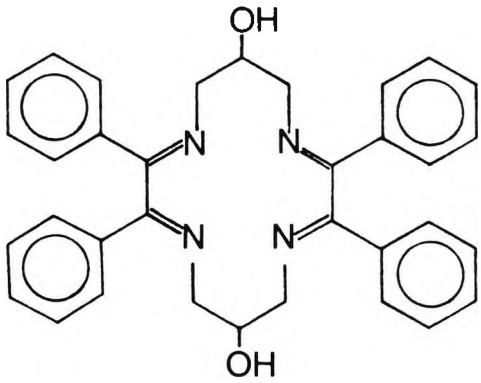
following a private communication from David Parker at Durham University was stopped having made the substituted diethyl malonate.



The only further work carried out with chromium was the attempt to proudce a compound with a higher quantum yield (5.23).

5.23 Attempt to make a more strongly luminescent complex of chromium

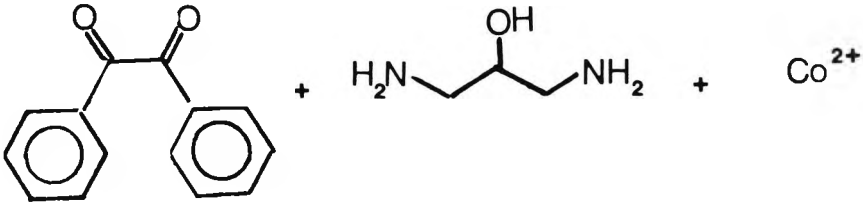
A derivatised macrocycle similar in shape to cyclam has been prepared previously [17] as a ligand for colbalt. Its structure is as follows:



C10

This ligand was seen to have two distinct advantages. Firstly the two hydroxyl groups may have been derivatised by phosgene to make chloroformates. This would enable the complex to be attached to protein. Secondly the phenyl groups may have produced a similar effect to what is seen in ruthenium complexes:- an increase in quantum yield and emissive lifetime.

The ligand was "built" around colbalt by the following reaction:



An attempt was made to replace colbalt acetate by chromium acetate C9. Chromium acetate was prepared as follows: potassium dichromate was reduced with zinc and hydrochloric acid. A blue solution formed that was passed through a filter into sodium acetate solution. A red precipitate, $Cr(OAc)_2$, formed which was filtered and dried for 2 hours. It was then used in the reaction with 1,3-diaminopropan-2-ol and benzil. After 4 days of refluxing the mixture under nitrogen no solid material was formed (unlike the colbalt preparation) and so some solvent was removed. Excess aqueous sodium perchlorate was added but only benzil was recovered.

It was unlikely that chromium would work as the central metal but it seemed worth a try as the complex, if formed, had such potential. No more time was spent on chromium chemistry after this.

5.24 Conclusion

The use of $Crphen_3^{3+}$ and Cr Cyclam $^{3+}$ systems looked promising from the literature. Problems with the synthesis of derivatives and their low quantum yields forced a decision to stop work on them. The best way to progress in this field will be in the synthesis of a strongly luminescent chromium (III) complex. The failure to make the phenyl and hydroxyl substituted complex after only one quick attempt should not discourage further work of this type.

5.30 Introduction to Aluminium Oxine Chemistry

Aluminium complexes of 8-hydroxyquinoline (oxine) have been used for assays of aluminium [18]. The method is for the determination of aluminium by gravimetric analysis according to the following reaction:

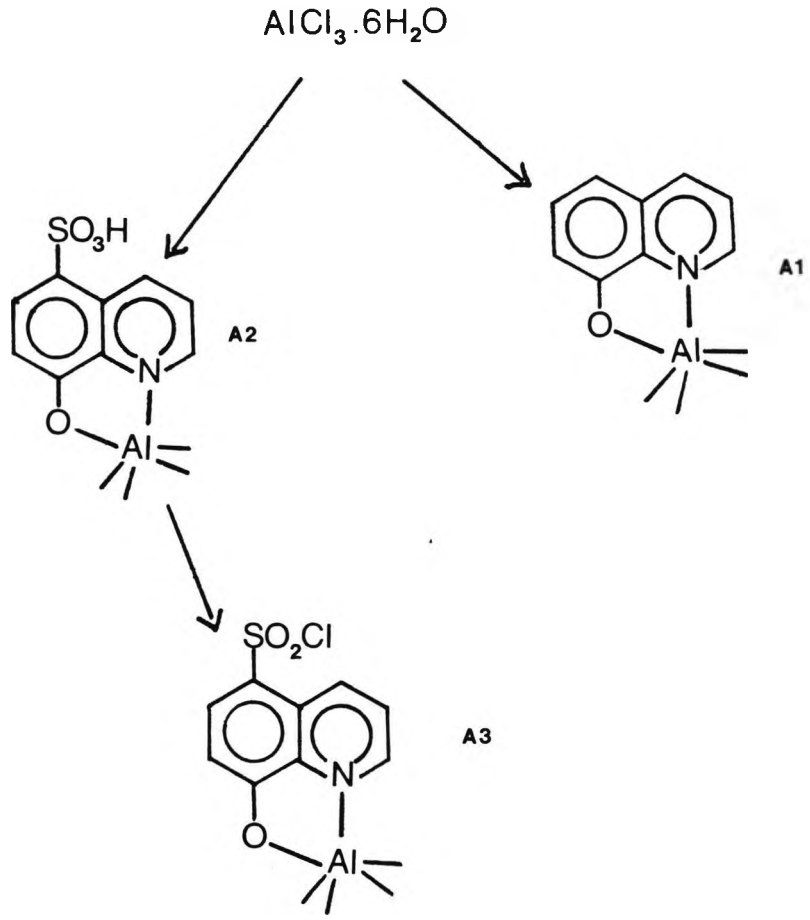
$$Al^{3+} + 3\ oxine^{-} \longrightarrow Al\ (oxine)_3$$

The product is insoluble in water between pH 4 and 9.

What is also known [18] is that the compound is strongly fluorescent with a large Stoke's Shift (90 nm). However, no report of an emission lifetime could be found and so the compound was made and its lifetime measured. The oxine complex, Al, was made using a three fold molar excess of 8-hydroxquinoline in dilute hydrochloric acid. The mixture was stirred and heated until all the 8-hydroxyquinoline had dissolved. Neutralisation to a slightly basic pH gave a lime coloured precipitate that was filtered, washed and dried. The product was confirmed by mass spectroscopy and microanalysis. The fluorescence spectrum showed λ_{max} excitation at 432 nm and λ_{max} emission at 522 nm in dichloromethane. The lifetime of the emission was 276 ns in acetonitrile. This reading was both interesting and encouraging and will be discussed in the next chapter.

The result prompted the strategies that follow to attempt to prepare a complex that would covalently link to an antibody.

5.31 Strategy 1



This success of using sulphonyl chlorides for protein labelling in the ruthenium phenanthroline compounds made it seem sensible to follow a similar path for oxine derivatives. 8-Hydroxyquinoline-5-sulphonic acid was purchased from Aldrich and linked to aluminium by the same method as above (5.30). A bright yellow precipitate was collected that was recrystalised from water to give lime coloured needles.

The fluorescence spectrum was similar to $Al(oxine)_3$ and the lifetime of the emission was 145 ns in DMF:H_2O, 1:1. The compound was crystalline but did not give an "in spec" microanalysis although it was quite close. IR spectroscopy showed the presence of the SO_3H group at 1189 cm^{-1} and 1040 cm^{-1}. FAB mass spectroscopy did not give a coherent result. It was assumed that this compound was A2 ie. $Al(oxine\text{-}5\text{-}sulphonic\ acid)_3$.

Preparation of the sulphonyl chloride, A3, was attempted by two methods.

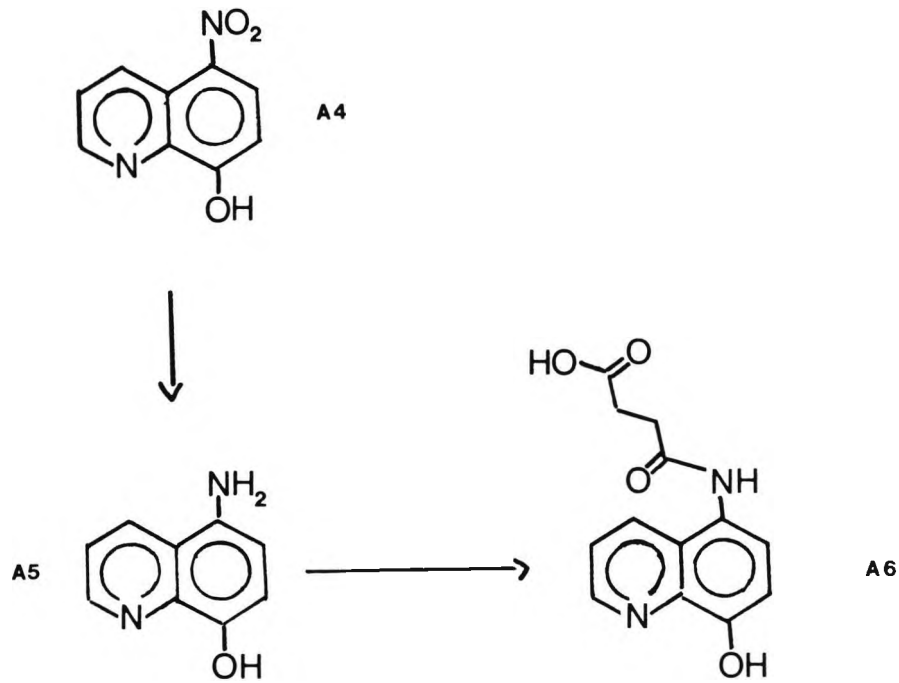
(i) The sulphonic acid was dissolved and heated at 120°C for 2 hours in chlorosulphonic acid. After carefully adding the cooled solution to ice (dropwise) a yellow precipitate formed. This was recoverd and dried. The product was soluble in acetone and the IR spectrum showed the presence of SO_2Cl groups at 1370 cm^{-1} and 1213 cm^{-1}. This yellow solid was unstable to air decomposing in 2 days to a grey brown oily solid. The fresh product was added to antibody in phosphate (pH 7.5) and carbonate (pH 9.4) buffers. The addition of the solid to carbonate buffer produced an effervescence presumably from acid and carbonate releasing carbon dioxide. The compound did not label antibody when added in carbonate (pH 9.4) or phosphate (ph 7.5) buffers.

(ii) The parent acid complex was mulled together with

an excess of phosphorus pentachloride. No reaction was observed. The unco-ordinated ligand was also taken and mulled with phosphorus pentachloride to see if the unreactivity was due to complexation. However, the free ligand showed no reactivity. This was in stark contrast to the reaction observed when dansyl chloride was prepared from its parent acid (see Chapter 7.13), but was similar to the attempt to form the sulphonyl chloride of the ruthenium phenanthroline disulphonic acid complex (5.15). Due to the failure to label protein with the sulphonyl chloride derivative an attempt was made to link the sulphonic acid complex A2 to antibody. The sulphonic acid complex was mixed with a water soluble carbodiimide (see 5.15 and 5.13) and this was added to an antibody sample in carbonate buffer. No labelling was observed by this method for this complex - this will be discussed further in the next chapter.

5.32 Strategy 2

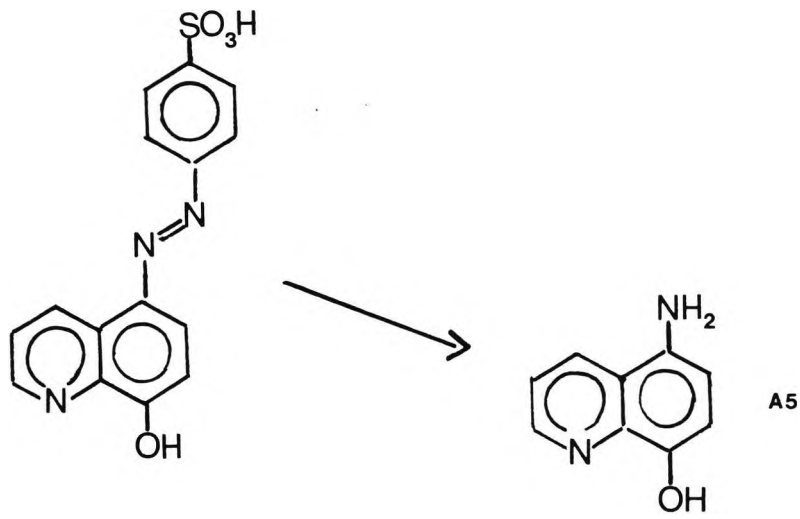
Following the lack of success with the sulphonic acid derivatives it was decided to make a primary amine derivative of oxine, A5. This has been previously prepared [19] as its dihydrochloride. For this work the free base would have to be made and reacted with succinic anhydride to form A6.



5-Nitro-8-hydroxyquinoline was purchased from Aldrich and was reduced with palladium on carbon (10%) and hydrazine in methanol. The reaction was successful - a wet sample of the product was analysed by mass spectroscopy and showed complete absence of starting material and formation of the desired product. However, on trying to remove all the solvent (rotary evaporation over hot water) the product decomposed from an orange solid to a black oily tar. The mass spectrum showed a wide variety of species from m/z 493 to 160. The preparation was repeated twice using less heat but the same happened each time.

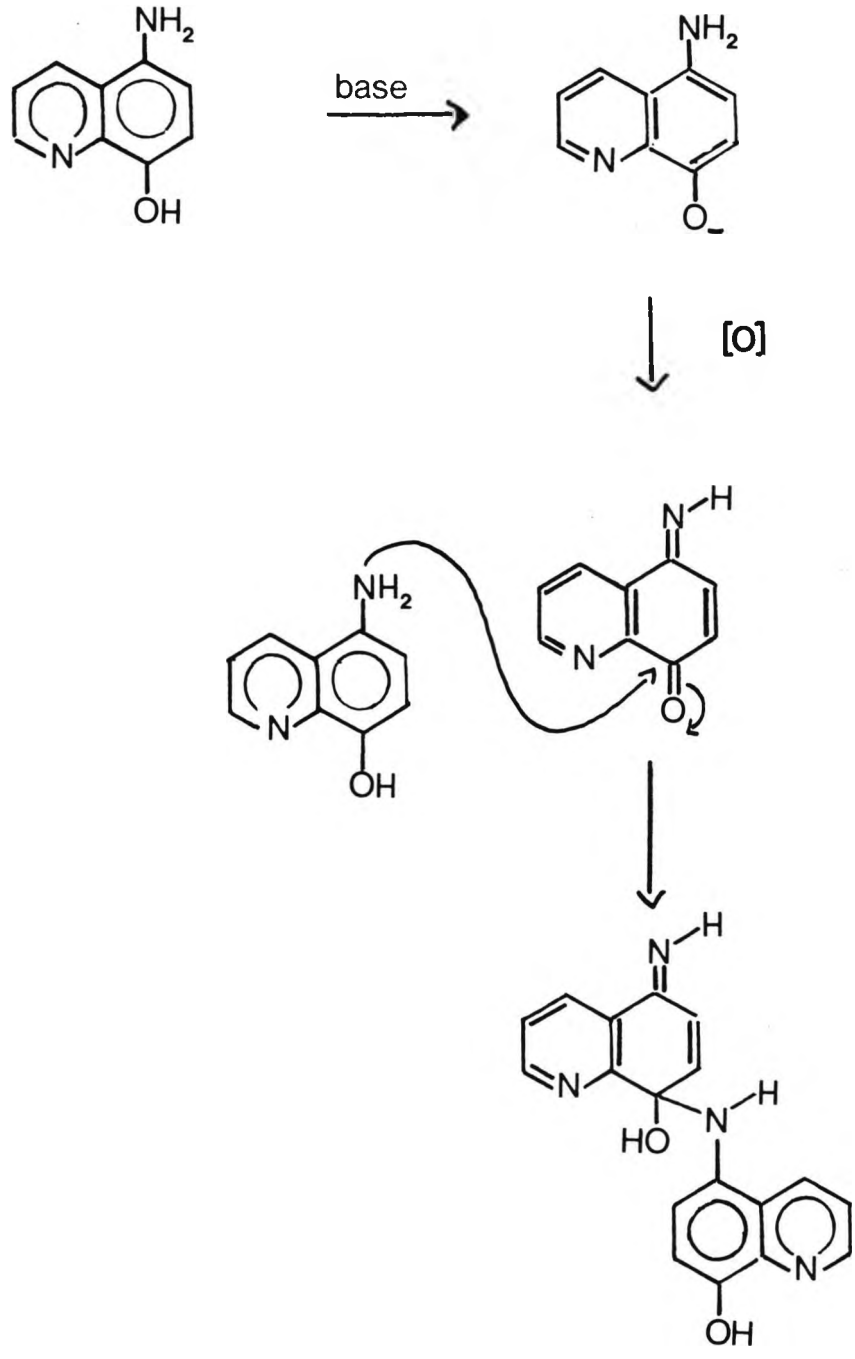
Another method for making the 5-amino-8-hydroxyquinoline follows the method of Hollingshead detailed in a book called "Oxine and its Derivatives" [19]. This involves

making the azodye of 8-hydroxyquinoline and then reducing with tin chloride. This is outlined as follows:



The diazonium salt of para-aminobenzene-sulphonic acid was prepared using hydrochloric acid and sodium nitrite at 5°C. This was added to 8-hydroxyquinoline to give a dark red precipitate. The solid was then reduced with tin(II) chloride and hydrochloric acid to give an orange solid. The product was the hydrochloride salt. Sodium acetate was added to an aqueous solution of the hydrochloride but a black tar formed just as before. Literature preparations do not include any mention of a free base only preparations of the salt.

