THE USE OF LONG WAVELENGTH FLUORESCENCE IN THE STUDY OF LIGAND-PROTEIN INTERACTIONS.

By

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To My Parents...

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AIM OF THE PROJECT

Drug-protein binding is of obvious importance both therapeutically and analytically. The aim of this project is to investigate the possibility of studying such interactions using long wavelength fluorescence and all the benefits associated with it.

The research will be carried out in four phases:

a) Investigation of the fluorescence properties of a number of long wavelength dyes.

b) Study of the possibility of using any suitable long wavelength fluorescent compounds as probes for protein. These proteins will be bovine and human serum albumin, α_1-acid glycoprotein and ß-lactoglobulin among others.

c) Determine both quantitatively and qualitatively the binding of these probes to proteins and investigate the possibility of using them in a study of the binding of drugs and other ligands to these proteins.

d) If successful, carry out a preliminary investigation using long wavelength fluorescence and flow injection analysis to produce a rapid and economical way for determining such interactions.

ABSTRACT

The binding of a drug or other ligand to plasma proteins can effect their absorption, metabolism and excretion which can lead to a change in its toxicity and therapeutic action. Fluorescence is a technique that has been used to study such interactions and has the advantages of extreme sensitivity and specificity. Previously fluorescence has been monitored in the UV/vis range of the spectrum. However, a new development is long wavelength fluorescence (600-1000nm), which has the added benefits of a lower background, decreased scattering, decreased photodecomposition and the availability of inexpensive, solid state, optical components.

Certain dyes including polymethines, xanthenes and phenoxazines that fluoresce in the long wavelength region (600-1000nm) of the spectrum were investigated for use as fluorescence probes.

Nile Red, a strongly hydrophobic phenoxazine dye, was found to have an emission wavelength and intensity which was strongly dependent on the polarity of its environment. Consequently, it was bound to certain proteins including bovine and human serum albumin, α_1-acid glycoprotein and ß-lactoglobulin and provided both qualitative and quantitative information on the nature and type of binding site on the protein. It was also used in the study of ligand protein binding interactions in which competition for a binding site on the protein occurs between the probe and other ligands such as drugs or fatty acids.

The project also involved a preliminary investigation into a novel double probe technique for the study of drug-protein interactions and the development of a flow injection analysis method involving gradient titration of a drug against a probe:protein system.

PRESENTATION OF WORK.

1.	April 1991:	Third International Symposium on Pharmaceutical and Biomedical Analysis (Lecture). Boston, Massachusetts, U.S.A.
2.	July 1991:	Research and Development Topics in Analytical Chemistry. Royal Society of Chemistry (Poster). University of Aberdeen, Scotland.
3.	July 1992:	Research and Development Topics in Analytical Chemistry. Royal Society of Chemistry (Poster). University of Birmingham, England.
4.	September 1992:	Autumn Meeting and Pre Doctoral Chemistry Symposium (Lecture and Poster). Trinity College, Dublin, Ireland.
5.	March 1993:	The Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy (Poster). World Conference Center, Atlanta, Georgia, U.S.A.
6.	May 1993:	5th International Symposium on Quantitative Luminescence Spectrometry in Biomedical Sciences (Lecture). University of Ghent, Belgium.
7.	July 1993:	Research and Development Topics in Analytical Chemistry. Royal Society of Chemistry (Poster). University of Bradford, England.
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PUBLICATIONS

1. Biomedical Applications of Near Infra Red Fluorescence Spectrometry. M.B. Brown, J.N. Miller, D.P. Riley and N.J. Seare. *Anal. Proc.*, (1993), **30**, 157.

2. Novel Instrumentation and Biomedical Applications of Very Near Infra Red Fluorescence.
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CHAPTER ONE

INTRODUCTION

1.1 DRUG-PROTEIN INTERACTIONS.

The biological activity of a drug is profoundly influenced by its interactions with protein molecules. Studies on the interactions of drugs with various kinds of sites or proteins including receptor sites in genetic apparatus neurons, transport systems, membranes and enzymes have provided important information for the mechanisms of the pharmacological and therapeutic effects of the drugs.

A number of other molecular interactions between drugs and sites in the biological system, such as tissues and plasma proteins like albumin, α_1-acid glycoprotein and lipoproteins have also been studied a great deal. Such interactions frequently influence the rates at which drugs are absorbed, distributed and eliminated. This in turn effects the rate and duration of action of the drug and hence its therapeutic effect. Sites at which drugs are bound, but do not give rise directly to a pharmacodynamic action are sometimes known as acceptor sites and the convenience with which these types of interactions can be studied has led to their use as models for drug-receptor binding.

Comprehensive reviews already exist on drug-protein binding[1-4], the aim of this part of the introduction is to give an overview of its biological significance. The importance of the more specific areas covered later may then be more easily understood.

1.1.1 Nature of Drug-Protein Interactions.

Drug-protein binding is essentially an interaction between a biological macromolecule, the protein, and a small drug molecule. The interaction is analogous to enzyme substrate binding except that the complex formed does not dissociate to yield a product. There are a few instances

in which drugs are reactive species which form chemical bonds to unite them to a site in an irreversible fashion such as dimethylnitrosamine, penicillin, phenoxybenzamine. However, in the majority of instances the drug forms a non-covalent molecular complex with protein, which involves the establishment of electrostatic, Van der Waals, hydrogen and hydrophobic bonds or a combination of these.

Hydrophobic bonding makes up a major contribution of the interactions of protein with most neutral drugs and is usually significantly involved in the binding of ionic drugs. Hydrophobic bonding is dependent on the solvent (water), in which the reaction takes place. It is broadly defined as the tendency of non-polar molecules, especially hydrocarbons to cluster together in aqueous solution[5]. The basis of the interaction is the entropy gain resulting from the enhanced freedom of the released water molecules and the lower energy environment of the non polar molecules when together.

Since the forces described are universal and will probably to some extent be distributed over all the atoms of any drug, the basis of binding to any site will depend on how these atoms and their forces are arranged in space, compared with those of the amino acids in the protein binding site. Since many of the interatomic forces are only significant at short distances the match may need to be very good and hence very selective. However, the stability of the bonding interaction is not only dependent on the nature of the drug and amino acids concerned, but also by the environment created by the peptide chain in the vicinity of the bonding amino acid. Any external factor which modifies the nature of the binding system e.g., pH[6], temperature and the presence of other molecules like detergents or lipids can also cause changes in the degree of binding.

Another influence on drug-protein binding is that sometimes when bound a drug may induce a conformational change in the protein

molecule, altering the ability of the protein to associate with the drug molecules at other binding sites. This concept is analogous to that of allosteric inhibition for enzyme substrate binding[5] and may explain why although there are many areas of amino acids which are possible binding sites for both anionic and cationic drugs, the actual number of primary (high affinity) and secondary (low affinity) are few. This is supported by the fact that normal drug-protein complexes are formed between milliseconds and microseconds, the rates of which have been studied by modern high speed relaxation methods. However, if a change in the conformation of the protein is involved binding may take several seconds or even minutes.