It is suspected that the free base is unstable and forms products by similar mechanisms to the one shown below:



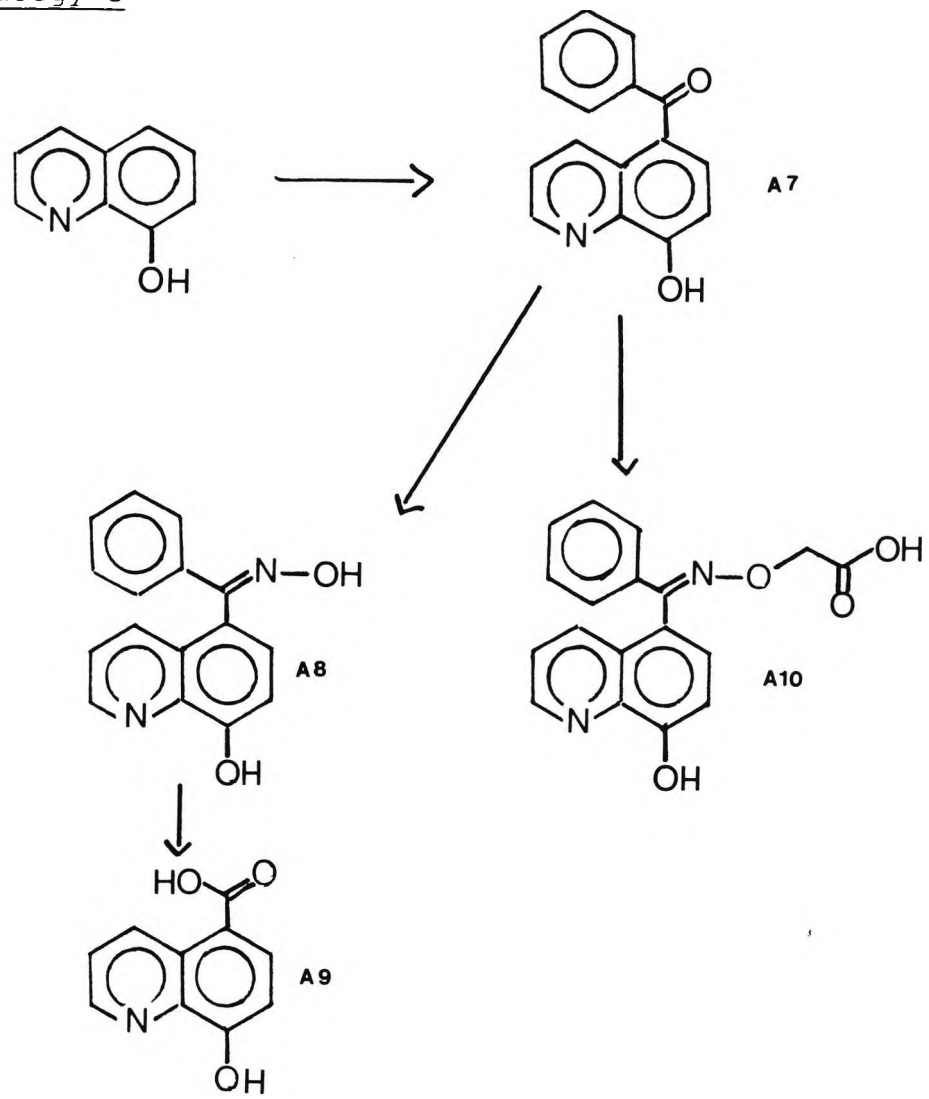
The above product would give a m/z of 319 but does not feature in the mass spectrum of the oil but the imine (-H_2O) m/z 300 is present. We suspect a polymeric compound of irregular composition. This strategy was then stopped.

For this work to succeed a new approach will be required.

5.33 Conclusion and Strategy 3

The results of the above syntheses were very disappointing. Ideally a carboxylic acid functionality needs to be introduced. The following strategy is a suggestion for further work. The first step, the synthesis of A7 was completed before I stopped work. 5-Benzoyl-8-hydroxyquinoline, (A7), was made by the Friedel-Crafts acylation of 8-hydroxyquinoline [20]. Two strategies may then be followed. Either oxine formation, A8, with hydroxylamine followed by a Beckmann rearrangement (by reflux in thionyl chloride) would give the carboxylic acid, A9. This has been done before [20]. Alternatively the ketone may be reacted with carboxymethoxylamine to give a carboxylic acid functionality in one step, A10, [21].

Strategy 3



Carboxymethoxylamine has been used for drug derivatisation for immunogen preparation [21]. At the time of carrying out the practical work for this project we were unaware of its existence but it may prove useful in the future. In conclusion, this work remains unfinished and requires a suitable compound for antibody labelling.

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Chapter 6

ANTIBODY LABELLING AND LUMINESCENT PROPERTIES

6.00 Introduction

This chapter discusses the covalent linking of the compounds described in chapter 5 to antibodies. Several of the compounds were synthesised with reactive groups capable of forming covalent bonds with protein.
eg. $Rubipy_2bipy(CONCS)_2^{2+}$ R9b.
Others were linked to protein by forming active esters "in situ" and added to antibody.
eg. $Rubipy_2bipy(CONHS)_2^{2+}$ R7.

In practice antibody labelling involves 3 stages:

(i) Addition of the label in 10-100 fold molar excess to the antibody. For all this work 2 mg of antibody (Human γ-globulin) was dissolved in 1 ml of a carbonate buffer pH 9.4-9.5. 1 mg of the labelling compound was dissolved in 0.1 ml DMF and this was added dropwise to the antibody solution. Initially it was thought that the labelling compounds would have to be water soluble to avoid using organic solvents with the antibodies. However, a 1:10 ratio of DMF, DMSO or acetonitrile to aqueous base may be used

without denaturing the antibody. Provided care was taken in a dropwise addition of the solvent no problems were encountered. In this work all the ruthenium and aluminium compounds that were used to try to label antibodies required DMF to be used in the labelling reaction.

(ii) The label-antibody mixture was then left at $0^{o}C$ for 24 hours. Also some G25 sephadex was suspended in PBS and left at $0^{o}C$ overnight to allow it to swell.

(iii) The antibody-label mixture was then passed through a G25 sephadex column eluting with PBS. The antibody fraction (mw of IgG $\sim$ 150000) moved on the solvent front and the unreacted label (mw $\sim$ 1000) moved slowly down the column. Unreacted FITC would stay at the top of the column whereas most of the compounds made in this work would have travelled ½ to 2/3 down the column when the IgG fraction was collected. This fact necessitated using a longer column (20 cm) for this type of work than was commonly used for FITC or radioactive labels ($\sim$10 cm).

The antibody fraction was collected and if it was labelled reasonably well it would be coloured red for ruthenium complex-antibody

conjugates. The presence of the label and the antibody were checked by fluorescence spectroscopy. Antibodies have distinct luminescent emissions at 333 nm (λ_{ex} 279 nm) and 520 nm (λ_{ex} 469 nm). The emission is very weak at 520 cm and is due to groups formed from degraded protein [1]. The complexes linked to protein emit at higher wavelengths in a characteristic manner. This method was used to check all antibody fractions even if a visual check looked promising.

Non-specific binding was checked by adding $Rubipy_3Cl_3.6H_2O$ and $Rubipy_2bipy(CO_2H)_2^{2+}$ (R6) to antibody samples. The above procedures (i)-(iii) were followed and the fluorescence spectra showed no emission due to the complex in the antibody fractions. However, problems were encountered with $Rubipy_2bipy(CONCS)_2^{2+}$ (R9a) and $Ru(dpphen)_2phen(\phi SO_3H)_2^{2+}$ (R22) which are discussed later (6.11).

An alternative method of removing unreacted label from antibody is dialysis. This was not used as it takes longer ($\sim$ 24 hrs).

The following table, 6.10 shows the results of the attempts to label antibody with the compounds described in chapter 5, sections 5.10 - 5.19.

6.10 Ruthenium Complex-Antibody Conjugates

CODE	LABELLING COMPOUND	LABEL PROPERTIES				FORMED ANTIBODY CONJUGATE ?	CONJUGATE PROPERTIES			COVALENT BOND STRUCTURE
		λ_{em}	λ_{em}	τ	solvent		λ_{ex}	λ_{em}	τ	
R7 5.11	$Rubipy_2bipy(CONHS)_2^{2+}$ made "in situ"	457	647	----	DMF	Yes Human Yes Goat Yes anti-TSH	450	630	410	Amide
R9b 5.13	$Rubipy_2bipy(CONCS)_2^{2+}$	461	621	275	CH_3CN	Yes Human	470	620	125	Acyl thiourea
R14 5.13	$Rubipy_2bipy(COAnthr)_2^{2+}$ +CMCMT	492	660	653	CH_3CN	Yes Human	482	674	625	Amide
R6 5.11	$Rubipy_2bipy(CO_2H)_2^{2+}$ +CMCMT	456	638	----	DMF	No Human	---	---	----	Amide if formed
R20 5.15	$Ruphen_2phen(\phi SO_2Cl)_2^{2+}$	475	620	260	CH_3CN	Yes Human Yes anti-TSH	470	630	1200	Sulphonamide
R19 5.15	$Ruphen_2phen(\phi SO_3H)_2^{2+}$ +CMCMT	480	615	1160	$H_2O:CH_3CN$ 1:1	Yes Human	468	610	----	Sulphonamide
R22 5.16	$Rudpphen_2phen(\phi SO_3H)_2^{2+}$ +CMCMT	462	650	800	$H_2O:CH_3CN$ 4:1	Non-specific with Human	---	---	----	-----

CMCMT is 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluenesulphonate.
TSH is Thyroid stimulating hormone.

Complex-antibody conjugates in PBS pH 7.5.

τ in ns. (XeF laser excitation). λ_{ex} and λ_{em} in nm.

6.11 Discussion of Results

A - Methods of Labelling

Two main approaches for linking the compounds to antibodies were followed. Either the complex was able to directly react with antibody or the complex required "in situ" activation. Two compounds, $Ruphen_2phen\ (\phi SO_2Cl)_2^{2+}$ and $Rubipy_2bipy(CONCS)_2^{2+}$ were able to be mixed with antibody directly. The rest of the compounds required activation using carbodiimides.

$Rubipy_2bipy(CONHS)_2^{2+}$ was made in dry DMF and required DCCI and N-hydroxysuccinimide. The rest of the carbodiimide activated compounds used 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluenesulphonate (CMCMT). This water soluble carbodiimide activates carboxylic acids to nucelophilic attack without being hydrolysed like its better known relative DCCI. However, water soluble carbodiimides can cause protein cross-linking and so this set of compounds were never preferred to the two compounds which could be used without activation.

The use of carbodiimides to activate sulphonic acids was novel and is certainly interesting. Both the carbodiimide and a sulphonic acid are good leaving

groups. Hence nucleophilic attack could occur at two sites. The arrows below (fig 1) illustrate the points of attack by nucelophiles on a complex linked to CMCMT.

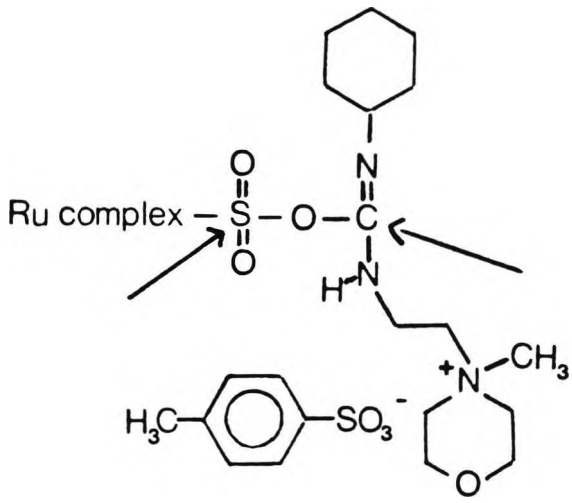


Fig 1

For the ruthenium complex $Ruphen_2phen(¢SO_3H)_2^{2+}$ this method seemed to link the complex to antibody. However, as will be discussed later, Al(oxine-5-sulphonic acid)$_3$ was not linked to antibody by this method (6.31). If a prediction was made as to which of these two would work before the experiments were done it is likely that the ruthenium complex would be chosen as the one to fail. This is because the complex is strongly electron withdrawing and so would stabilise the sulphonate leaving group. This contrasts with the neutral aluminium complex. Perhaps the factor that caused the ruthenium complex to bind was that the metal ion is distant from the sulphonic acid groups whereas the aluminium ion is relatively close.

As has been mentioned before "in situ" preparations are not preferred. Their problem lies in not knowing how much activated compound has been formed so that reproducible labelling of antibody can be performed. This is important because by adjusting the level of labelling the activity of the antibody can be preserved and the quantum yield of emission optimised. Hence the two compounds that could be used as prepared solids were preferred for further use by Boots-Celltech.

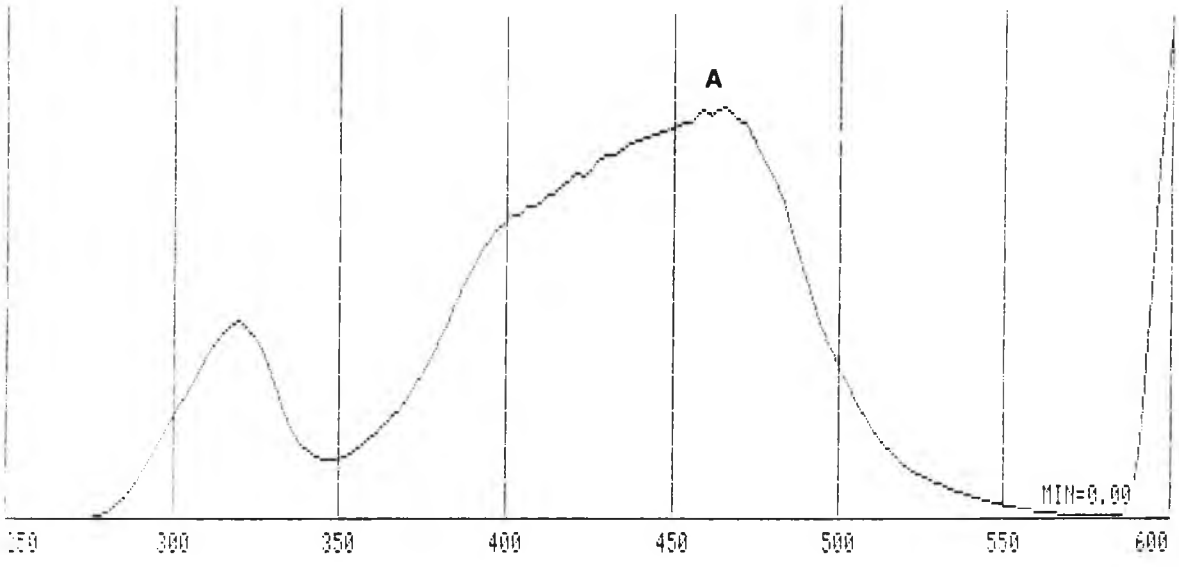
Unusual results were obtained for $Rudpphen_2phen(\phi SO_3H)_2^{2+}$ when added to antibody. The complex appeared to bind non-specifically. This was observed as a red streaking down the G25 separation column. Perhaps an ionic bond formed between the complex and the antibody but this is unlikely as $Ruphen_2phen(\phi SO_3H)_2^{2+}$ did not do the same. More probably a hydrophobic bond between the phenyl groups and the antibody or some sort of interchelation effect occurred. (cf J Barton [2])

Another compound that showed unusual results was $Rubipy_2bipy(CONCS)_2^{2+}$ (R9a). This is not listed in the table 6.10 as it was prepared by another strategy to give R9b. R9a appeared to have successfully labelled antibody until it was removed by electrophoresis. A monoclonal antibody that is labelled with a fluorophore will give a smear instead of a sharp band on polyacrylamide gel electrophoresis. This is because the

label alters the net charge of the monoclonal antibody. Different numbers of labelling molecules label the monoclonal so the protein smears out on the gel. Using R9a the monoclonal antibody ran as a sharp band and the red coloured label moved away from this band. This experiment was performed by Hishani Williams at Boots-Celltech Diagnostics Ltd. A similar experiment was performed on the $Ruphen_2phen(\phi SO_2Cl)_2^{2+}$ - antibody conjugate. This gave a smeared band of protein and no loss of label.