1.1.2 The Effect of Drug-Protein Interactions on Absorption.

Many drugs administered orally are insoluble in water and if it were not for interaction with the plasma proteins like albumin, globulins and lipoproteins they could not be transported in the blood. Most drugs remain mainly in their ionised forms at all levels in the gastrointestinal tract, and the pH of the plasma water is at a level to prevent bases from penetrating the intestinal wall. The reversible binding of the drugs to the plasma proteins assists the transfer of drug molecules across from the intestinal lumen into the blood because a large concentration gradient is maintained between the drug at the absorption site and that in the plasma.

1.1.3 The Effect of Drug-Protein Interactions on Drug Distribution.

Whatever the route of administration, almost all drugs reach their receptors via the systemic circulation. The capillary wall or membrane, through which the plasma is in contact with the tissue fluids is therefore

the initial barrier to drug distribution. Since only unbound drug can diffuse through the capillary membrane, drug binding to plasma proteins influences the distribution of the drug within the body and hence its effect because the concentration of the drug in the extravascular space is only equivalent to that of the free drug in the plasma. However, it is by no means certain that a protein bound drug is therapeutically and toxicologically inactive. For example, some drugs bound to serum albumin often cause the production of antibodies when they are injected into animals which implies that they are still interacting with receptors. In addition, if a drug is highly plasma protein bound at a concentration where the sites are not saturated, the protein bound drug can act as a reservoir[7,8], replacing by dissociation any free drug lost in metabolism and excretion. Thus maintaining the concentration of the free drug at a therapeutically useful level, over a varied range of the total concentration of the drug in plasma. Plasma protein binding also reduces the concentration of some drugs below a toxic level making the drug safe for therapeutic use.

As already stated the greater the binding to plasma proteins the lower the concentration of free drug distributed at equilibrium between the tissue and plasma. It has also been shown[7] that strongly plasma bound drugs seem to have a low volume of distribution and that for drugs with a high value of $K_a(>10^4M^{-1})$ there exists a dose range within which a small increase will produce a disproportionately large increase in the concentration of the free drug. This is due to the fact that all the binding sites have become saturated. This model is highly simplified but it shows that there is only a narrow controllable therapeutic dose range for highly bound drugs. However as with some other studies[9,10] on the effects of drug protein binding it assumes that drug-protein complexes are completely and rapidly reversible and that they only occur in the intravascular systems, neither of which may be the case[2].

It is known that some of the interactions that occur in plasma also occur in the tissue. Both albumin and ligandin have affinity for drugs in the tissue, in fact 55-60% of the total exchangeable albumin is located outside the plasma. In addition, some planar aromatic drugs like chloroquine are found to bind to melanin granules in the eye and skin; planar drugs like chlorpromazine combine with DNA by intercalation between the base pairs of the double helix; lipid soluble drugs are known to become localised in adipose tissue and several drugs persist in the lungs long after they have left the rest of the body[11]. Therefore, it is not only plasma binding which affects the distribution of the drug.

1.1.4 The Effect of Drug-Protein Interactions on Metabolism.

The main organ concerned with metabolism is the liver[12], although the kidney, lungs, intestinal mucus, plasma and nervous tissue also contain important drug metabolizing enzymes. The enzymatic changes undergone by the drug in these parts of the body usually results in the loss of its pharmacological activity. The term detoxicification describes the result of such metabolic changes. However this is not the only possibility, a pharmacologically active metabolite may be formed from an inactive precursor or prodrug, or the metabolites of a drug may have a different type of action to that displayed by the administered drug.

Generally the metabolism of a drug decreases its lipid solubility by making it more polar and therefore more hydrophilic, thereby facilitating its excretion. Drugs which are water soluble but not lipid soluble are more usually excreted unchanged in the urine and have only a short lifetime in the body. While drugs that are lipid soluble and are not metabolised at all or at any appreciable rate have a long persistence in the body.

The influence of drug-protein binding on metabolism is uncertain as it is not known whether free or total drug is available for metabolism for a lot of drugs[2]. A theoretical model of the effect of drug-protein binding on metabolism was put forward by Gillette[1]. He suggested that if the activity of the metabolizing enzyme is so high that nearly all the drug is cleared from the blood as it passes through the liver e.g. propranolol[13], then an increase in binding of the drug to plasma proteins will result in an increase in its metabolism by increasing the rate at which it is carried to the liver. Thus the plasma proteins have a transport function. However, if little of the drug is metabolised in the liver an increase in binding will have little effect on its metabolism i.e. protein binding has a storage function. If, as is likely, that some drugs depend on a passive uptake mechanism and only the free drug will be available for the metabolism, protein binding will have a protective role for the drug[14].

1.1.5 The Effect of Drug-Protein Interactions on Excretion.

The main route of excretion of drugs and drug metabolites is in the urine via the kidneys, although excretion can occur in the faeces and sweat. The two main processes involved in renal filtration are glomerular filtration and tubular filtration. Glomerular filtration relies on the passive diffusion of unbound drugs across the glomerular membrane. Plasma proteins do not pass through the membrane but free drugs are readily filtered and so the concentration of drug in the glomerular filtrate generally equals the free drug concentration in the plasma. For this reason plasma protein binding has a retaining effect.

Tubular secretion is primarily an active process and unlike glomerular filtration it is not limited by protein binding. Only free drug can be secreted by the tubules, however the drug-protein complex dissociates rapidly and any free drug secreted is replaced almost instantaneously by

free drug from this dissociation. As a result of this, even drugs which are 90% bound to a plasma protein can be removed completely by tubular secretory mechanisms in the kidney[15]. Drug-protein binding therefore accelerates the elimination of a drug, which is excreted by this method, as it increases the plasma drug concentration and carries the drug to the sites of secretion.

1.1.6 The Clinical Significance of Drug-Protein Interactions.

The effect of drug-protein binding on the activity of the drug is varied and is dependent on the pharmacokinetic properties of the particular drug. For instance, it is known that drug-protein binding inhibits the activity of a drug but also depending on how the drug is eliminated, protein binding can increase or shorten the duration of action of the drug. However, it is generally agreed that only the free drug or the free drug metabolite concentration in the plasma would be expected to correlate with the expected therapeutic and toxicological effects of the drug. It is therefore necessary when designing drug therapy to study the free drug and bound drug concentrations from the initial dosing to the final elimination of the drug. In cases where only the total plasma level of the drug is known decreased binding will increase the concentration of free drug and it may fall within the toxic range. For this reason, determination of the extent of binding is necessary for drugs with a narrow therapeutic range like digitoxin or where the metabolism varies in individuals as with amitriptyline.