B LUMINESCENCE SPECTRA

All the compounds before and after linking to antibody showed in outline the luminescence excitation and emission expected. One aspect of the excitation spectrum and one aspect of the emission spectrum are of interest. The excitation spectrum below is typical (fig 2)



Excitation spectrum of $Ru(dpphen)_2phen(\phi SO_3H)_2.2Cl$ in methanol, OD=0.1, em=605nm, slits 5/10nm.

The position of the excitation maxima (A) varied from compound to compound and between parent compound and conjugate but only by a few nanometers.

The emission spectra were all simple broad peaks without shoulders. The one shown below (fig 3) is of $Ru(dpphen)_2phen(\phi SO_3H)_2.2Cl$ and corresponds to the excitation spectrum shown earlier.

Emission spectrum of $Ru(dpphen)_2phen(\phi SO_3H)_2.2Cl$ in methanol, OD=0.1, ex=475nm, slits 5/10nm.

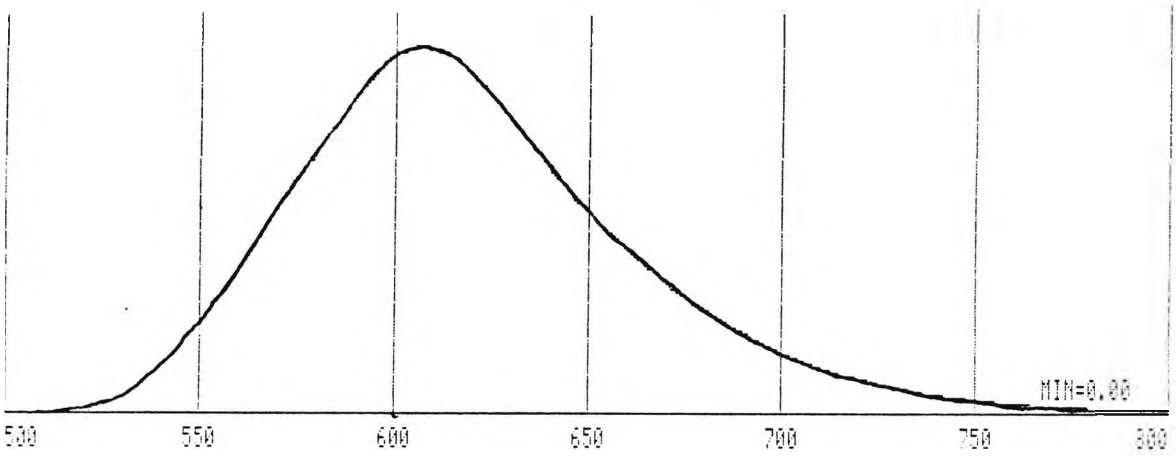


fig 3

The position of the emission maximum would be dependent on the amount of conjugation in the ligands and the type of solvent. It is hard to draw any conclusions as the solvent for the labelling compounds varied.

The most satisfying fact from all the results was that the requirement for a complex-antibody conjugate with a large Stoke's Shift had been met in all cases.

LUMINESCENT LIFETIMES

Most of the luminescent lifetimes were recorded using a xenon-fluoride laser which excites the sample, at 353 nm. Although this was not the ideal excitation wavelength for these compounds the power of the laser pulse was sufficient to see the emission decay pattern. The samples were all observed in non-degassed solvents. This was done because an immunoassay would not be practical if a freeze-thaw cycle or argon gas purge was necessary.* The key results are of the complex-antibody conjugates. The lifetimes depended on five main factors.

1. Quenching by oxygen.
2. Quenching by atoms or groups covalently linked to the label.
3. Quenching by the antibody.
4. Solvent effects.
5. Competing photochemistry.

*Also the use of freeze-thaw cycles would denature the antibody and argon bubbling was found to be impractical with proteinacious solutions.

Each of these will now be discussed in turn.

1. Oxygen in the solution was by far the most important quenching agent of the lifetime of these compounds. The following luminescent lifetime readings illustrate this point well for $Rubipy_3Cl_2.6H_2O$ in water.

$Rubipy_3Cl_2.6H_2O$

SOLVENT	LIFETIME
H_2O degassed by argon	557ns
H_2O not degassed	369ns

Oxygen quenching was mentioned in chapter 4 as significant and as mentioned above immunoassays would not be able to include argon purges or freeze-thaw cycles. However, towards the end of this project another way of degassing water was tried. This was to use a solution of 12 g/litre sodium sulphite. This reacts with oxygen and quickly clears the solution of dissolved oxygen. Both peak heights of the emission and the lifetimes were recorded as follows for $Rubipy_2Cl_26H_2O$ in the following solutions.

$Rubipy_3Cl_2.6H_2O$

SOLUTION	EMISSION PEAK HEIGHT	LIFETIME
H_2O not degassed	24%	369ns
H_2O degassed by argon	34%	557ns
H_2O 12g/l Na_2SO_3	40%	718ns

The lifetime in the sulphite solution is unusually long when compared with other degassed solutions [10]. It may be that the SO_3^{2-} counter ion increases the stability of the excited state.

This method was also tried for $Rudpphen_2phen\ (\phi SO_3H)_2^{2+}$ (R22)

$Rudpphen_2phen(\phi SO_3H)_2^{2+}$

SOLUTION	LIFETIME
$H_2O:CH_3CN$ 4:1 not degassed	800ns
$H_2O:CH_3CN$ 4:1 12g/l Na_2SO_3	4050ns

Here a helium-cadmium laser was used to excite the sample (440 nm). Clearly sodium sulphite addition was a very effective way of increasing lifetimes and quantum yields for these systems.

The next piece of work that needs to be done is to see how a complex-antibody conjugate is affected by sodium sulphite. The use of sodium sulphite for immunoassays was first described by A M Sidki [3,4] in a PhD thesis (London 1984). There the use was to enhance phosphorescence and utilised in a time resolved immunoassay. For all the compounds mentioned above the use of sodium sulphite would probably be advantageous and certainly practical.

A similar study at Boots-Celltech diagnostics found that for iridium complexes (of phenanthroline and bipyridyl) the sodium sulphite in fact quenched the lifetimes and quantum yields. This may perhaps be explained by a redox reaction occurring with the excited state of iridium complexes.

2. The quenching by atoms and groups attached covalently to the label was best observed when $Ruphen_2phen(\phi SO_2Cl)_2^{2+}$ and $Ruphen_2phen(\phi SO_3H)_2^{2+}$ were compared (see table 6.10). The sulphonic acid had an emission lifetime of 1160 ns whereas the addition of chlorine atoms caused the lifetime to drop to 260 ns. On conjugation to antibody involving the loss of chloride ions the lifetime increased to 1200 ns. Chlorine atoms along with bromide and iodine are well known quenching groups. Fluorescein has many "relatives" with chlorine, bromine or iodine atoms

included[11].These compounds are strongly quenched by comparison with fluorescein due to the formation of triplet states and radiationless pathways by intersystem crossing. Chlorine atoms cause a further mixing of multiplicities in the ruthenium compounds and may lead to photochemistry such as S-Cl bond cleavage [12].

The other compound that showed quenching of this type was $Rubipy_2bipy(CONCS)_2^{2+}$ with its lifetime of 275 ns. Fluorescein isothiocyanate does not emit as strongly as fluorescein- ϕ_{em} Fluorescein = 0.8-0.9 and ϕ_{em} FITC = 0.1. The formation of the thiourea when the complex-antibody conjugate forms perhaps increases the quenching of the emissive lifetime. Hence the complex-antibody conjugate gave a lifetime of 125 ns.

3. This short lifetime may be evidence of quenching of the lifetime by the antibody. The complex centre is close to the antibody (by centre we mean the $Rubipy_3^{2+}$ part) and so the protein may be able to quench the excited state. This is feasible as proteins are known to be good electron transfer systems [5]. A speculative idea for antibody quenching of luminescence is that the complex promotes photooxidation or reduction of disulphide bonds[1]. This may depend on whether the complex is bound to a residue close to such bonds. We are not aware of any such work done on antibodies to

give any substance to these thoughts.

In contrast to the possibility of close range quenching by antibody the lifetime of compounds with "spacer" groups between the luminescent centre and the antibody seem to be unaffected,

ie. $Ruphen_2phen(\phi SO_3H)_2^{2+}$ = 1160 ns

[$Ruphen_2phen(\phi SO_2Cl)_2^{2+}$ = 260 ns]

$Ruphen_2phen(\phi SO_2NHIgG)$ = 1200 ns

and $Rubipy_2bipy(CO\ Anthr)_2^{2+}$ = 653 ns

$Rubipy_2bipy(CO\ Anthr\ IgG)$ = 625 ns

The distance of the luminescent centre of the molecule from the antibody may be the explanation. The other possible explanation for the short lifetimes of $Rubipy_2bipy(CONCS)_2^{2+}$ and its conjugate is that the acylisothiocyanate quenched the lifetime less than the acylthiourea of the conjugate.

To solve this problem, a whole series of compounds would have to be made with the same functional group for antibody conjugation. The compounds would each have different spacer groups and so be compared. Perhaps a series of carboxylic acids with their amide derivatives being compared with their antibody conjugates would be useful.

4. The solvents used to make the measurements depended upon what was the most convenient. Two factors operate that would affect lifetimes. Firstly different solvents contain different amounts of dissolved oxygen and this will have a bearing on the rate of quenching. Secondly the lifetime of an excited state can be lengthened if the solvent stabilises it. Polar aprotic solvents tend to stabilise excited states and so help to increase the luminescent lifetimes. All the solvents were polar, often polar aprotic and so hopefully helped to increase lifetimes of the emissions. Evidence for this is seen in the table below [6].

$Rubipy_3Cl_2.6H_2O$ at 25°C (N_2 purged)

SOLVENT	water	methanol	ethanol	acetonitrile	pyridine
LIFETIME	650ns	720ns	740ns	890ns	920ns

5. Finally, the other main cause for the shortening of a lifetime is competing photochemistry, commonly known as fading. Here an excited state leads to the breakdown of the lumophore. For $Rubipy_3^{2+}$ the photoaquation or photoanation of the complex has been studied. This is where a bipyridyl ligand is removed and substituted by water (the bulk solvent) or by anions in the solution. Hoggard and Porter [7] conclude in their studies that "although the quantum yield for photosubstitution is small, the $Rubipy_3^{2+}$ often absorbs a large fraction of the total absorbed light, and the small concentration may be quickly depleted in a sensitisation experiment".

Our purpose would not be for sensitisation work but for immunoassay. Although this only would involve small amounts of label in practice the argument above would not directly apply as only a few pulses of light would be required for a measurement as opposed to the many for a sensitisation experiment. Hence photofading of the label should only play a minor role in quenching the lifetime and quantum yields of these complexes.

6.12 Conclusion to the Ruthenium Complex-Antibody Conjugates

This reconnaissance into the use of ruthenium luminescent compounds as antibody labels has been fruitful in several ways. Compounds can be made and linked to antibody giving suitable Stoke's Shift and lifetimes. Studies suggest that with more work to optimise labelling that a reasonable quantum yield could be observed. In particular the use of sodium sulphite to remove oxygen from the solution promises much.

Studies at Boots-Celltech showed, on labelling, a monoclonal antibody (anti TSH) with $Ruphen_2phen(\phi SO_2Cl)_2^{2+}$, that only 12% - 15% of the binding activity was lost. This is similar to what is observed for other labels like FITC. Hence this work is worth pursuing further. The only hurdle to be overcome

is a sufficiently inexpensive and reliable excitation source. If lasers become cheap enough these labels have a promising future. In the past phosphorescent labels with quantum yields about 1000 times less than fluorescein (ϕ = 0.002) have been able, by time resolution, to achieve similar sensitivity to a fluorescein labelled immunoassay [4]. Some of the complex compounds have quantum yields only 10 times less than fluorescein. This means that with the added advantage of the laser's power over a xenon flashlamp these labels could be ideal for very sensitive immunoassays. An argon-ion laser (emission at 482 nm) may prove to be the best option.

6.20 Luminescent Characteristics of the Chromium Compounds

The chromium compounds $Crphen_3.3ClO_4$ and $Crcyclam(CN)_2ClO_4$ were investigated to see if they would be suitable for further work as antibody labels. The strength of the emission was very poor. Normally the emission from a 10^{-5}M solution of a ruthenium compound was easily detected but a 10^{-2}M solution was required to observe the chromium compounds.

The excitation and emission spectra suggest that the luminescence mechanism for the two chromium complexes is similar. The Stoke's Shift and the "shape" of the excitation and emission bands closely resemble one

another. This confirms the theory currently held and described in chapter 3 that the mechanism involves the "metal" electrons only. The ligands do not play a part except in tuning the energy of the metal orbitals and if polydentate, restricting the competing photochemical pathways.

The following data was obtained for the two complexes:

	$Crphen_3.3ClO_4$	$Crcyclam(CN)_2ClO_4$
λ_{ex}	397 nm	416 nm
λ_{em}	728 nm	715 nm
τ	25 μs	104 μs

all solutions not degassed and $H_2O:CH_3CN$, 4:1

A helium-cadmium laser (ex 440 nm) was used to excite the samples. $Crphen_3.3ClO_4$ in degassed $(Ar)CH_3CN$ was also excited by a Xe-F laser but gave an emission lifetime of only 165 ns. This may be evidence of the shorter (high energy) wavelength leading to competing photochemistry or other radiationless pathways. The main difference between $Crphen^{3+}$ and $Crcyclam(CN)_2^+$ is that $Crphen_3^{3+}$ may be more easily substituted than the trans cyclam complex. This would explain the shorter emission lifetime of the phenanthroline compound. Both compounds have an emitting excited state from which competing photochemistry is possible. The trans cyclam complex is very difficult to substitute (unlike its

analogue where the CN^- anions are CIS to one another - [8] - the quantum yield is lower). On the other hand the phenanthroline ligands may be substituted by water or other solvent molecules. However, both compounds have luminescent lifetimes that are excellent and lend themselves to time resolution. These non-degassed results prompted trying sodium sulphite. A $Crphen_3.3\ ClO_4$ solution instantly turned purple on contact with sodium sulphite and gave no emission anywhere between 500 and 700 nm. A $Crcyclam(CN)_2.ClO_4$ solution made up to 12 g/litre of sodium sulphite gave an emission at about 660 nm with a very short lifetime. It would seem that $Crphen_3^{3+}$ reacted with sodium sulphite and so destroyed the compound. There are several possible reactions that could have occurred. The chromium(III) may have been reduced to its chromous(II) state. This would have made a green $Crphen_3^{2+}$ complex. If this formed it was immediately dissociated to a purple coloured product. Another possibility was that the perchlorate anion reacted with the sulphite to give the following reaction:

$$ClO_4^- + 4SO_3^{2-} \longrightarrow 4SO_4^{2-} + Cl^-$$

The chloride ions formed would have displaced the phenanthroline ligands from the chromium ion and formed purple aqua-halide complexes.

eg. $Cr(H_2O)_5Cl^{2+}\ 2Cl^-$

Certainly many chromium complexes of this type are purple in colour. It is unlikely that $CrCl_3.3H_2O$ is

formed as this is green in concentrated solutions and this solution was concentrated. The change brought about by adding sulphite to the chromium cyclam complex was also unusual. It was unlikely that any dissociation of the ligands occurred but a change of oxidation state is possible. Perhaps Cr cyclam^{2+} complexes are also weakly luminescent.

6.21 Conclusion to the Chromium Complex Work

The two compounds both showed great promise for time resolved analysis. Their massive Stoke's Shifts and long luminescent lifetimes even in aerated solutions made them appear good candidates for further study. The relative instability of chromium bipyridyl and phenanthroline complexes - in particular their photochemistry - could be overcome by using cyclam type compounds. However, all the compounds in the chromium complex family have low quantum yields of luminescence. The failure of sodium sulphite to increase the lifetime (and hence the quantum yield) means that unless an alternative compound can be found to remove oxygen from solution there is no hope for the use of these compounds as antibody labels. Other compounds have been used to remove oxygen from solution such as sodium metabisulphite, but at present these compounds do not look promising for further work of this type.

6.30 Luminescent Properties of the Aluminium Compounds

The luminescent characteristics of two of the compounds described in chapter 5 were investigated. $Al(oxine)_3$ and $Al(oxine\text{-}5\text{-}sulphonic\ acid)_3$ gave the following results.

	$Al(ox)_3$	$Al(ox\text{-}5\text{-}SA)_3$
λ_{ex} (nm)	432	432
λ_{em} (nm)	522	512
τ (ns) *	276	145
	in CH_3CN	in DMF:H_2O, 1:1

* excitation at 353 nm, Xe-F laser.

The most interesting results are the luminescent lifetimes. The molecules, should give a short lifetime as the excitation involved a simple $\pi - \pi^*$ mechanism. This is due to the lowering in energy of the nitrogen lone pairs on co-ordination.

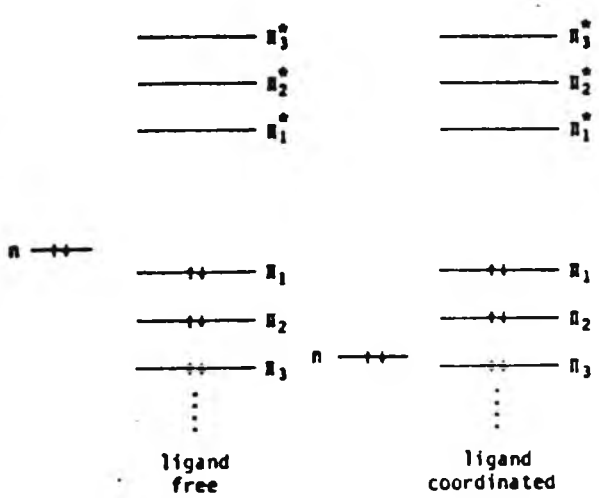


fig 5

Typically $\pi - \pi^*$ fluorescence is a rapid process with a lifetime of about 5 ns. Hence some explanaton must be given for the long lifetimes. From the data shown above it is not possible to do more than speculate. It may be that there is some similarity to pyrene which has a π-π^* mechanism and a luminescent lifetime of about 500 ns [9]. Pyrene is thought to have an excited state that makes the return of the electron to the ground state difficult. Perhaps the efficient π-π^* excitation of pyrene is followed by a rearrangement of electronic states to achieve stability. This new configuration of states could make return to the ground state difficult and so a long lifetime results. This argument could also be applied to the aluminium systems . The Stoke's Shift of 90 nm suggests some relaxation of the excited state is involved before emission. This work simply opens up this field for a more systematic and detailed study.

6.31 Labelling Proteins with Aluminium Compounds

This section reviews what has been stated in chapter 5 and earlier in this chapter. Two compounds were mixed with antibody solutions without success. Firstly the crude Al(oxine-5-sulphonyl chloride)$_3$ compound was mixed with antibody samples dissolved in carbonate (pH 9.4) and phosphate (pH 7.5) buffers. There was no evidence

of labelling of the protein by an aluminium oxine complex when the protein fraction was examined by fluorescence spectroscopy. The compound also caused the carbonate solution to effervesce. This was due to the carbonate as the same occurred when adding the sulphonyl chloride to pure buffer without antibody. Either the sulphonyl chloride was hydrolysed to hydrochloric acid and this reacted with the carbonate to give carbon dioxide gas or the crude compound was contaminated by an acid.

The second compound that was mixed with antibody was Al(oxine-5-sulphonic acid)$_3$ using the water soluble carbodiimide approach. This was unsuccessful and was discussed in 6.11.

6.32 Conclusion to the Aluminium Compounds

It was a pity that an aluminium oxine complex was not successfully linked to protein. The Stoke's Shifts are good and lifetimes reasonable. The major problems with the use of these compounds are two-fold. Firstly the emission wavelength is close to where degraded proteins fluoresce (ch 2.15) [1]. Emission above about 600 nm is sufficient to avoid this. Secondly the lifetime is only just long enough and so most of the quantum yield will be lost in the time-resolved "gate". However, having stated the problems these compounds are still worth investigating as alternative protein labels.

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Chapter 7.

EXPERIMENTAL

7.00 Preparative Chemistry of Ruthenium Compounds

Codes R1-R24 correspond with the strategies in Chapter 5

R1 4 Methyl pyridine

This was purchased from BDH and was distilled before use.

R2 4,4'-Dimethyl-2,2'-bipyridine

600 mls of R1 was refluxed with 10 g of 10% palladium on carbon for 5 days. The mixture was then refluxed for a further hour after addition of 100 mls of hot benzene. The palladium on carbon was then removed by filtration to give a clear yellow solution. The volume of the solution was reduced to about 100 mls by rotary evaporation and was left to stand for 24 hours. White crystals formed and were recoverd by filtration. These were recrystalised from ethyl acetate to give a nearly colourless solid. Yield 17.0 g.

100 MHz 'H n.m.r. : $CDCl_3$, TMS ref.

δ = 2.5s(6H), 7.1 d(2H), 8.3 s(2H), 8.6 d(2H).

CHN: Theory - C 78.22%, H 6.56%, N 15.20%.

Found - C 78.20%, H 6.73%, N 15.21%.

R3 2,2'-Bipyridine-4,4'-dicarboxylic acid

This preparation was carried out a large number of times. A typical example is given. 4.0 g of R2 and

25 g of potassium permanganate were added to 400 mls of water. This mixture was refluxed for 14 hours. Brown manganese dioxide precipitated and was removed by filtration. The filtrate was washed with ether (3 x 200 mls) to remove any unreacted R2. Concentrated hydrochloric acid was then added dropwise to the aqueous solution. This was done until a white precipitate persisted. The precipitate was collected by filtration using glass fibre filter paper and was dried over silica under vacuum. Yield 1.70 g (24%).

I.R. Arom CH 3100 cm^{-1} H bond 3700-2200 cm^{-1}

Carbonyl 1720 cm^{-1}.

CHN: Theory - C 59.00%, H 3.30%, N 11.50%.

Found - C 59.33%, H 3.72%, N 11.58%.

R4 Ruthenium trichloride trihydrate

This was purchased from Aldrich and Lancaster.

R5 Bis-Bipyridyl ruthenous chloride dihydrate

A typical preparation. 2.50 g of R4, 3.0 g of 2,2' bipyridine and 5.0 g of Lithium chloride were placed in 50 mls of DMF under a nitrogen atmosphere. This mixture was stirred and refluxed for 7 hours. The purple solution was cooled to below 15°C with stirring. 250 mls of acetone was then added with plenty of stirring and the flask was stoppered under nitrogen. The solution was left at 0°C for 24 hours. A black

microcrystaline solid was recovered by filtration and was washed with water (3 x 200 mls) and ether (3 x 100 mls). Yield 3.0 g.

I.R. Arom CH 3100 cm^{-1}, H bond peak at 3500 cm^{-1}

CHN: Theory C - 46.15%, H 3.85%, N 10.77%

Found C - 46.27%, H 3.19%, N 10.53%.

R6 2,2'Bipyridyl-4,4'-dicarboxylic acid-bis-2,2'-bipyridyl ruthenous hexafluorophosphate

0.52 g of R5, 0.30 g of R3 and 0.30 g of sodium bicarbonate were placed in 15 mls of water and 10 mls of ethanol. This mixture was refluxed for 2 hours with stirring. The colour of the solution changed from purple to bright red. The solution was cooled and acidified with 1M hydrochloric acid. 25 mls of a saturated aqueous solution of ammonium hexafluorophosphate was added and a red precipitate formed. This was collected by filtration and dried over silica under vacuum for 24 hours. Yield 0.20 g.

I.R. H bond 3500 cm^{-1} broad, Arom CH 3100 cm^{-1}, carbonyl 1720 cm^{-1} PF 840 cm^{-1}.

UV. pH1 : peaks at 418 and 484 nm

pH12 : only one peak at 460 nm.

cf literature pH0.6 : peaks at 420 and 475 nm

pH12.5 : peaks at 455 (chapter 5.11 [7]).

Fluorescence: λ_{max} excitation 456 and 466 nm broad.

emission 638 nm broad.

MS: FABS Durham University.

M^+, $Rubipy_3(CO_2H)_2\ PF_6^+$ m/z 803 present.

$Rubipy_3(CO_2H)_2^+$ m/z 658 present

(see Appendix A 8.70)

100 MHz 'H n.m.r. DMSO d^6, TMS ref.

δ = 7.6, 8.1, 8.7, 7.4.

R7 N-hydroxysuccinimide derivative of R6

This was an "in situ" preparation and so the above named complex was not isolated.

0.038 g of **R6** , 0.018 g of N-hydroxysuccinimide and 0.032 g of dicyclohexylcarbodiimide were stirred together at room temperature in 20 mls of dry DMF for 2 hours. 0.1 ml aliquots were taken from this solution and added to 1-2 mgs of antibody dissolved in 1.0 mls of a labelling buffer (see later). This was done immediately after the 2 hours to avoid hydrolysis of the ester. This method proved unreliable and only worked on 2 occasions out of 4.

R8 2,2'-bipyridyl-4,4'-bis(carbonyl chloride)-bis-2,2'-bipyridyl ruthenous hexafluorophosphate

0.40 g of R6 was placed in 20 mls of thionyl chloride and 20 mls of toluene. This mixture was refluxed for 24 hours. The solvents were removed by rotary evaporation to give a dark brown solid. This was freeze dried for 48 hours. Yield 0.40 g.

I.R. Arom CH 3100 cm^{-1},

carbonyl - not clear, PF 840 cm^{-1}.

Easily soluble in acetone - unlike RC5.

R9a 2,2'bipyridyl-4,4'-bis(carbonyl-isothiocyanate)-bis-2,2'-bipyridyl ruthenous hexafluorophosphate

0.50 g of R8 and 0.1 g of potassium thiocyanate were dissolved in acetone. This was stirred for 0.5 hours and a white precipitate formed. This precipitate (KCl) was removed by filtration and the clear red solution evaporated to dryness. Yield 0.50 g.

I.R. Arom CH 3100 cm^{-1}, H bond 3500 cm^{-1} broad,

NCS 2050 cm^{-1}, carbonyl - not clear, PF 840 cm^{-1}.

Insoluble in H_2O unlike R6.

R9b 2,2'-bipyridyl-4,4'-bis(carbonyl-isothiocyanate-bis-2,2'-bipyridyl ruthenous hexafluorophosphate

0.25 g of R5 and 0.31 g of R12 were dissolved in 100 mls of HPLC grade acetonitrile under nitrogen. This mixture was refluxed for 2 days and gave a red solution. The acetonitrile was then removed by rotary evaporation until about 3 mls remained. To this 200 mls of water was added and the resulting red solution was filtered to remove unreacted starting materials. (R5 and most bipyridine compounds are insoluble in water). A clear red filtrate was obtained and 0.50 g of ammonium hexafluorophosphate was added. A brick red precipitate

formed which was collected by filtration. Yield 0.85 g.

I.R. H bond 3420 cm^{-1} broad, Arom CH 2921 cm^{-1}

-NCS 2103 cm^{-1}, Arom CC 1603 cm^{-1}, PF 845 cm^{-1}

Carbonyl - not clear.

Note: CH_3CN 2260-2240 cm^{-1}

-SCN 2170-2135 cm^{-1}

-NCS 2050-2150 cm^{-1}

R10 2,2'-bipyridyl-4,4'-bis(carbonyl azide)-bis-2,2'-bipyridyl ruthenous hexafluorophosphate

This was unsuccessful - 0.05 g of R8 was dissolved in 5 mls of acetone. To this solution an excess (0.026 g) of sodium azide was added in 0.50 mls of water. The mixture was stirred at room temperature for 1 hour and a red precipitate formed. This was collected by filtration. Yield 0.02 g.

The product was clearly impure - it was a mixture of red and white powders.

It did not label antibody at all.

R11 2,2'-bipyridyl-4,4'-bis(carbonyl chloride)

1.0 g of dry R3 was suspended in 200 mls of sodium dried toluene and 25 mls of distilled thionyl chloride. This mixture was refluxed until the white solid had disappeared. The solution (tinged orange) was the fractionally distilled (30 cm column) to remove the

thionyl chloride. A further 100 mls of tolune was added and the solution was again fractionally distilled until 50 mls remained. The solvent was then evaporated to dryness by rotary evaporation and oil pump. A pale grey solid was recovered. Yield 0.50 g.

This material had to be used very quickly as it was very hydroscopic. It sometimes decomposed (even if left in a dessicator) after 2-3 days.

I.R. 3100 cm^{-1}, Arom C-H, 1750 cm^{-1} C=O,
1600 cm^{-1} Arom C-C, major peaks 1200 cm^{-1},
900 cm^{-1}, 700 cm^{-1}.

<u>R12 2,2'-bipyridyl-4,4'-bis(carbonyl isothiocyanate)</u>

0.2 g of R11 and 0.42 g of potassium thiocyanate (1:6 mols) were dissolved in 800 mls of acetone. The solution was heated to boiling and filtered to remove some solid material. TLC (60_{F254} Silica Acetone eluent) showed two products present. The acetone was removed by rotary evaporation to give a yellow solid that is very soluble in acetone (unlike the starting materials). This crude solid was used without further purification. Yield 0.31 g crude material.

MS: M^+ 326-327 not present but bipy(CO)$(CONCS)^+$
m/z 268-269 is present.

I.R. -NCS at 2064 cm^{-1}

<u>Note</u>: -NCS 2050-2150 cm^{-1} range
-SCN 2170-2135 cm^{-1} range.

R13 4,4'-bis[(2-carboxyphenylamino)carbonyl]-2,2'-bipyridyl

0.20 g of R11, dissolved in 75 mls of acetonitrile, was added to 0.20 g of anthranilic acid, also dissolved in 75 mls of acetonitrile. On mixing the 2 solutions a yellow precipitate formed which was recovered by filtration and dried over silica under vacuum. Yield 0.30 g.

360 MHz 'H nmr: DMSO d^6, TMS ref.

δ = 12.42 s (2H), 9.00 d (2H), 8.96 d (2H), 8.66 d (2H), 8.07 d (2H), 7.07 d (2H), 7.72 m (3H), 7.25 dd (3H), 6.80 d (1H), 6.58 dd (1H).

'H decoupling at all above signals except at 12.42 and 7.72 show the following:

8.96 and 9.00 are coupled to 7.97

8.66 is coupled to 7.72 and 7.25

8.07 is coupled to 7.72 and 7.25

and 6.80 and 6.58 are coupled to 7.72 and 7.25 as well.

It was concluded that the main product was the disubstituted product with some unreacted anthanilic acid present. If the 4H integration for anthranilic acid is subtracted from the rest of the spectrum a perfect fit is obtained (ie. remove 1H at δ = 6.58, 6.80, 7.25 and 7.72).

MS: M^+ not present, (m/z 482).

Spectrum 464, 446 (7.2%), 420, 419, 392, 391 and 300.