1.1.7 The Competition of Drugs for Binding Sites.

The plasma proteins that bind drugs may have more than one binding site. The sites that avidly bind one drug may be different from those that

bind another and so not all highly bound drugs displace one another from proteins. Even so, when two drugs bind to the same site, displacement will depend on the affinities of the drugs for the binding sites and also on the concentrations of the competing drugs[2]. The drug with the larger association constant may displace the other drug, but the binding of both drugs will be decreased. The displacement results in more of the displaced drug being available for diffusion to its site of action and other tissues leading to an increase in its volume of distribution and pharmacological effect. The rate of elimination may also be increased since a larger fraction of the drug is available for metabolism and excretion[8]. The displacement is more likely to be clinically significant and sometimes produce a toxic effect, when the drug displaced is highly protein bound. Consider two drugs, warfarin which is 97% bound and phenobarbital which is 50% bound to plasma protein. If 3% of the Warfarin is displaced from its receptor the amount of free warfarin increases two fold from 3 to 6%. If 3% of phenobarbital is displaced however it increases to 53% a change that is inconsequential. In fact if phenylbutazone is administered to patients already treated with warfarin, severe hypoprothrombinemia and bleeding may result due to the increased action of the displaced warfarin[16]. However, displacement reactions can also be used beneficially, for example some sulphonamides are known to compete with warfarin for binding sites. The resultant potentiation of toxicity of warfarin is put to use by combining it with a sulphonamide in rodenticide preparations[17]. Also the antirheumatic activity of certain drugs may be mediated through their ability to displace plasma bound tryptophan and other small peptides[18].

Drug displacement interactions can also occur between compounds which bind to different sites on the protein. This is known as non-competitive binding inhibition and takes place when the drug binding to the protein causes a conformational change in the protein structure modifying other binding sites on the protein perhaps rendering them

inactive. One such example of this is warfarin which acetylates residues of albumin and therefore inhibits the binding of drugs such as flufenamic acid and phenylbutazone[19]. Non-drug substances can also cause inhibition of drug-protein binding, fatty acids inhibit the binding of many acidic drugs to albumin[20], while bilirubin effects albumin and inhibits sulphonamides[8].

As the apparent volume of distribution of most drugs far exceeds the plasma volume, the bulk of the total drug must be in the tissue. Tissue binding displacement will give rise to an increase of the free drug in the tissue which will diffuse into the plasma. This will cause an equilibration of the displaced drug and there will be an increase in both free and bound concentrations in the plasma. As the volume of the plasma is so small, the distribution of the displaced drug in the plasma cannot buffer the impact of increased amounts of free drug in the body. For the majority of drugs, then, tissue binding displacement approximates to a reverse of the effects seen with plasma protein displacement.

1.1.8 The Influence of Disease on Drug-Protein Interactions.

Many disease states affecting drug-protein binding have been discussed, renal failure[21,22], liver disease[23], nephrotic syndrome[24], thyroid disease[25], and diabetes[26] are just a few mentioned in the literature. The common factor in these disease states is that they reduce binding to plasma proteins. Alteration in binding may involve the binding protein, the drug or the conditions required for binding.

Hypoalbuminaemia is associated with all the above diseases and is characterised by a decrease in the plasma albumin concentration which results in a decrease of bound drug. Uraemia is also associated with decreased protein binding of certain weakly acidic drugs such as

salicylate, warfarin and sulphonamides. The defect is related somewhat to decrease in plasma albumin but cannot be accounted for by hypoaluminaemia alone and is probably due to certain metabolites like urea and uric acid which would not be ordinarily present in healthy individuals binding to albumin and interfering with the acidic drug binding sites. The phenomena of decreased drug binding is only important for highly bound drugs.

Basic drugs also bind to lipoproteins and α_1-acid glycoprotein (α_1-AGP). The lipoproteins show large fluctuations due to both physiological and pathological conditions. Hyperlipoproteinaemia is known to occur with hypothyroidism, liver diseases and alcoholism. α_1-AGP, the acute phase protein is elevated in certain situations and diseases, like stress, heart disease, hepatic damage, malignant tumours and acute and chronic inflammatory disease[27], while in hepatic disease, pregnancy, nephrotic syndrome and malnutrition it decreases[28]. Associated with these changes in binding proteins, both increases and decreases in the plasma protein binding of basic drugs have been recorded[29].

Another explanation for the variations in protein-drug binding during disease is that of pH. Variations in plasma pH, that occur in disease could influence the protein binding site or in fact the drug itself. For example, it is known that the affinity of basic drugs to α_1-AGP increases with increasing pH[30,31]. Also for weakly acidic drugs like sulphonamides, the presence of a negative charge on these molecules (in an alkaline medium) adds an electrostatic bond to the hydrophobic bond between the two molecules and again results in an increase in their affinity. Generally, acidosis and alkalosis produces changes in the duration and intensity of action of a wide range of drugs.

Physiological inhibitors of drug-protein binding also occur in the diseased state. Palmitate and oleate are the major free fatty acids in

human plasma, the concentrations of which increase as a result of renal failure, stress and fasting[32]. At high enough concentrations these fatty acids are known to inhibit the plasma protein binding of certain non-steroidal anti-inflammatory drugs, antibiotics, hypolipidaemic agents and oral coumaric anticoagulants[33].

1.2 THE STUDY OF DRUG-PROTEIN INTERACTIONS.

1.2.1 The Significance of Drug-Protein Interaction Measurements.

The information obtained from binding measurements i.e., binding constants and the nature and number of binding sites, provide a broad foundation for the understanding of the theoretical aspects of drug binding to proteins and therefore help in recognizing any therapeutical and pharmacological consequences it may have.

The vast majority of studies reported on drug-protein binding are in-vitro investigations. This is not ideal because the conditions used in-vitro can never be comparable to that of which actually occurs in the body. Often isolated proteins are considered alone and the calculation of kinetics based on pure systems alone may poorly reflect the in-vivo events that involve competition between multiple drugs, metabolites and endogenous substances all over the body. Also, complexities in interactions, unknown to in-vitro studies do arise in the body which may radically alter the drug-protein binding interaction. For example, in-vivo, induction or sensitization to future binding as a result of prior contact with a drug may influence the conformation of a binding protein to a more active shape. A drug, or perhaps a drug-protein complex may influence the protein synthesizing machinery in such a way that it induces synthesis of a more or lesser amount of a particular binding protein. Also, in-vitro, the concentrations of drug or protein may be different to those in-vivo,

and so binding at receptors may occur which would never be apparent in the body. For example, at high concentrations of protein, dimerisation may occur and produce hydrophobic bonding sites which would not occur at lower concentrations.

Despite the problems involved in vitro studies they continue to provide invaluable information of drug-protein interactions which is the keystone to understanding the behaviour of drugs in living animals. However, in attempting to apply the in vitro findings to drug kinetics in vivo, the complexities involved need to be examined and investigated.

1.2.2 Methods of Studying Drug-Protein Interactions.

The methods which can be used to investigate drug-protein binding cover a diverse range of chemical and physical techniques. The choice of method depends on the concentration of the component/or components at equilibrium which is/are to be measured. There are many techniques that have been devised to study drug-protein interactions and they can generally be divided into two main groups. Classical techniques which involve the determination of the concentrations of free and bound drug and spectrophotometric techniques where a change in physical property of the protein or drug is detected.