446 $M^+ - 2H_2O$

464 $M^+ - H_2O$

391 $M^+ - 2(CO_2H) + H$

R14 4,4'-bis[(2-carbonylphenylamino) carbonyl]-2,2'bipyridyl-bis-2,2'-bipyridyl ruthenous hexafluorophosphate

0.10 g of R5 and 0.10 g of R13 were dissolved in 150 mls of DMF. The purple solution was refluxed under nitrogen and stirred. No colour change was observed after 1 hour and so 50 mls of ethanol were added. The colour began to turn red after another hour and was bright red after 24. The solvent was removed by rotary evaporation until only 5 mls of DMF remained. Then 600 mls of water was added. The red solution was filtered to remove any unreacted R5 and 0.80 g of ammonium hexafluorophosphate was added (20 fold excess) to the clear filtrate. A red precipitate instantly formed which was filtered and dried under vacuum over silica for 24 hours.

Yield 0.11 g (48%).

MS: FABS Durham University

M^+ m/z 895 present

895 at centre of Ru isotope pattern and there are no peaks higher than these in the spectrum.

M^+ is $Rubipy_3(CONHC_6H_4CO_2H)_2^+$

I.R. H bond 3406 cm^{-1} broad, Arom CH 2920 cm^{-1},

carbonyl 1667 cm^{-1}, Arom CC 1589 cm^{-1}, PF 843 cm^{-1}.

Fluorescence: λ_{max} excitation 492 nm broad

λ_{max} emission 660 nm broad

solvent CH_3CN

Lifetime of emission 653 ns (aerated).

R15 2,2'-bipyridyl-4,4'-bis(ethyl ester)

Prepared by F Henderson.

R16 2,2'-bipyridyl-4,4'-bis(ethyl ester)-bis-2,2'-bipyridyl ruthenous hexafluorophosphate

0.05 g of R17 and 0.08 g of R5 were dissolved in 30 mls of ethanol. The solution was refluxed under nitrogen for 2 days and then allowed to cool. 100 mls was added to the ethanolic solution and the solution was filtered. 0.16 g of ammonium hexafluorophosphate was added and a red precipitate formed. This was recovered by filtration. Yield 0.020 g (13%).

MS: M^+ $Rubipy_2bipy(CO_2Et)_2^+$ m/z 714 present with 713 and 716. No higher peaks observed. Peaks at 410-415 corresponding to loss of $bipy(CO_2Et)_2$.

R17 Ruthenium-bis-1,10-phenanthroline dichloride dihydrate

The same method was used as for R5 except 3.0 g of bipyridine was replaced with 3.46 g of 1,10-phenanthroline. Yield 2.10 g of black crystals.

CHN: Theory - C 54.14% H 3.02% N 10.52%

Found - C 53.45% H 2.82% N 10.41%

R18 4,7-diphenyl-1,10-phenanthroline disulphonic acid disodium salt hydrate

Purchased from Aldrich.

R19 4,7-diphenyl-1,10-phenanthroline disulphonic acid-bis-1,10-phenanthroline ruthenous chloride trihydrate

0.20 g of R17 and 0.30 g of R18 were dissolved in 30 mls of water and 5 mls of methanol. The solution was refluxed for 2 hours and gave a red precipitate. The mixture was cooled and the precipitate recovered by filtration. The red solid was washed with ether and then recrystallised from water. The product was dried over P_2O_5 under vacuum for 24 hours. Yield 0.20 g (56%)

CHN: Theory C 53.43%, H 3.54%, N 7.78%.

Found C 53.53%, H 3.10%, N 7.71% for

$RuC_{48}H_{38}N_6S_2O_9Cl_2$

MS: FABS Durham University

M^+ $phen_2Ruphen(\phi SO_3H)_2{}^+$.m/z 948-954 isotope spread present

m/z 772 (loss of phen) present.

m/z 490-496

(loss of $phen(\phi SO_3H)_2$ from cation parent + Cl) present

IR: H bond 3445 cm^{-1} broad, Arom CH 3100 cm^{-1},

Arom CC 1628 cm^{-1}, RSO_2O 1200 cm^{-1}, S = O 1030 cm^{-1}.

Fluorescence λ_{max} excitation 480 nm broad

emission 615 nm broad

lifetime 1.20μs $H_2O:CH_3CN$, 1:1 (aerated)

Quantum yield ϕ = 0.19 in $H_2O:CH_3CN$ 1:1 degassed by freeze thaw x 3. std. $Rubipy_3Cl_2.6H_2O$

R20 4,7-diphenyl-1,10-phenanthroline disulphonyl chloride-bis-1,10-phenanthroline ruthenous chloride

0.05 g of R19 was carefully dissolved in 10 mls of chlorosulphonic acid. This solution was heated to 120°C for 2 hours under nitrogen. The colour changed from red to green indicating oxidation from RuII to RuIII. The mixture was cooled to 0°C and then was carefully added, dropwise, to 300 mls of ice. A red solution formed immediately and this was extracted with dichloromethane (2 x 150 mls). The extract was dried for 2 days with calcium chloride. The drying agent was then removed by filtration and the solvent removed by rotary evaporation. A red solid was recovered and was dried over P_2O_5 under vacuum for 24 hours. Repeated preparations gave varying yields betweeen 1 and 15 mgs (2 - 30%).

IR: H bond 3400 cm^{-1} broad, C-H 3050 cm^{-1}
unknown 1720 cm^{-1}, Arom C-C 1620 cm^{-1},
RSO_2Cl 1380 cm^{-1} and 1180 cm^{-1}.

The peak at 1380 cm^{-1} is a distinct RSO_2Cl and is not present in **R19** .

Fluorescence λ_{max} excitation 475 nm broad
λ_{max} emission 620 nm broad
lifetime 0.25μs CH_3CN (aerated)

It was also attempted to synthesise this compound by reacting solid PCl_5 with solid **R19** (see D1 prep). However, no reaction occurred on mulling the two solids.

R21 Ruthenium-bis-(4,7-diphenyl-1,10-phenanthroline)-dichloride dihydrate

The same method was used as for RC2 except: 3.0 g of bipyridine was replaced with 2.0 g of 4,7-diphenyl-1,10-phenanthroline.

2.50 g of RCl was replaced by 0.78 g of RCl

5.0 g of LiCl was replaced by 4.50 g of LiCl.

Yield 1.23 g of black crystals.

(Product was not washed with ether).

CHN: Theory - C 66.05%, H 4.15%, N 6.41%.

Found - C 65.91%, H 3.71%, N 6.50%.

R22 4,7-Diphenyl-1,10-phenanthroline disulphonic acid-bis-4,7-diphenyl-1,10-phenanthroline ruthenous chloride dihydrate

0.50 g diphenylphenanthroline disulphonic acid sodium salt and 0.79 g of R21 were dissolved in 20 mls of water and 5 mls of methanol. The solution was stirred, under nitrogen at reflux temperature for 16 hours. However, no colour change (purple to red) was observed. 25 mls of ethanol were added and within 10 minutes a red colour was observed. After 2 hours a red suspension had formed and on cooling was recovered by filtration.

Yield 1.05 g (85%).

CHN: Theory - C 63.33%, H 3.83%, N 6.15%.

Found - C 63.48%, H 3.94%, N 6.29% for $RuC_{72}H_{52}N_6S_2O_8Cl_2$.

R23 4,7-diphenyl-1,10-phenanthroline disulphonyl chloride-bis-4,7-diphenyl-1,10-phenanthroline ruthenous chloride

Preparation of this complex was attempted by adding R22 to (i) $ClSO_3$ as in prep of R19

or (ii) PCl_5 as in prep of D1

Both reactions failed to give a product that could either be characterised or would label antibody.

(ii) produced no reaction - it was tried 3 times. Addition of a small amount of $POCl_3$ was also not successful.

R24 Alanine-alanine-lysine derivative of R20

4-(3-sulphophenyl)-7-[3-(lysyl alanyl alanyl sulphonyl)phenyl-1,10-phenanthroline-bis-1,10-phenanthroline ruthenous chloride.

Introduction

This work was carried out using the "FMoc" strategy for peptide synthesis. 20 mgs of R20 were available hence the small scale of the work. The tripeptide was HO-lys ala ala-NH_2.

Day 1: (a) 200 mls of dichloromethane was dried and distilled.

(b) 1000 mls of DMF was placed over a 3A molecular sieve.

Day 2: (a) The DMF was filtered to remove the sieve and then stirred under reduced pressure for 1 hour to remove amine impurities.

(b) 200 mls of 20% piperidine solution in DMF was prepared.

(c) 50 mls of 10% Diisopropylethylamine (DIPEA) in DMF was prepared.

(d) 83 mgs of solid support was weighed out - Celite pepsyn KA, functionality OH 0.11 mmols/gram give 0.0091 mmols.

(e) N-methyl morpholine (NMM) was made up in DMF so as 0.1 ml portions contained 0.018 mmols.

0.43 mls of NMM was made up to 20 mls with DMF.

(f) 4-Dimethylaminopyridine (DMAP) was made up in DMF so as 0.1 ml portions contained 0.0018 mmols. 40 mgs of DMAP was dissolved in 20 mls of DMF.

(g) 17 mgs of FMoc lys (Boc)OH (0.036 mmols) was weighed out twice. (First aa attachment is always repeated).

(h) 11 mgs of F(Moc)AlaOH (0.036 mmols) was weighed out twice.

ALL SOLUTIONS ABOVE WERE KEPT COLD AND IN THE DARK. ALL SOLIDS WERE DESSICATED OVERNIGHT UNDER VACUUM OVER SILICA.

Day 3: (a) 2.0 g of fresh DCCI was dissolved in 50 mls of dichloromethane (1a). This gave 0.018 mmols in 0.1 ml for symmetrical anhydride (SA) preparations.

(b) The solid phase (2d) was placed on a small scintered funnel with a tap below the scinter. The solid was washed with DMF 3 times - the first wash was allowed to stand to 10 minutes to expose the OH groups to the solvent.

(c) The first symmetrical anhydride (of lysine) was prepared (see later) and added to the solid phase so that the solvent covered the beads. 0.1 ml of the DMAP solution (2f) and 0.1 ml of the NMM solution (2e) were added with the symmetrical anhydride. The mixture was stirred carefully with a small spatula for 100 minutes.

(d) The solution was removed through the filter and then the solid was washed with DMF 3 times.

(e) Part 3c was repeated except that the mixture was stirred for 40 minutes.

(f) A PSA test was carried out on 2 grains of solid (deprotected), see "PSA test" and section 3g.

The test showed the presence of amines on

the solid phase and so it was decided to proceed with the bulk of the material.

(g) Some of the 20% piperidine solution was added to the solid (enough to cover it) and this was stirred gently for 10 minutes. This deprotects the NH_2 by removing the FMoc group.

(h) The solution was removed through the filter and the solid was carefully washed with DMF 3 times. Care was taken to wipe the lip of the funnel and to fill it right up with DMF so as to remove all the piperidine.

(i) The PSA test was carried out on 2 grains from the bulk.

(j) The second symmetrical anhydride (of alanine) was prepared (see later) and was added to the solid phase. No DMAP or NMM was used and the mixture was stirred for 30 minutes.

(k) The PSA test was carried out on the protected and deprotected products.

(l) Deprotection was carried out (3 g) as before.

(m) The third symmetrical anhydride (of alanine was prepared (see later) and was added to the solid phase as in 3(j).

(n) This was tested, deprotected and washed (3f, 3g, 3h).

(o) The solid was washed with dichloromethane and then with ether.

Day 4: (a) The solid phase was washed with DMF cf 3(a)

(b) The solid was washed with t-Amylalcohol 3 times.

(c) The solid was washed with hydrochloric acid solution (aqueous pH 3.0) for 10 minutes.

(d) The solid was washed with t-Amylalcohol 3 times.

(e) The solid was washed with DMF 3 times.

(f) The solid was washed with 10% DIPEA solution.

(g) 20 mgs of the Ruthenium complex (R20) was added in DMF and was stirred gently with a five-fold excess of triethylamine for 4 hours.

(h) The solution was removed through the filter and washed with DMF very thorougly. The solid was coloured red and was washed with dichloromethane (twice) and ether (3 times)

Day 5: "Deblocking" procedure.

(a) The solid particles were suspended in 95% aqueous trifluoroacetic acid (tfa) for 90

minutes at room temperature.

(b) The tfa was collected by filtration and the solid was washed with more 95% tfa. The washings and the filtrate were combined and reduced to a small volume by rotary evaporation.

(c) Ether was added and removed by rotary evaporation twice. This was done to azetrope off the tfa. On further addition of ether a red solution formed.

(d) The ether was completely removed by rotary evaporation and the solid was dissolved in 1 ml of water to give a red solution.

Purification

(a) The solid was dissolved in 1 ml of water to give a red solution.

(b) 150 l injections of this were passed through a TSK gel CM 25 W ion exchange column. The eluent programme was as follows:

A = H_2O, B = 2M NaCl pH 6, C = MeCN

A	89%	1%	
B	1%	89%	
C	10%	10%	over 40 minutes

λ_{abs} = 230 nm

Peaks were observed at 1.3, 2.9 and 13.0 minutes.

The peak at 13.0 minutes was red and luminescent.

Reverse phase was attempted: sepherisorb 50 DS2

A = 0.1% TFA in H_2O

B = MeCN

A 100% 0%

B 0% 100% over 30 minutes.

This gave lots of peaks.

The product from the ion exchange programme at 13.0 minutes was evaporated to dryness. Half of this mainly white solid (salt) was dissolved in water and passed down a G10 sephadex column, eluting with water. Unfortunately the product dispersed on the column and diluted. No further purification was tried as there was too little material to work with. The dilute product was passed through the same ion exchange programme as used earlier and gave a small red peak at 13.0 minutes. The other peaks had disappeared.

Symmetrical Anhydride Formation

(a) The blocked amino acid (Cambridge Research Biochemicals) was dissolved in 1 ml of dichloromethane with DCCI dissolved in it (0.1 ml of 3(a) was added to 1ml of CH_2Cl_2). A drop of DMF may have been required to dissolve all the amino acid.

(b) The solution was gently swirled until a white precipitate of DCU formed.

(c) The dichloromethane was removed by rotary evaporation taking care not to heat the mixture.

(d) The solid was resuspended in about 2 mls of DMF and added to the solid phase through a cotton wool plugged pasteur pipette. Most of the DCU was caught on the plug and the DMF solution passed through. The pipette was washed through with more DMF so that all the solution was on the solid phase. DMF was added as necessary to ensure all the solid phase was covered.

PSA test

(a) A freshly made solution of picryl sulphonic acid dihydrate in 10% DIPEA (ie. a little on the end of a microspatula in 1-2 mls DIPEA solution) was made.

(b) 2-3 beads of solid phase were placed in the PSA solution.

(c) The beads were white initially and were watched to see if they went red.

(d) If the beads remained white then no free amine was attached to the solid phase. If the beads went red then free amine groups were attached to the solid phase.

eg.	SP-lys(Boc)FMoc	white
	SP-lys(Boc)NH_2	red

(e) The first amino acid took about 3-5 minutes to turn red. The others took 1-2 minutes.

Glassware

All "deblock" glassware was soaked in chromic acid for at least 2 hours. This was washed in distilled water and oven dried. All other glassware was carefully washed and oven dried.

7.10 Preparative Chemistry of Chromium Compounds

Codes C1-C10 correspond to the strategies in Chapter 5

C1 Chromium metal - BDH

C2 Chromium dichloride - $CrCl_2$ solution.

0.52 g of chromium metal, C1, was dissolved in a mixture of 2.5 mls of concentrated hydrochloric acid and 2.5 mls of distilled water. This was done under a nitrogen atmosphere at room temperature. The acid solvent was purged with nitrogen before use. The mixture was sonicated for 3 hours to dissolve the metal and then 40 mls of nitrogen purged water was added with stirring to give a blue/green solution. This solution contained $CrCl_2$ and was very unstable in air.

C3 Tris-1,10-phenanthroline chromic perchlorate

$Crphen_3.3ClO_4.2H_2O$

5.40 g of 1,10-phenanthroline (Aldrich gold label) was dissolved in 20 mls of ethanol and purged with nitrogen for 5 minutes. This was added to the solution C2 and the resultant mixture was cooled in an ice bath while care was taken to ensure the nitrogen atmosphere was

maintained. 2.0 g of sodium perchlorate was then added and a dark green precipitate formed. This solid was recovered by filtration under nitrogen. The solid was then placed in a 1M aqueous solution of perchloric acid and stirred. Oxygen was bubbled through this mixture for about 5 hours. The green solid turned yellow. The product was placed in a filter funnel and washed with water. A pink residue was left in the funnel while a yellow precipitate formed in the filtrate. This yellow precipitate was dried over silica under vacuum for 24 hours.

CHN: Theory C 46.64%, H 3.04%, N 9.07%.

Found C 46.26%, H 2,73%, N 8.84%.

Fluorescence: λ_{max} excitation 397 nm broad

λ_{max} emission 728 nm sharp

Solvent: H_2O: CH_3CN 1:4 mixture

Lifetime: H_2O:CH_3CN, 4:1 (aerated) 440 nm He-Cd laser excitation 25μs.

C4 and C5

Attempted preparations of $Cr(dpphen)_3.3ClO_4$ (C4) and $Cr(phen(\phi SO_3H)_2)_3.3ClO_4$ (C5).

The method was followed as for C3 with the following modifications:

C4 2.0 g of 4,7-diphenylphenanthroline was added in 100 mls of ethanol: chloroform (1:1). A green solution formed and 2.0 g of sodium perchlorate was added in 10 mls of water. No complex formed in either of the layers.

C5 2.5 g of 4,7-diphenyl-phenanthroline disulphonic acid disodium salt in 50 mls of water was added. This gave an olive green solution. No precipitate formed on addition of 1 g of sodium perchlorate and 5 mls of perchloric acid.

C6 Chromium trichloride. THF complex

Purchased from Alfa Chemicals. $CrCl_3.3THF$

C7 Chromium Cyclam complex

cis/trans-Cr cyclam $Cl_2.ClO_4$

cyclam = 1,4,8,11-tetraazacyclotetra-decane

2.80 g of cyclam (Fluka) and 1.59 g of C6 were weighed out under a nitrogen atmosphere. The solids were dissolved in 150 mls of hot, dry DMF. The solution was heated at reflux temperature, under nitrogen for 2 hours and then filtered to give a mixture of solids. The solid was washed with 200 mls of hot, dry DMF to leave one product. This pink solid (0.8 g) was washed with acetone and then dissolved in 100 mls of water. The solution was heated to 50°C and 2.0 g of sodium perchlorate was added and gave a pink/mauve precipitate. The suspension was allowed to cool and the solid was recovered by filtration. The solid was washed with acetone and dried over silica under vacuum.

Yield = 0.90 g

C8 Chromium cyclam complex

trans - Cr cyclam$(CN)_2.ClO_4$

0.50 g of C7 was dissolved in 8 mls of DMSO and heated at 62°C to give a red solution. 0.46 g of finely ground sodium cyanide was added and the temperature maintained at 62°C for 2 hours. A yellow precipitate formed and the solution was allowed to cool. The precipitate was filtered and washed with ethanol and ether. The solid was then dissolved in 800 mls of water and filtered (G4 scinter) to remove some impurities. 7.5 g of sodium perchlorate was added and a fine yellow precipitate formed. This suspension was left overnight and then filtered. The solid was washed with ethanol and ether to give a fine lemon yellow powder.

Yield = 0.25 g

CHN: Theory C 35.69%, H 5.99%, N 20.81%.

Found C 35.42%, H 6.02%, N 20.73%.

(WO_2 combustion additive used).

Fluorescence: λ_{max} excitation 416 nm broad

λ_{max} emission 715 nm

shoulder 735 nm

Lifetime H_2O: CH_3CN, 4:1 excitation 440 nm, He-Cd laser, 104μs (aerated).

C9 Chromium Acetate

12.5 g of potassium dichromate and 12.5 g of zinc dust were placed in a flask under nitrogen. 75 mls of concentrated hydrochloric acid was added to 50 mls of

distilled water and the resultant solution was added slowly with vigorous stirring. A brown "sludge" formed giving out lots of heat. As the reaction proceeded the colour changed to green and then to blue. After the reaction was complete the solution was filtered under nitrogen into a saturated solution of sodium acetate (22.5 g or more required). A red precipitate formed which was collected by filtration under nitrogen and dessicated for 2 hours. This compound was then used in the next reaction.

Yield = 7.9 g.

C10 Attempted Preparation of a Chromium Macrocylic complex

2.78 g of 1,3-diaminopropan-2-ol was dissolved in 50 mls of methanol. 5.0 g of hydrogen bromide solution (48%, Aldrich) was then added. This solution was stirred and purged with nitrogen for 5 minutes. The temperature was increased to 65°C and then 6.4 g of benzil was added (a nitrogen atmosphere was maintained). After stirring the solution for 20 minutes, 2.82 g of chromium acetate was added and the solution was kept at 65°C for 4 days. No precipitate formed unlike the colbalt preparation (see chapter 5). The temperature was increased to 80°C for 24 hours but still no precipitate formed. The red solution was then cooled and some of the solvent removed. No precipitate formed for several days and the reaction was abandoned.

7.20 Preparative Chemistry of Aluminium Compounds

Codes A1-A8 correspond to the strategies in Chapter 5.

Aluminium trichloride hexahydrate purchased from Aldrich (gold label).

A1 Aluminium oxine complex

Tris-8-hydroxyquinoline aluminium

1.00 g of $AlCl_3.6H_2O$ and 1.80 g of 8-hydroxyquinoline were suspended in 200 mls of water. 10 drops of concentrated hydrochloric acid and a further 750 mls of water was added. This mixture was stirred and heated until all the 8-hydroxyquinoline had dissolved. The solution was then slowly neutralised with solid potassium hydroxide until a lime green precipitate persisted. This precipitate was recovered by filtration and washed with 200 mls of water. The solid was dried over silica under vacuum for 24 hours.

Yield = 2.50 g.

MS: M^+ $Al(ox)_3$ m/z 459, 21%.

$Al(ox)_2$ m/z 315, 100%

CHN: Theory C 70.58%, H 3.95%, N 9.15%

Found C 69.75%, H 3.84%, N 9.00%.

IR: H bond 3439 cm^{-1} broad

Arom C-C 1606 cm^{-1}, 1498 cm^{-1}, 1468 cm^{-1}, 1384 cm^{-1}, 750 cm^{-1}, 4 very strong peaks.

Fluorescence: λ_{max} excitation 432 nm

(also a peak at 346 nm)

λ_{max} emission 522 nm broad

Solvent CH_2Cl_2

Emission lifetime: 276 ns in CH_3CN (aerated)

A2 Tris-8-hydroxyquinoline-5-sulphonic acid aluminium

ox = oxine = 8-hydroxyquinoline

$Al(ox5SA)_3$ $AlC_{27}H_{18}N_3O_{12}S_3$

5.00 g of $AlCl_3.6H_2O$ and 16.20 g of 8-hydroxyquinoline-5-sulphonic acid were placed in 800 mls of water. This suspension was heated until all the solid had dissolved. On cooling, fine, bright lime yellow crystals appeared which were collected by filtration. These crystals were dessicated under vacuum over silica for 24 hours.

Yield = 13.80 g

Fluorescence: λ_{max} excitation = 432 nm

λ_{em} emission = 512 nm

Microanalysis: $C_{27}H_{18}N_3O_{12}S_3Al$

Theory C 46.35%, H 2.59%, N 6.00%.

Found C 46.89%, H 3.30%, N 5.93%.

IR: 3400 cm^{-1} broad, 3080 cm^{-1} C-H,

1603 cm^{-1} C-C Arom, 1189 cm^{-1} SO_3H.

A3 Attempted Preparations of Tris-8-hydroxyquinoline-5-sulphonyl chloride aluminium

$AlC_{27}H_{15}N_3O_9Cl_3S_3$

(1) 1.50 g of A2 was placed in 20 mls of chlorosulphonic acid. The solution was stirred at 120°C under nitrogen for 1 hour. After cooling the product was added dropwise (with great care !) to 500 mls of ice. A yellow precipitate formed which was filtered and dried over silica under vacuum for 24 hours. This product was soluble in acetone and chloroform unlike the starting material. The solid decomposes within 2 days (if left exposed to the air) forming a grey brown oily solid. When mixed with carbonate buffer (pH 9.5) gas bubbles are formed.

Yield = 1.35 g.

IR of solid: 3400 cm^{-1} H-bond, 3000 cm^{-1}, 2900 cm^{-1}, 1640 cm^{-1}, 1595 cm^{-1} Arom C-C, 1370 cm^{-1} SO_2Cl 1213 cm^{-1} SO_2Cl.

Fluorescence: λ_{max} excitation 430 nm

λ_{max} emission 530 nm (in CH_2Cl_2)

(2) 0.5 g of AC3 was mulled together with an excess of phosphorous pentachloride. No reaction occurred, cf D1 preparation.

A4 5-Nitro-8-hydroxyquinoline

Purchased from Aldrich.

A5 5-Amino-8-hydroxyquinoline

It was attempted to prepare this ligand by two methods:

(a) 3.0 g of A4, 4.0 g of hydrazine hydrate and 1.00 g of palladium on carbon (10%) were mixed in 1 litre of methanol. This mixture was refluxed by the heat of the reaction for about 30 minutes. A further 1.0 g of the palladium on carbon was added and the mixture kept at reflux temperature for a further 2 hours. After cooling the palladium on carbon was removed by filtration and the methanol removed by rotary evaporation. A sample of the solid was removed (not completely dry) for MS analysis. This showed the product was there: M^+ m/z 160.07 (100%), M^+-H_2O 132 (19%). However, on drying the compound thoroughly the orange solid degraded into a black oily solid. Repeated attempts (twice) proved unsuccessful (using very low temperatures for rotary evaporation) in isolating the orange solid before it decomposed. The black solid gives the following:

MS: m/z 493, 430, 380, 331, 317, 316 (4.1%), 300, 243, 230, (3.79%), 200 (16.4%), 190 (5.6%), 185 (23%), 181 (7.9%), 169 (10.6%), 160 (67.9%). This shows a wide variety of species.

(b) 86 g of sulphanilic acid and 26 g of sodium carbonate (anhydrous BDH) were dissolved in 750 mls of water. A solution of 35 g of sodium nitrite in 100 mls of water were added and the mixture kept at $0^{o}C$. 75 mls of concentrated hydrochloric acid was added to this solution and the temperature was maintained at $0^{o}C$. The HCl addition was done dropwise and the hydrochloric acid had been kept refrigerated overnight before use. 73 g of oxine dissolved in 50 mls of hydrochloric acid (conc) and 50 mls of water and this was added dropwise to the diazonium solution with thorough stirring. A heavy deep red precipitate formed and was filtered and dried as well as possible between filter papers. The damp solid was then reduced by treatment with 500 mls of hydrochloric acid and 550 g of stannous chloride (hydrated). The reduction occurred rapidly. The mixture was then heated to $95^{o}C$ and kept there for 35 minutes. The red solution so formed was cooled to $0^{o}C$ and left overnight. A yellow precipitate - the dihydrochloride salt was filtered and then dissolved in 2.5 litres of water. Hydrogen sulphide gas was bubbled through the solution to remove any tin chloride as a tin sulphide precipitate. The precipitate was filtered and a clear yellow solution resulted. This solution

was reduced in volume by rotary evaporation to about 1 litre and cooled. The dihydrochloride salt of 5-amino-8-hydroxyquinoline formed as a precipitate. However, any attempt to isolate the free base led to a rapid decomposition to a dark green oil. This was the same as the above preparation (a).

A7 5-Benzyl-8-hydroxyquinoline

9.0 g of benzoyl chloride, 8.7 g of 8-hydroxyquinoline and 20 g of dry aluminium trichloride (anhydrous) was dissolved in nitrobenzene. The $AlCl_3$ was weighed out under nitrogen. The mixture was refluxed under nitrogen for 12 hours. On cooling ice was added followed by 10% hydrochloric acid (100 mls). The solvents were then removed by rotary evaporation until only the nitrobenzene was left. The mixture was filtered and the solid washed with 10% hydrochloric acid (1000 mls). The resultant solid was recrystalised from 10% hydrochloric acid and then dissolved in water. On addition of sodium acetate a yellow/white precipitate appeared. This was recovered by filtration and dried.

Yield = 2.5 g

MS: M^+ m/z 249 (66.5%), 248 (6.4%), $M^+-C_6H_5$ 172 (89.1%).

IR: H-bond 3457 cm^{-1}, C=O 1637 cm^{-1}, Arom C-C 1612 cm^{-1}.

'H nmr 100 MHz: δ =8.0 m(7H), 8.3 dd(1H), 9.5d, 9.65d.

60 MHz decoupling shows signals at 8.3, 9.5 and 9.65 are coupled.

A8 Oxine derivative of 5-benzyl-8-hydroxyquinoline

1.0 g of hydroxylamine hydrochloride, 2.0 g of sodium acetate and 0.5 g of A7 were dissolved in 125 mls of water (some solid remains). This mixture was refluxed for 3 days and gave a clear solution. On cooling and removal of half the solvent a precipitate formed.

MS: M^+ m/z 264 (90%), M^+-OH 247 (100%), 231 (10.2%).

IR: H-bond 3413 cm^{-1}, Arom C-C 1616 cm^{-1}.

This preparation was also attempted using 0.5 g of A7, 0.5 g $NH_2OH.HCl$, 0.5 mls of pyridine and 20 mls of ethanol. After 1 hour of reflux and work up (Vogel 3rd Ed) a mixture of A7 and A8 was produced.

MS: M^+ m/z 264 (5.3%), 249 (71.8%).

IR: C=O still at 1637 cm^{-1}.

D1 Dansyl Chloride

2.0 g of 5-dimethylaminonaphthalene-1-sulphonic acid (Aldrich) and 1.66 g phosphorus pentachloride were mulled together as solids.

Quickly the solids effervesced and mixing was continued for 10 minutes. Water (25 mls) was added, causing more effervescence and a yellow oil/precipitate was formed. Chloroform was added to extract the non-aqueous soluble material and on separation and reduction in volume a yellow oil was collected. This was left to stand overnight and yellow crystals were collected.

Yield = 0.65 g.

7.30 Labelling of Antibodies

The procedure is described in 6.00. The Human IgG was supplied from Birmingham Medical School. PBS was made up as follows:

NaCl 8.00 g/l, KCl 0.20 g/l, KH_2PO_4 0.20 g/l and Na_2HPO_4 1.15 g/l.

Carbonate buffer was made up as follows:

Na_2CO_3 1.59 g/l and $NaHCO_3$ 2.93 g/l.