CLASSICAL TECHNIQUES.

The most common classical techniques are equilibrium dialysis, ultrafiltration and gel filtration[34]. The only difference between any of these methods is the way that the physical separation of the larger particles of protein and the complexes from the smaller drug particles is achieved. The separation is usually obtained by use of a barrier or

membrane that has pores large enough to allow the passage of drug molecules but not the larger protein and drug-protein molecules. The methods are described in brief below.

Equilibrium Dialysis.

With this technique the separation is achieved by the use of a semi-permeable membrane. The membrane is only permeable to the free drug molecules (Fig. 1.1) and their passage through is solely due to a concentration gradient[34]. After an equilibration period, the concentration

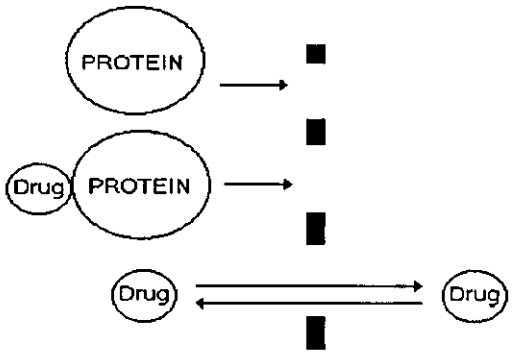


Fig. 1.1: Behaviour of protein molecules, drug molecules (D), and drug-protein complexes toward a dialysis membrane.

of the free drug in the buffer compartment will equal that in the macromolecular protein solution. By measuring the free drug concentration on either side of the membrane the amount of bound drug can be determined by subtraction from the total drug added to the reaction mixture. Equilibrium dialysis is the most commonly used method because it is simple, inexpensive and it approximates to in-vivo conditions, the conditions of which can always be regenerated. However, there are drawbacks, for example in-vivo, the binding process occurs within a fraction of a second, but with this technique, depending on the drug, equilibration can take many hours and even with the developments to decrease the time involved for equilibrations[35] the method is still time

consuming. The prolonged time for the method can also result in adsorption of the drugs by the dialysis membrane, drug degradation, protein denaturation and microbial growth in the buffer[36]. There is also the problem of the "Donnan Effect"[37] which may cause a difference between drug concentration in the two compartments and is mostly related to drugs that are both ionized and slightly bound. In addition, it is sometimes necessary to perform many separate dialysis experiments to obtain sufficient binding data and this can prove tedious.

Ultrafiltration.

Like dialysis, ultrafiltration uses a semi-permeable membrane which allows the passage of water and drug molecules only (Fig. 1.2).

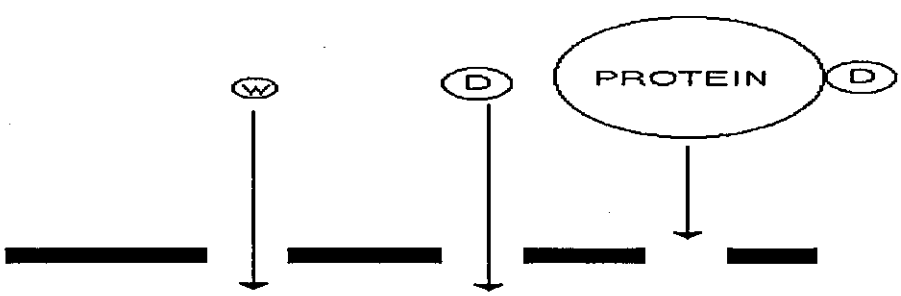


Fig. 1.2: Behaviour of water molecules (W), drug molecules (D), and drug-protein complex at an ultrafilter membrane.

However, with this technique preparation of a protein free solution is achieved by forcing the reaction mixture through the membrane under pressure. This pressure can be from a compressed nitrogen source[38], centrifugation and even by a vacuum[39]. Ultrafiltration is probably not as common as dialysis because although it has the advantage of being economical with time and materials its problems are numerous. The major problem of ultrafiltration is the 'sieve effect'[36]. This is caused by the fact that although there are pores wide enough for the passage of

free drug molecules, there are also smaller pores allowing just the passage of water molecules. Thus, with time more water molecules will be filtered than drug molecules, hence the drug concentration in the ultrafiltrate becomes lower than in the unfiltered solution. This effect may be reinforced if protein molecules partially obstruct the pores, hindering the passage of drug molecules but not the water molecules. The error caused by the 'sieve effect' increases with increasing pressure of filtration and therefore the pressure should be kept as low as possible to minimize its effect. Efficient stirring can also help the problem as this will remove the polarization of the protein on the membrane. The uptake of drug molecules by the membrane and the Donnan Effect also cause problems with this technique.

Gel Filtration.

Separation in gel filtration is achieved by the basic principle that gel particles are permeable to small molecules but not to macromolecules. The gel structure contains pores of varying diameter up to a maximum size. The sample is washed through a column of the gel and molecules larger than the largest pores in the gel are excluded from the gel structure. Smaller molecules, however, penetrate the gel to a varying extent depending upon their size and this retards their progress through the column. Thus, the proteins and the drug-protein complexes appear first in the eluate whereas the free drug molecules follow later. There are a wide variety of products available for the gel column, Sephadex (cross-linked polydextrans), Biogel (cross-linked polyacrylamides), agarose gels and porous glass beads are all used for biochemical work[40].

Gel filtration has been used infrequently for the study of drug-protein interactions because it has a number of problems associated with it. For example, specific interactions of certain drugs with the gel, together with calculating the binding constant from results obtained as peaks and

troughs make it difficult to use. In addition, it is of no use for weak drug-protein complexes and with small samples as large sample volumes are necessary. It is also sometimes necessary to make a preliminary study to find the optimal conditions for each drug. However, if all the conditions are already known, gel filtration can be faster than equilibrium dialysis for high molecular weight drugs[36].

Determination of Free Drug.

The above techniques simply involve the physical separation of bound and free drug. The subsequent calculation of free drug can be achieved by a number of methods regardless of what method of separation was used. The most common method involves the use of radiolabelled drugs. For example, in equilibrium dialysis, tracer amounts of the radiolabelled drug are added on either side of the membrane but more usually on the buffer side. After equilibrium has been reached the free fraction of drug can be calculated, from liquid scintillation counting. Although this method is very sensitive and simple, it is fairly expensive and adds the complication of radiation and all the necessary safety requirements. For those drugs which strongly absorb in the ultraviolet/visible regions, absorbance measurements can be used, although this method lacks sensitivity. Other spectroscopic methods and enzymatic techniques have also been used[35].

SPECTROSCOPIC METHODS.

The second group of methods used in drug-protein binding determinations are those of the spectroscopic techniques. Binding of the drug to a protein will often be accompanied by a change in the spectroscopic property of the drug or protein, the extent of the change is often a measure of the bound drug or protein. These techniques

require no physical separation of the bound and free drug and are therefore referred to as homogeneous or direct methods unlike the classical methods which are the heterogeneous or in-direct methods.