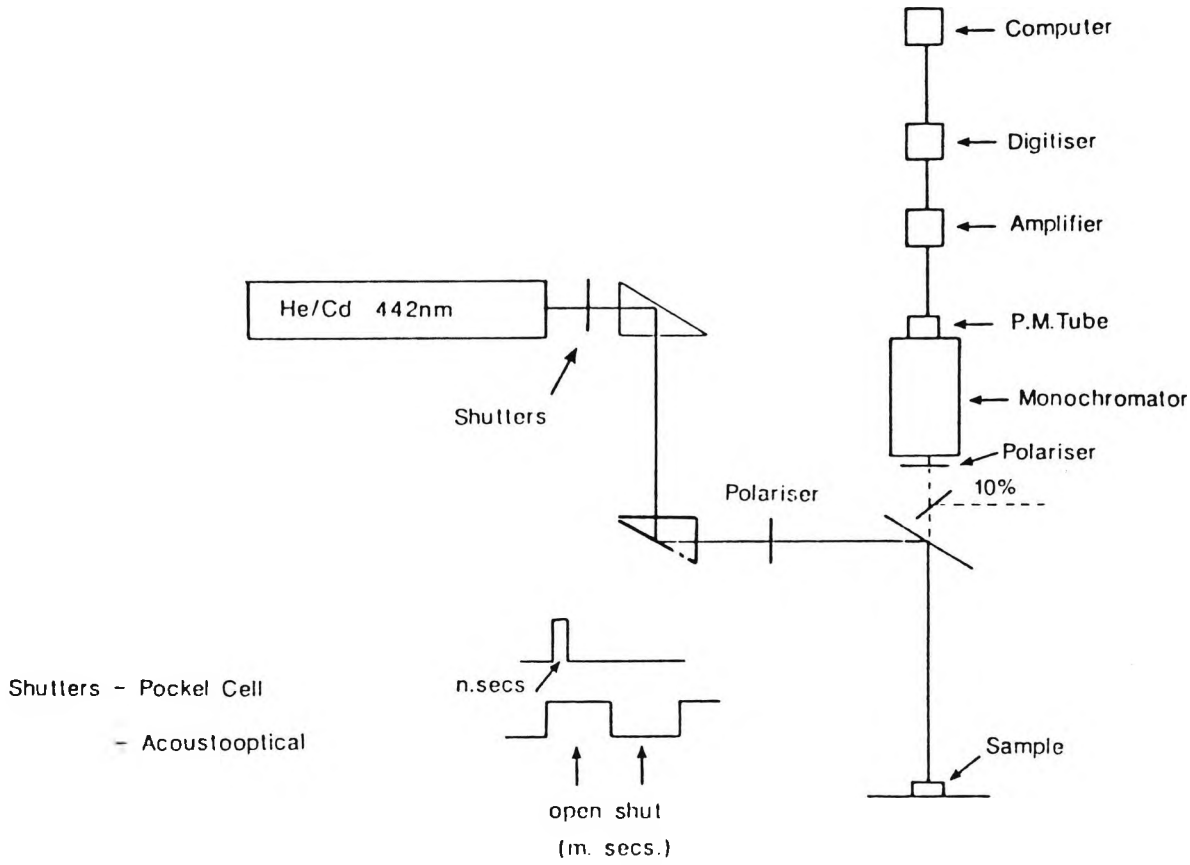
DMF was stirred under reduced pressure before use to remove amine contaminants. G25 sephadex was purchased from Sigma and the 20 cm columns were made up in a 10 ml pipette with a cotton wool plug. Care was taken to ensure a reasonable flow rate before the antibody/label mixture was added.

7.40 Luminescent Lifetime Measurements

Luminescent lifetimes were recorded using either a xenon-fluoride excimer laser or a helium-cadmium laser. The xenon-fluoride laser (Oxford Instruments) gave a pulse of light at 353 nm wavelength, of about 23 ns duration and of 114 mJ energy. The emission from the sample was observed by a Hamamatsu (Tube R928) photomultiplier tube tuned to the wavelength of the emission maximum. The decay of the emission signal was recorded on an oscilloscope and photographed. The picture was traced, enlarged and the natural log of the intensity plotted against time. The lifetime of luminescence was the gradient of the line T/In Intensity.

The helium-cadmium laser used a photoacoustic shutter to create the pulse of exciting light. This was reflected into a fluorescence microscope as shown below.

Fluorescence Microscope



The microscope was a Carl Zeiss LAB 16 fluorescence microscope with a 40/0.75 Neofluar objective. The helium-cadmium laser, model 4240NB was made by Liconix, Sunnyvale, USA. The wavelength of the light was 442 nm and had a power output of 50 mW. The laser was gated by

an acousto optical shutter device, rise time 50 ns (AA France) controlled by a microcomputer C64 (Commodore, Braunschweig, FRG). The diameter of the laser beam impinging on the sample was 4 μm. The emission spectra were recorded with a monochromator, grating 1200 grooves/mm (Bausch & Lamb, USA) and the fluorescence intensity was determined with a Hamamatsu R446 photomultiplier tube connected to the computer. Drops of the solutions to be tested were put on a glass slide (avoiding air bubbles) and this was placed on the microscope.

7.50 Recording of Spectra

Infra Red: Recorded on a Perkin-Elmer 983G IR Spectrophotometer.

Fluorescence: Recorded on a Perkin-Elmer MPF-4 Spectrophotometer.

UV: Recorded on a Perkin-Elmer Lambda 5 UV/VIS Spectrophotometer.

'H nmr: Recorded on a Jeol JNM-MH-100 (100 MHz) and on a Jeol JNM-PMX-60 (60 MHz) at City University. A 360 MHz Bruker WH360 was used for R13 (Edinburgh University).

^{13}C nmr: Recorded on a Jeol-JNM-FX60, Alan Osborne

operator.

Mass Spectra: Recorded on a Kratos MS30 (electron impact) Chris Whitehead operator.

FAB Mass Spectra were recorded at Durham University.

Microanalysis: Performed at City University on a Carlo Erba.

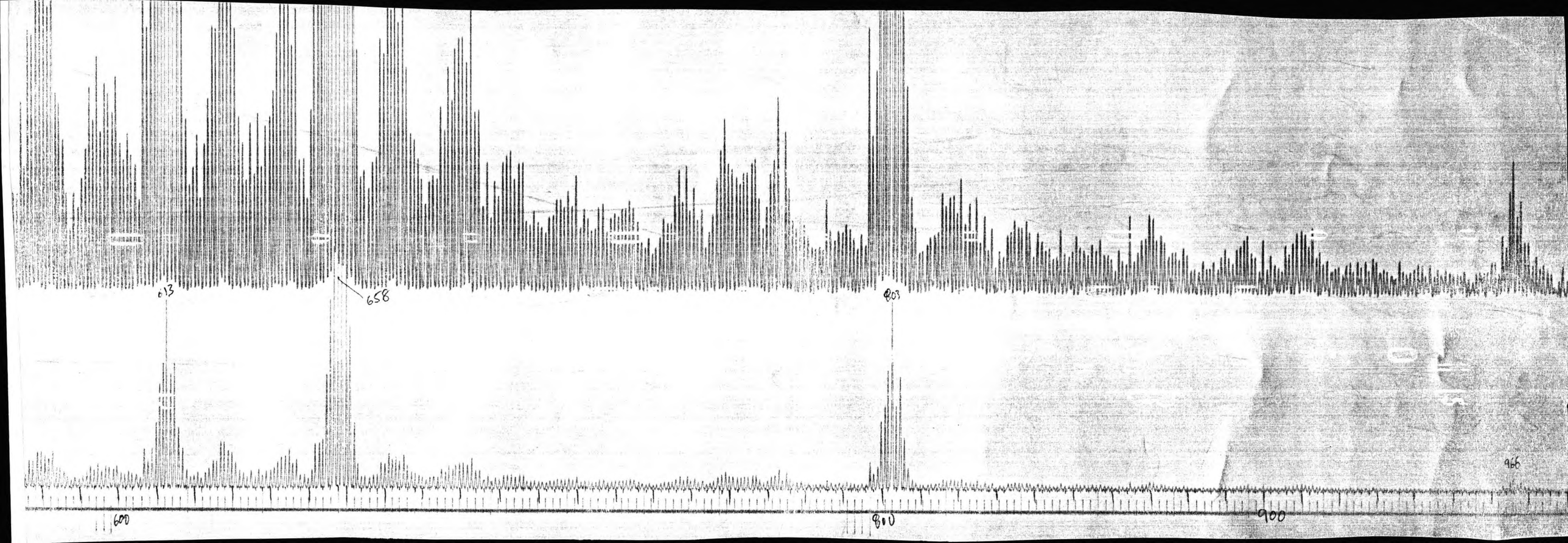
7.60 Appendices A and B

Appendix A. The FAB Mass Spectrum of R6

R6 is $Rubipy_2bipy(CO_2H)_2.2PF_6$

This compound gave the clearest spectrum of any of the compounds tested by FAB mass spectroscopy. The sample gave a series of intense ions with ruthenium isotope clusters [ruthenium has five isotopes: masses 99 (13%), 100 (13%), 101 (17%), 102 (31%) and 104 (18%)]. The highest major cluster of peaks has the most intense peak at m/z 803. Assuming that this is due to ^{102}Ru this would correspond to the diacid complex with two positive charges plus a PF_6^- counter ion. The fragment ion m/z 658 represents the complex with no PF_6^- ions and the ion m/z 613 the loss of 45 atomic mass units ie. the loss of a $^{\bullet}CO_2H$ group. This pattern is compatible with the structure. There is a weak cluster at m/z 966 which

is not easy to explain. $[Rubipy_2bipy(CO_2H)_2.2PF_6]^+$ only gives 944 atomic mass units. The species $Rubipy_3bipy(CO_2H)_2PF_6^+$ would give an m/z of 959 but this is highly unlikely to form and is not close enough to the cluster centre at m/z 966. No other compound reported gave a peak higher than the expected molecular ion. All other compounds tested by FAB mass spectroscopy that gave clear molecular ions are reported in the experimental along with any major diagnostic peaks.

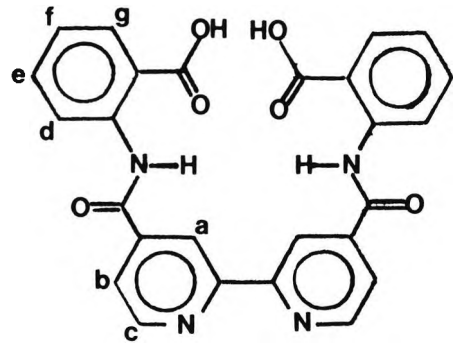

613
658
803
966
600
800
900

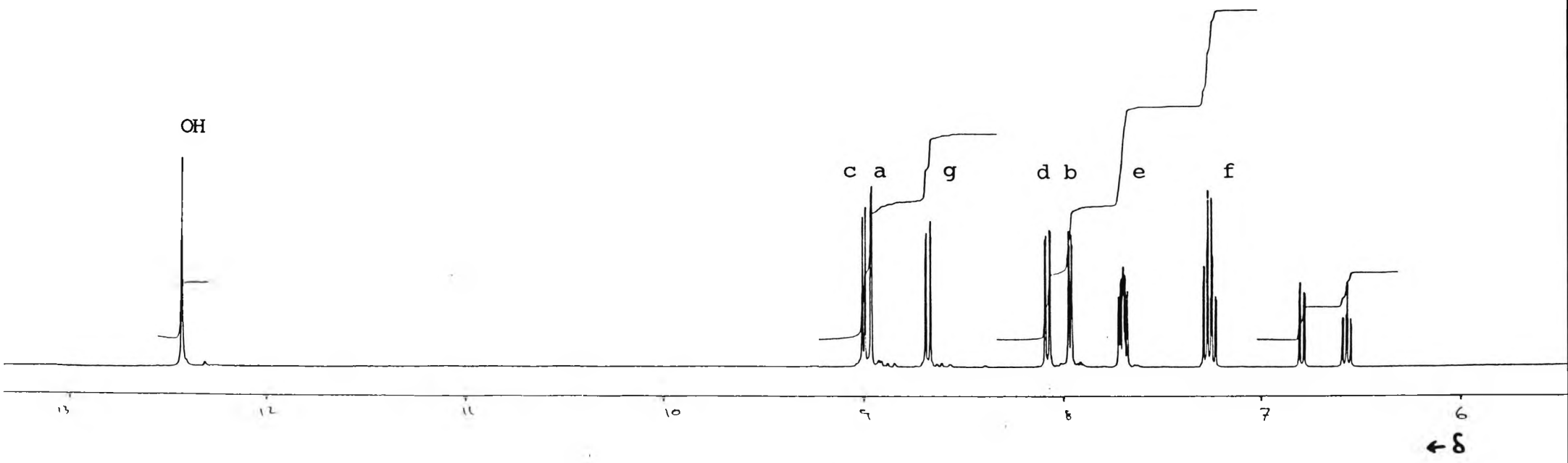
Appendix B The 360 MHz Spectrum of R13

R13 is 4,4'-bis[(2-carboxyphenylamino)carbonyl]-2,2'-bipyridyl.

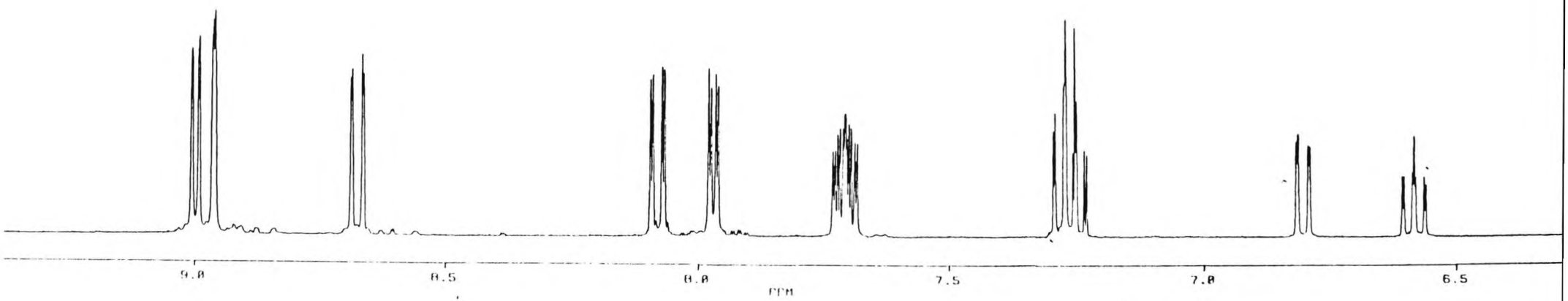
The spectra described in the experimental are here recorded without further comment.

COMPLETE SPECTRUM

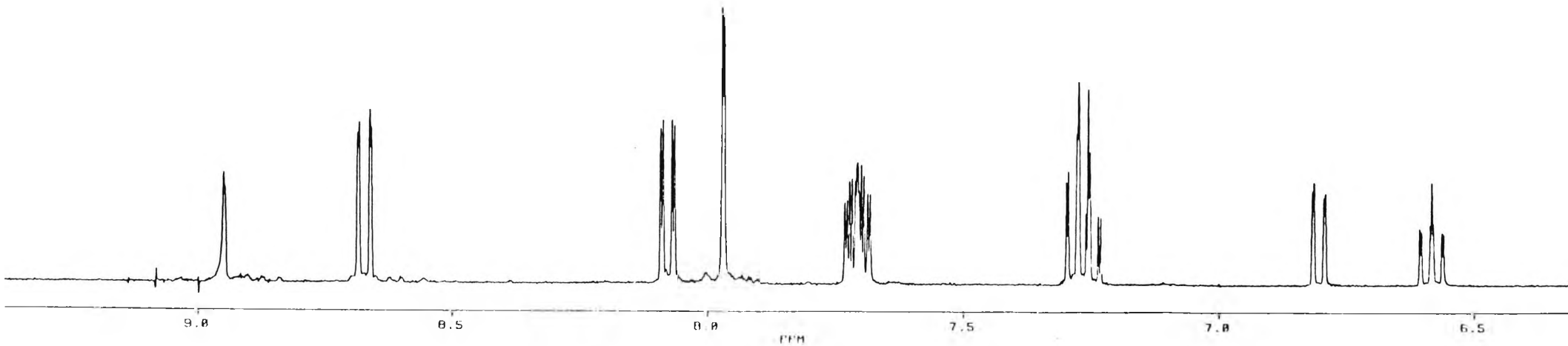




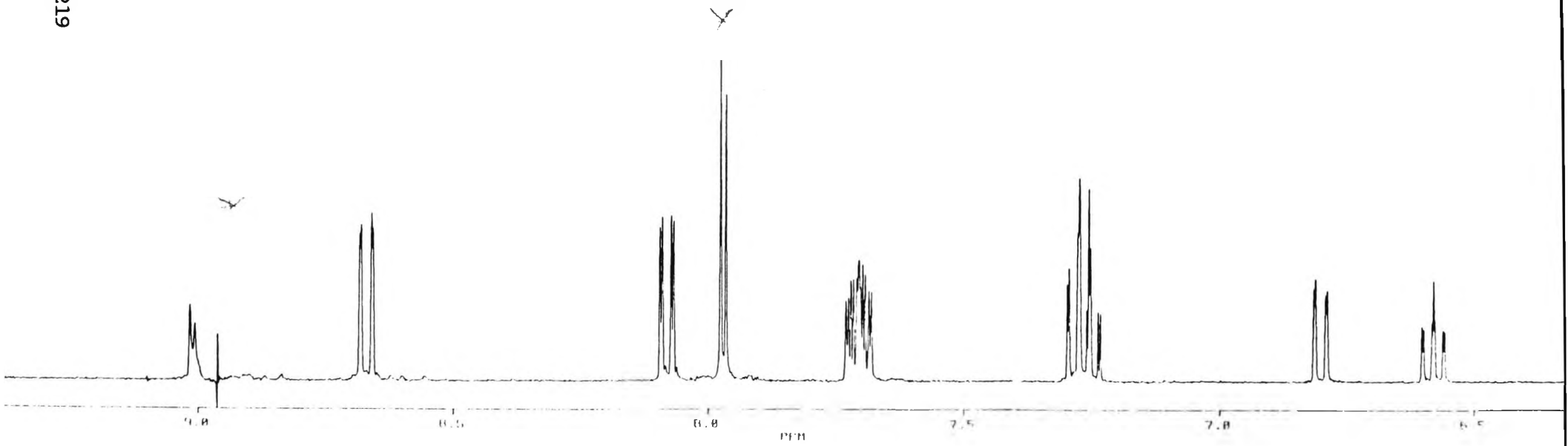
NON-DECOUPLED SPECTRUM $\delta = 6.5-9.5$.