Compared to the classical methods available, spectroscopic methods have a number of distinct advantages:

1. They are quicker than classical methods as no separation is required and therefore the long equilibrium periods are unnecessary.
2. Measurements are performed on the whole reaction mixture that is, the drug-protein complex in contact with the free drug and the protein at equilibrium.
3. Not only can they be used to measure the amounts of free and bound drug in a given system but also they provide information on the nature and type of the drug-protein interaction.

Most spectroscopic methods can be adapted for drug-protein studies and only the common ones will be discussed.

Nuclear Magnetic Resonance (NMR) and Electron Spin Resonance (ESR).

When two electrons are present in the same atomic orbital they have opposite spins and as a result neutralize any magnetic effects each might have. In a similar manner, pairs of protons in the nucleus of an atom do not show any overall magnetic effect. However, an unpaired proton or an unpaired electron will impart a magnetic moment to the species which will then act in a manner comparable to a magnet and will interact with an applied magnetic field. Such interactions form the basis of NMR and ESR spectroscopy.

Due to their relatively high abundance and good sensitivity for detection,

protons are the nuclei most commonly used in NMR and therefore in drug-protein studies. The unpaired proton will assume one of two possible positions when an external magnetic field is applied, in the same direction as the magnetic field (low energy state) or directly against the magnetic field (high energy state). The difference in energy levels will not only depend upon the nucleus but also on the magnitude of the magnetic field applied. In such a situation there will be a majority of nuclei in the low energy state, the equilibrium proportions depending upon the difference in energy levels between the two states. Transitions between these two states may be induced by the adsorbent radiation usually from the short wavelength region of the spectrum and this is how NMR is observed[34].

Electron spin resonance spectroscopy is comparable to NMR spectroscopy but depends on an unpaired electron providing the magnetic moment. However, the fact that an electron is smaller than a proton results in a magnetic moment which is greater and microwaves rather than lower energy radiowaves are necessary to induce the transition. However, in a drug-protein complex there is no group bearing an unpaired electron and so often a nitroxide free radical is covalently bound to the drug[41] and is known as a spin label or probe.

NMR and ESR provide a wide range of information on the nature, structure and conformation of drug binding sites on the protein. However, the intricate nature of their spectra makes interpretation difficult and the stoichiometry of the binding is often unavailable from the data taken and must be obtained from other techniques. In addition, with ESR spectroscopy, care must be taken that the spin labelled drug is binding to its correct site and that the native state of the protein is not significantly changed with the addition of the extra spin label[40].

Optical Rotary Dispersion (ORD) and Circular Dichroism (CD).

The phenomena of optical activity has been known for more than a century. In the absence of a magnetic field, optical activity is supported in a molecular sample only if the constituent molecules lack mirror symmetry, particulary from the presence of asymmetric carbon atoms and from the effect these atoms have on nearby chromophores. Optical activity can be defined in a general sense as the differential interaction with left versus right circulary polarised light[42]. Of the various forms of optical activity, the two best known are Optical Rotary Dispersion (ORD) and Circular Dichroism (CD). ORD is defined as the spectrum of the angle of rotation of a plane of polarized light that is passed through the sample. The rotation is due to a difference in the index of refraction for light having left versus right circular polarization i.e., $n_L = n_R$ where the angle of rotation is proportional to $\Delta n = n_L - n_R$. CD on the other hand is defined as the spectrum of $\Delta\epsilon = \epsilon_L - \epsilon_R$ where ϵ_L and ϵ_R are the absorption coefficients of left and right circular polarized light respectively. It can be demonstrated that the complete CD and ORD spectrum for a molecule are related by a set of integrals known as the Kronig-Kramers transformations[40]. These equations imply that if the ORD is known at all wavelengths than the CD spectrum can be calculated and vice versa.

The ORD and CD of samples depend strongly on the wavelength of light used to perform the measurement. For absorbing samples, the ORD and CD are typically determined over the same wavelength range used to record an absorption spectra. In fact, if the sample contains only strongly allowed electronic transitions, the shape of the CD spectrum (often called a Cotton Effect[43]) can be directly related to the absorption spectrum. Originally ORD was the preferred experimental technique since it could be measured in all parts of the spectrum while CD can only be observed directly in the absorption band. However, recent improvements in instrumentation has elevated the measurement of CD

to a favoured status over that of ORD. CD is preferred because it is much easier to interpret than ORD as with CD the measurement of $\Delta\epsilon$ is related only to the transition properties of the molecule at that wavelength. With ORD however the transition properties exist throughout the entire spectrum.

Circular Dichroism can be used to study drug-protein interactions when the drug involved is optically active, particulary those of natural origin. The drug may show a change in CD upon binding to the protein, either because of electronic interactions with its binding site or because it may undergo a conformational change when it binds. These changes can be detected easily because most common proteins have no CD for visible light. It is especially convenient to study optically inactive drug molecules. Here an induced CD will occur on binding for the same reasons mentioned for the optically active drug molecule. However, CD measurement in the absorption band of the small molecule will reflect properties of the bound protein.

On the whole the use of ORD and CD for drug-protein binding studies has been mainly used to provide information on the three-dimensional confirmation of binding sites and protein. However, in some cases, quantitative calculations of the binding characteristics can be obtained.

1.3 FLUORESCENCE.

The application of fluorescence techniques to the study of drug binding to proteins has been extremely successful for both quantitative and qualitative characterisation of binding. The fluorescence of the drug and/or protein will be modified when bound. This change in fluorescence will normally be characterised by excitation wavelength, emission wavelength, quantum yield, fluorescence lifetime and degree of polarization. If such changes occur it is

possible to evaluate the extent of binding and the nature of the interaction. If neither the drug or protein is fluorescent, the interaction can be measured by using a suitable covalent label or a probe, both of which will be discussed later.

In order to understand the sensitivity and applications of fluorescence to the study of drug-protein interactions a general overview of the theory of fluorescence will be given.

1.3.1 The Theory of Fluorescence.

A hot body that emits radiation only because of its high temperature is said to show *incandescence*. Any other form of light emission is called *luminescence*. With *luminescence*, for emission to be continuous there must be some source of energy supply. It is in this area where the different types of *luminescence* are classified. With *radioluminescence* the energy is supplied by a radioactive source, with *electroluminescence* it is an electric current. Energy from a chemical reaction is the source for *chemiluminescence*, when the reaction takes place in a living system *bioluminescence* occurs. If the energy is supplied by absorption of infra red, ultraviolet or visible light then the process is called *photoluminescence* which is what occurs in any fluorometric analysis.

In the last year, of the last century, Planck[44] advanced the revolutionary hypothesis that radiant energy can only be absorbed in discrete units or quanta. The energy carried by a quantum of radiation was proportional to the frequency of oscillation, and the two were related by the equation,

$$E = h\nu$$

where:

E = Energy (Joules)

v = frequency (Hz)

h = Planck's Constant (6.62×10^{-34} J/s)

which can be adapted to,

$$E = h \frac{c}{\lambda}$$

where:

λ = wavelength (cm)

c = speed of light invacuo, $2{,}9998 \times 10^{10}$ cm/s

The various regions of the electromagnetic spectrum (Fig. 1.3) can now be discussed in terms of wavelength or energy.

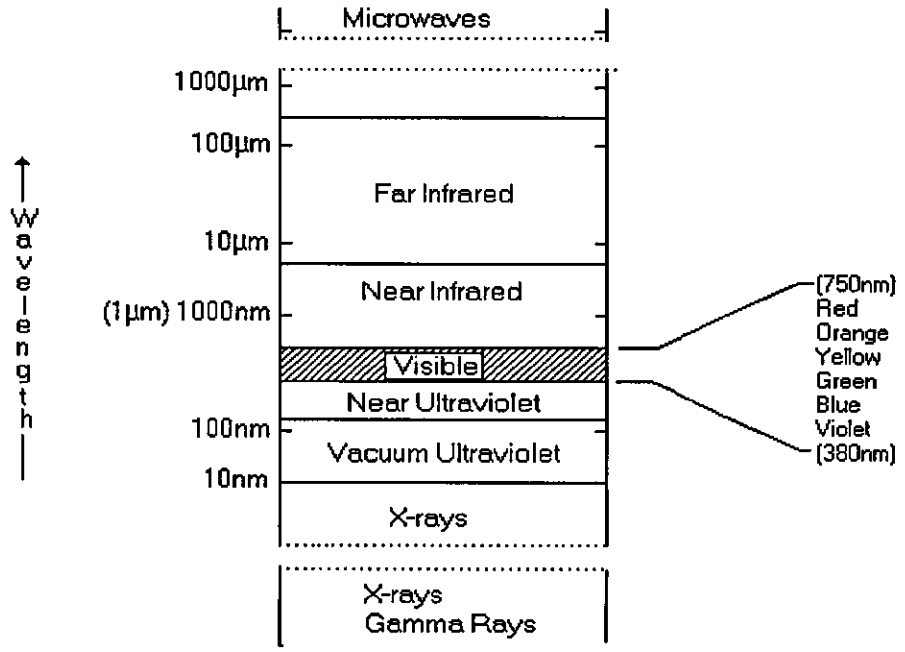


Fig. 1.3: The Electromagnetic Spectrum.

At the top are microwaves and radiowaves both with very long wavelengths and very small energies. These are followed by the infrared region which consists of both far and near infra red subregions. After the infrared comes the visible region (light), the radiation perceived by the human eye, which occupies only a very small range in the electromagnetic spectrum. On the other side of the visible region is the ultraviolet region, followed by X-rays and gamma rays both with very short wavelengths and high energies (Fig. 1.4).

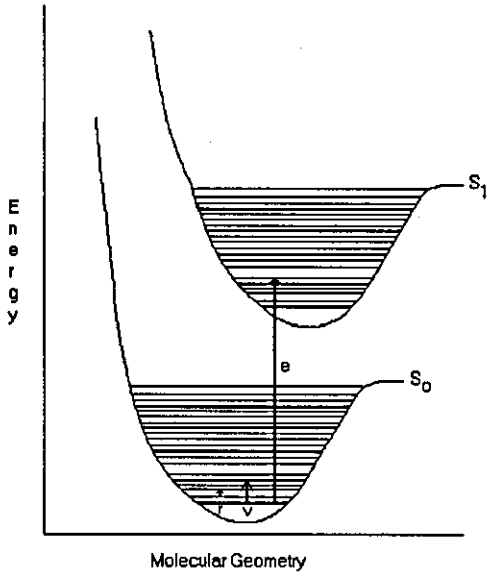


Fig. 1.4: Energy levels of small molecule. Transitions corresponding to electronic (e), vibrational (v), and rotational (r) spectra are indicated.

Fig. 1.4 shows a section through the potential energy surfaces of the two lowest electronic states of a typically simple molecule. Superimposed on each of these states is a series of vibrational levels that, are themselves divided into rotational levels. The energy spacing between the lowest rotation-vibration states of the two electronic states S_o and S_1 is much greater than the thermal energies at room temperature[45]. Therefore, in the absence of radiation, that excite a transition, all molecules are in the lowest electronic state S_o. The energy difference between the vibrational levels is fairly large and so the lowest vibrational level of S_o can be considered to be the only one appreciably populated. However, the

rotational energy spacings are small and so many rotational levels are populated. The process by which a molecule reaches the excited state by photon absorption is referred to as photoexcitation or excitation. In this process, radiation is absorbed, resulting in an n-π^* or π-π^* transition[46].

The spin of the excited electron in such a transition is also important. In all systems electrons have spins of either -½ or +½. Molecular species in the ground state generally have an even number of electrons, at least two electrons are needed for each covalent bond. In addition, the spins of these electrons are paired, one electron of the pair has a spin -½ and the other has a spin of +½, so the net spin is zero[47]. Such a state is called a singlet state and is symbolised as S_o. In the excited state, the excited electron in the molecule retains its spin and is still paired with the electron in the ground state (Fig. 1.5). A given molecule may have several excited states symbolised as S_1, S_2 etc.

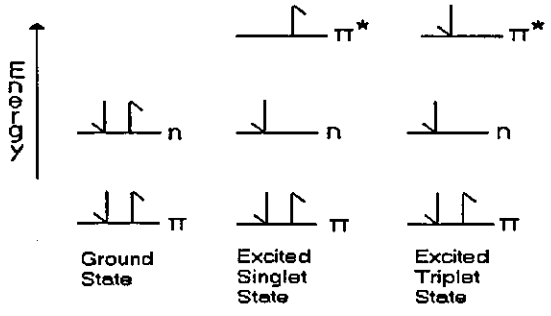


Fig. 1.5: Energy level diagram for the ground state and for the excited singlet and triplet states resulting from an n→π^* transition. Each arrow represents an electron; the direction of the arrow indicates the spin direction.

The amount of light absorbed was found to follow the Lambert-Beer Law[48],

$$A = a\ b\ c$$

where:

A = absorbance

a = absorptivity constant

b = path length of light through sample (cm)

c = concentration of absorbing species

If the Lambert-Beer law is formulated using molarity to express concentration then:

$$A = \epsilon\ b\ c$$

where:

ϵ = molar absorptivity, which like 'a' is characteristic of a chemical species at a given wavelength ($Lmol^{-1}cm^{-1}$)

It would be expected that the absorption/excitation process would result in a set of definite absorption bands as the energy is absorbed as a simple quanta. This is not the case however as it is not possible to resolve the bands and normally a broad absorption spectra results.