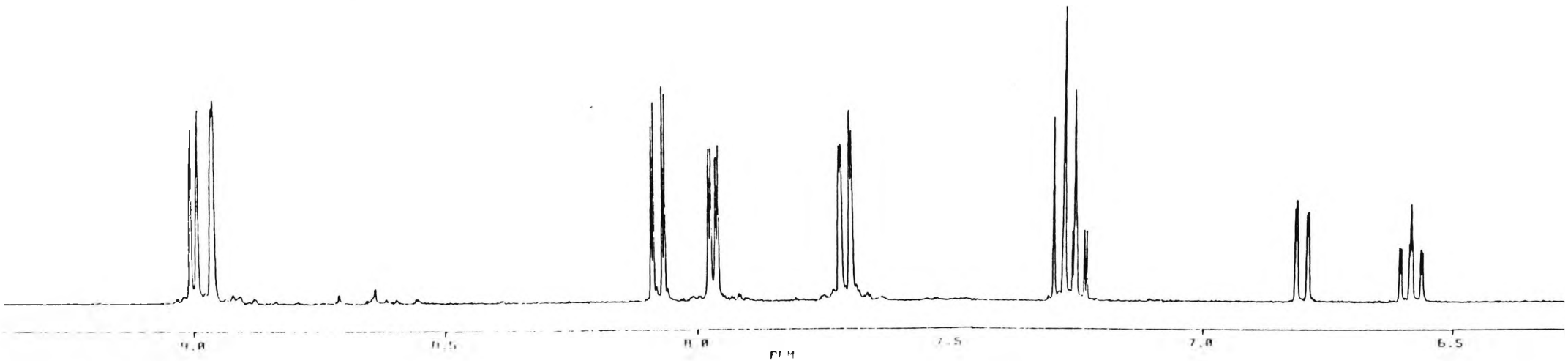
DECOUPLED AT $\delta = 9.1$



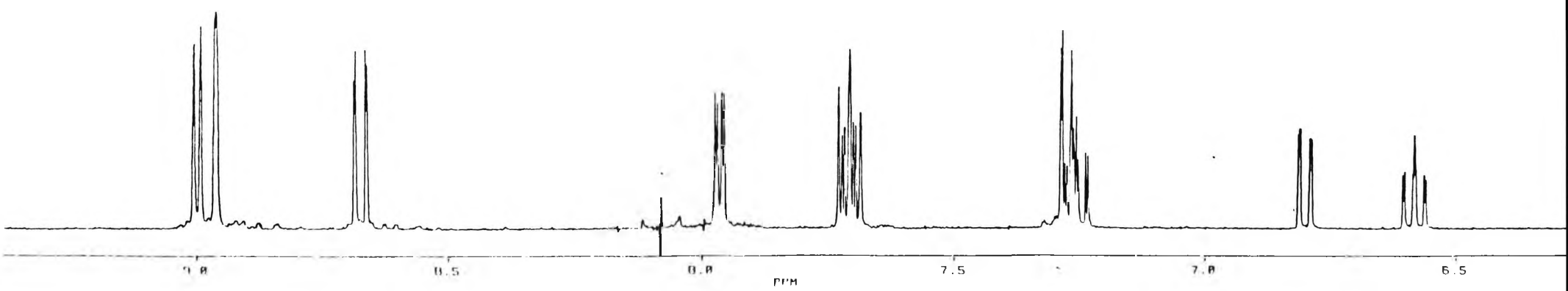
DECOUPLED AT $\delta = 8.9$



DECOUPLED AT δ = 8.7



DECOUPLED AT δ = 8.1



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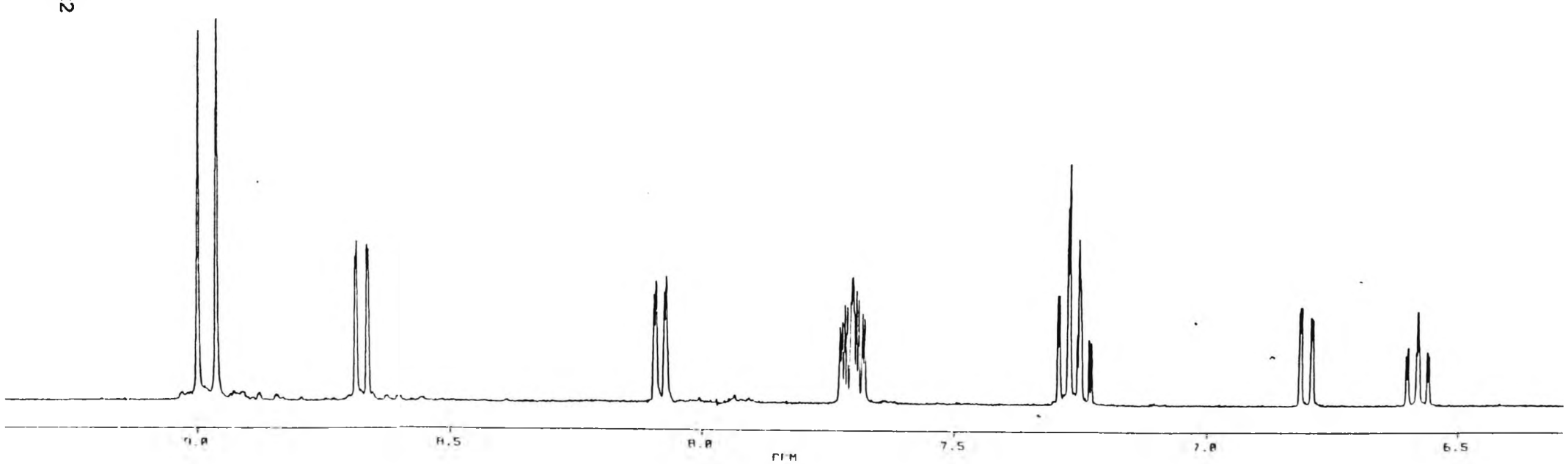

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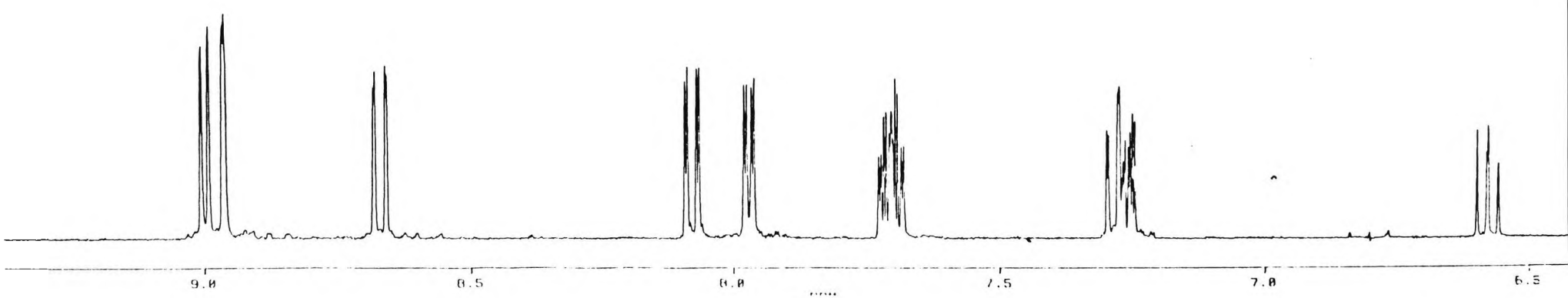

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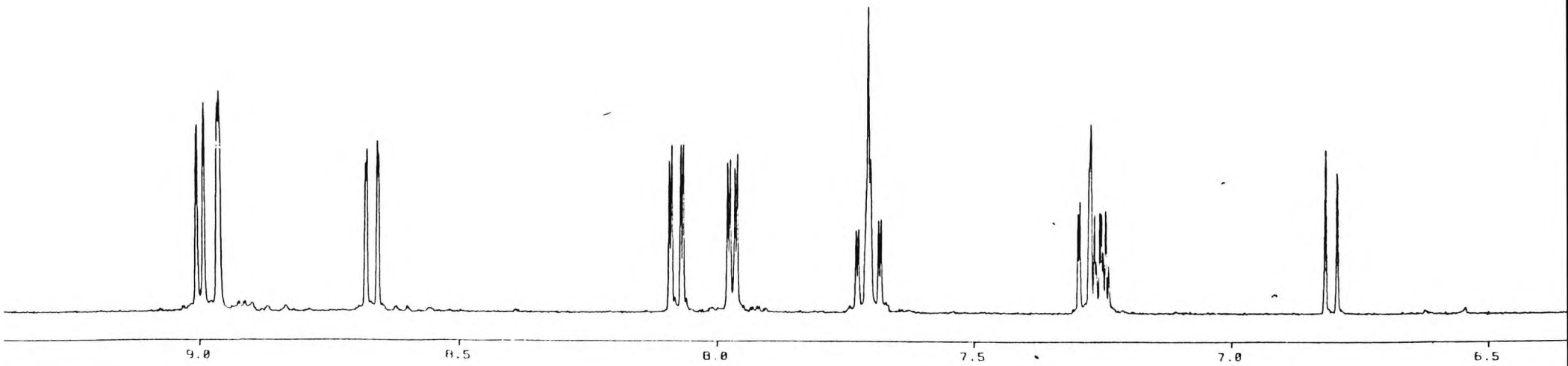

Thesis Summary

Several new ruthenium complex compounds were synthesised that, when mixed with antibody, formed complex-antibody conjugates. The luminescent properties of these conjugates were investigated showing comparable spectra and lifetimes to the unconjugated compounds. The most promising conjugate for trials as a label for an immunofluorometric assay was made from $Ruphen_2phen(\phi SO_2Cl)_2^{2+}$ and IgG. It gave the following luminescent properties.

λ_{ex}	470 nm
λ_{em}	630 nm
τ	1200 ns

EPILOGUE - SCIENCE IS A GODLY ACTIVITY

It may seem strange towards the close of the twentieth century to write about God and science in a chemistry thesis. In earlier centuries, theology and science often joined hands - Newton felt he was thinking God's thoughts after Him - and even in this century Einstein disputed the uncertainty principle on the basis that "God doesn't play dice". My reason for concluding my work is out of gratitude for God's gift of science. Please forgive me if it seems to you out of place.

The early chapters of Genesis are full of words like create and make. God is creating the universe and in particular mankind.

> "So God created man
> in his own image,
> in the image of God
> he created him,
> male and female
> he created them."
>
> Genesis 1:27

This passage is a vital part of the creation account and considers man as the peak of God's creative work. In Hebrew the repetition of a word is a deliberate way of emphasising a point. Hence man, male and female, is singled out as the pinnacle of God's creation. However,

even more significantly, God states in these verses that man is made in his image. This means that man, male and female, should be a representation of what God is like. Man, although not God, has been made to bear an image of what God is like. This image has several aspects, from moral conduct [1] to emotions [2]. For this study the most obvious aspect of God is that he loves to create. Hence man, by nature, will be creative and this is the Bible's explanation as to why art and science are pursued so avidly.

The Genesis account goes further;

> "God blessed them and said,
> 'be fruitful and increase in number;
> fill the earth and subdue it'."
>
> Genesis 1:28

> "The LORD God took the man
> and put him in the garden
> of Eden to work it and
> take care of it."
>
> Genesis 2:15

Here the ideas of man subduing and caring for the earth are stated. The force of these verses is a commission to harness the world's resources. The emphasis in Genesis 2:15 on care shows that whatever subdue means (1:28) it does not mean to waste or to rape the earth.

Much scientific work is devoted to harnessing the world's resources, especially in energy or food production. More recently the need to care for the earth has become a serious issue and so plenty of work will hopefully be done on pollution and other related topics in the future.

Another passage from the second chapter of Genesis is also relevant.

> "Now the Lord God had formed out
> of the ground all the beasts
> of the field and all the
> birds of the air. He brought them
> to the man to see what he
> would name them; and whatever
> the man called each living
> creature, that was its name."
>
> Genesis 2:19

In our culture names are not as significant as in Hebrew culture. To name a person or thing in the Hebrew culture (or many other ancient cultures) was to do far more than label it A instead of B. To name often meant to describe. For instance God named Abram, "Abraham" which means Father of multitudes [3]. In the above passage man is commissioned to "describe" the animals. This also is part of what science is all about - describing what we see.

Hence I would submit, that the Bible teaches that the pursuit of science by mankind has its roots in the way God made man. He has made man in his image to be creative and has commissioned man to harness the world's resources, to care for the earth and to describe what he sees. Science for all its tussles with religion is a truly godly activity.

However, the picture is not complete as Genesis chapter 3 records the fall of man. Here mankind rebels against God's rule and sins against God. Since then man has borne God's image in a fallen way. Good is mixed with evil. Morally man is left bankrupt and in need of forgiveness which is freely offered in Jesus. For this subject it means that science may not always be pursued for good. Creativity can turn to making weapons of terror as well as cures for disease. Harnessing the world's resources can become exploitation and the description of what we see (theories and observations) can be held so arrogantly that the limitation of being human and created is forgotten.

Perhaps a good illustration of this fallen image is seen in the use of atomic energy. Here there is a great harnessing of resources that reflects the creativity and power of God. However, this is sadly so often exploited to make bombs and to terrorise. Carelessness with the

waste, leading to terrible pollution, may also be another tragedy that occurs through selfishness.

In conclusion, the Bible encourages us to pursue science as a gift of God. However, it also warns us to carry it out with care and reverence, avoiding the arrogance of thinking we can "know it all" and the wickedness of using it for evil gain.

Thankfully the Bible tells us that God did not write man off after the fall but in his delight of creating, he began his "new creation" of a people who find his forgiveness and new life in Jesus [4]. To him be glory forever!

References

1. 1 Peter 1:15-16 "be holy in all you do"

2. Isaiah 49:15 "Can a mother forget the baby at her breast and have no compassion on the child she has borne? Though she may forget I will not forget you!"

3. Genesis 17:5 "No longer will you be called Abram your name will be Abraham for I have made you a father of many nations."

4. Romans 8:6 "But God demonstrates his own love for us in this: while we were still sinners, Christ died for us."

ABBREVIATIONS

tBoc or **Boc** = tertiary butyl oxycarbonyl.

FMoc = Fluorene methyl oxycarbonyl.

NMM = N-methyl morpholine.

DMAP = Dimethylaminopyridine.

FITC = Fluoresceinisothiocyanate.

IEMA = Immunoenzymetric assay.

ELISA = Enzyme linked immunosorbent assay.

DCCI = Dicyclohexylcarbodiimide.

DCU = Dicyclohexylurea.

NHS = N-hydroxysuccinimide.

bipy = 2,2'-bipyridine.

bipy$(CO_2H)_2$ = 2,2'-bipyridyl-4,4'-dicarboxylic acid.

phen = 1,10-phenanthroline.

dpphen = 4,7-diphenyl-1,10-phenanthroline.

Ru bipy$_3^{2+}$ = Tris bipyridyl ruthenium 2+

ala = alanine.

lys = lysine.

phe = phenyalanine.

PBS = phosphate buffered saline

DMSO = dimethylsulphoxide

DMF = dimethylformamide

IgG = immunoglobulin G, antibody

THF = tetrahydrofuran

MS = mass spectrum

FABMS = fast atom bombardment mass spectrum

IR = infra-red spectrum

CHN = carbon, hydrogen and nitrogen microanalysis