Having absorbed energy and reached one of the higher vibrational levels of the excited state, the molecule quickly loses this excess energy by collision and falls to the lowest vibrational level of the excited state. The non-radioactive process of losing energy by collision is known as internal conversion and is also how molecules in a higher excited state drop to a lower one. They pass from the lowest vibrational level of the upper excited state to a higher vibrational level of a lower excited state which has the same energy. From here the process is repeated until the lowest vibrational level of S_1 is reached. It is only from this level that the

molecule can return to any vibrational level of the ground state by emitting a photon in the form of fluorescence (Fig. 1.6). However, there are many non-radiactive processes that compete with fluorescence to depopulate this excited state including internal conversion, photodecomposition, quenching and intersystem crossing.

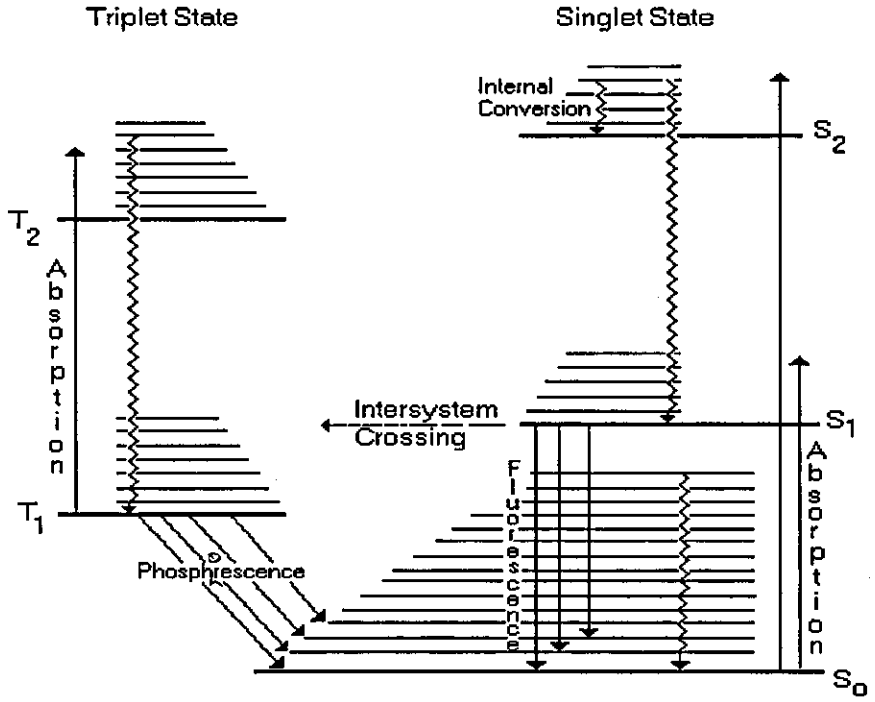


Fig. 1.6: Transitions giving rise to absorption, fluorescence and phosphorescence.

The phenomena of intersystem crossing involves a transition from the singlet to the triplet state as shown in Fig. 1.6. A triplet state is another type of excited state, the spin of the excited electron is not paired with the spin of the electron with which it is paired in the ground state (Fig. 1.5) hence the net spin is not zero. The quantum theory[46] shows such a molecule can exist in three forms (hence triplet state, T), each form having a slightly different but indistinguishable energy. According to Hund's rule for atoms[45], electrons with parallel spins are lower in energy than a corresponding pair with opposite spins. By analogy, a triplet state in a molecule will have a lower energy than the corresponding singlet

state. This drop in energy to the triplet state suggests another way by which the energy of the excited state may be dissipated. Intersystem crossing can occur in any substance where the lowest vibrational level has the same energy as the upper vibrational level at the triplet state T_1.

A radioactive transition from the lowest vibrational level of T_1 to S_o is forbidden since it involves a change in multiplicity[45]. However, as with intersystem crossing although unlikely the transition can occur. The process of transition from $T_1 \rightarrow S_0$ with the emission of a photon is known as phosphorescence. Due to the relatively long lifetime of the triplet state, molecules in this state are much more susceptible to radiationless, deactivation processes. For these reasons phosphorescence at room temperature is rare, but by dispersion of the sample in a rigid matrix or freezing the solution to a low temperature enables phosphorescence to be observed from many compounds[46]. The decay times for the processes mentioned associated with fluorescence are shown in Fig. 1.7.

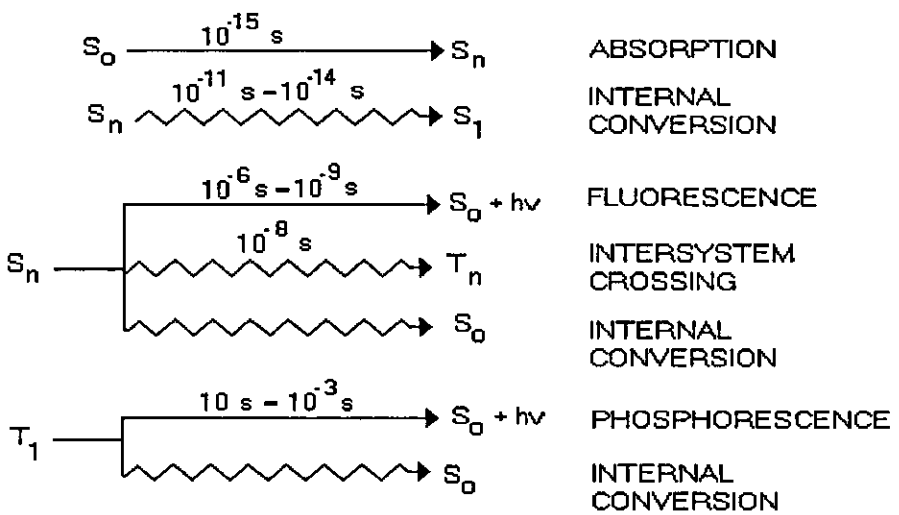


Fig. 1.7: Decay times for processes associated with fluorescence.

These processes obviously affect the efficiency of fluorescence as they limit the number of excited molecules returning to the ground state which fluoresce. This efficiency is known as the Quantum Efficiency[49] (QE) and is defined as:

$$QE = \frac{number\ of\ quanta\ emitted}{number\ of\ quanta\ absorbed}$$

The theoretical range for quantum efficiency is from zero to one, but in practice it ranges from about 0.01 to 0.9.

The fact that the QE efficiency is never one explains the Stokes' Shift associated with the molecules. The Stokes' Shift of a molecule is the difference in wavelength between its excitation and emission maxima. As energy is lost upon excitation before fluorescence occurs the emission wavelength will be of lower energy, hence higher wavelength compared to the excitation. This is supported by the fact that an even larger Stokes' Shift is associated with phosphorescence where even more energy is lost. However the Stokes' Shift for a molecule can be changed when necessary as a molecule can be excited away from its maxima. This is possible because fluorescence always occurs from the lowest vibrational level of S_1 to S_o. Thus, the shape and maxima of the emission spectrum will be independent of the excitation wavelength, only the fluorescence intensity of the emission spectra will be affected.

A plot of emission intensity against wavelength for any given excitation wavelength is known as an *emission spectrum*. If the wavelength of the exciting light is changed and the emission intensity from the sample is plotted against the wavelength of the exciting light the result is an *excitation spectrum*. In addition, if the intensity of the exciting light is kept constant as its wavelength is changed, the plot of emission against excitation wavelength is the *corrected excitation spectrum*. As the

quantum efficiency of most molecules is independent of the excitation wavelength, the emission will be directly related to the molecular extinction coefficient of the molecule i.e. *the corrected excitation spectrum* should be the same as the *absorption spectrum*[50]. This is seldom the case, the difference being due to instrumental artefacts.

1.3.2 The Relationship Between Fluorescence and Concentration.

The basic equation defining the relationship of fluorescence to concentration is again adapted from the Lambert-Beer Law and is[51]:

$$F = QE \times I_o(1 - e^{-\epsilon bc})$$

where:

F - Fluorescence

QE - quantum efficiency

I_o - incident radiant power

ϵ - molar absorptivity

b - path length

c - molar concentration

This basic equation indicates that there are three major factors other than concentration that affect the fluorescence intensity.

1. QE, as would be expected, the greater the QE the greater the fluorescence.

2. I_o, theoretically the greater the intensity of the incident radiation, the better the fluorescence. In practice, a very intense source can cause photodecomposition.

3. ϵ, for a molecule to fluoresce, it must first absorb radiation. Hence, the higher the value of e, the better the fluorescence.

For very dilute solutions where A<0.05 absorbance units, the above equation reduces to:

$$F = k\ QE\ I_o\ \epsilon\ b\ c$$

where:

k = an instrumental factor, it is a proportionality constant due to the fact that although fluorescence is emitted in all directions, only that of a limited aperture is measured.

Since all the terms on the right hand side, except 'c' are kept constant under analytical conditions, the instrumental fluorescence is a function of the concentration of the compound under examination. A plot of F versus c would give a straight line plot analogous to Lambert-Beer plot. However, at high concentrations quenching may become so great the curve may slope away. This is explained by the inner filter effect which is explained in section 1.3.5.

1.3.3 Advantages of Fluorescence.

Fluorescence is a very important analytical tool because of its extreme sensitivity and its good specificity. On average the sensitivity of fluorescence is one thousand times greater than any other spectrophotometric technique. The main reason for this is that in fluorescence the emitted radiation is measured directly and can be changed by altering the intensity of the incident radiation. So, an increase in signal over a zero background is measured in fluorescence. In other spectrophotometric methods, like absorbance, the measured quantity absorption is measured indirectly as the difference between the

incident and transmitted beam. Therefore, a small decrease in intensity of a very large signal is measured with a corresponding large loss in sensitivity.

The specificity of fluorescence is due to two main factors. The first being that there are fewer fluorescent compounds than absorbing ones. This is because all fluorescent compounds must absorb radiation, but not all the compounds that absorb radiation fluoresce. In consequence there are much fewer interferences when a fluorescence rather than an absorptiometric method is employed. Secondly, there are two wavelengths in fluorescence to characterise the sample, unlike spectrophotometry where there is only one. For example, two compounds that absorb radiation at the same wavelength will probably not emit at the same wavelength and so the compounds will have a different Stokes' Shift. Other factors that increase selectivity are the lifetime and polarity of fluorescence which help in the discrimination of compounds[45].

Fluorescence is also generally much more sensitive to the environment of the chromophore or molecule that is absorbing and emitting photons than is light absorption. This is a consequence of the relatively long time the molecule stays in the excited state before de-excitation. Absorption or even CD is a process that is over in 10^{-15} seconds and the solvent is the only thing really close enough to affect them. In contrast, during the 10^{-9} to 10^{-7} seconds that a singlet remains excited, all kinds of physical and chemical processes can occur like protonation, solvent reorientation, and changes in optical rotation. These processes can even involve other molecules which are over 10nm away from the fluorophore at the moment of excitation. It is therefore possible for fluorescence to provide dynamic information on its environment within nanoseconds.

Another fairly unique feature of fluorescence is the ability of other chromophores quite far away from an excited singlet to cause quenching. In a favourable case this permits distances between chromophores up to a range of 80Å to be measured[40]. The process is known as singlet-singlet resonance energy transfer. Even when the two chromophores are far apart an interaction exists between them called a very weak coupling limit. By the process of energy transfer resonance the excited chromophore (donor) transfers its energy to another chromophore (acceptor). The donor becomes quenched and the acceptor excited and consequently it can fluoresce. This process from the viewpoint of the acceptor is known as sensitized emission. A requirement for the resonance interaction producing energy transfer is that acceptor absorption must overlap the donor fluorescence. Therefore in any system capable of energy transfer an additional process may occur in which the donor emits a photon that is then reabsorbed by the acceptor. This is distinguishable from the true singlet-singlet energy transfer, because it leaves the rate of donor emission unchanged and it can be removed by dilution.

So, in general those materials that possess native fluorescence, those that can be converted to fluorescent compounds and those that quench fluorescence of other compounds can all be determined quantitatively by the use of fluorimetry.

1.3.4 Environmental Effects.

Environmental factors can seriously affect the fluorescence characteristics of molecules. The fluorescence of several dye molecules have been characterised in a large number of solvents and solvent mixtures[52]. Generally, transfer of a fluorescent molecule from a non-polar to a polar solvent results in a red shift in the fluorescence emission

spectrum and a drop in the quantum yield[53]. Transfer from a polar to a non-polar solvent has the opposite effect. However, it must be noted that specific quenching mechanisms also contribute to these effects (section 1.3.5).

The fluorescence intensity of some ionisable molecules, where the quantum yield of the dissociated and undissociated states are different is highly dependent on pH. For example, the anilinium ion ($C_6H_5NH_3+$) fluoresces very weakly while aniline ($C_6H_5NH_2$) is strongly fluorescent. The emission wavelength may also change due to the degree of ionisation of the molecule and hence the pH. For these reasons accurate pH control is essential and recommended buffers should never be changed without prior investigation.

A decrease in temperature usually produces an increase in fluorescence intensity[54] because there will be a decrease in collision rate between the excited state molecule and surrounding solvent molecules i.e., the radiationless deactivation processes will decrease. Normally unthermostatted room temperature measurements of samples are satisfactory, providing time is allowed for any heated or cooled samples to reach ambient temperature. However, a thermostatically controlled cell is necessary for elevated temperatures.

The viscosity of a sample also influences fluorescence. A higher viscosity reduces the number of collisions an excited molecule undergoes and the net effect is an increase in intensity. It is therefore possible to increase the fluorescence of some molecules with the use of viscous solvents like glycerol or gelatin.

1.3.5 Quenching.

Quenching, is the reduction of fluorescence by a competing deactivating process caused by an interaction between a fluorescent molecule and another molecule which is present and is common in fluorescent studies. There are four common types of quenching found in fluorescence[55], these are temperature, oxygen, concentration and impurity. The effects of temperature has been discussed in section 1.3.4.

The notorious quencher of fluorescence is probably molecular oxygen. In liquid solutions quenching of excited singlet states of organic molecules by dissolved oxygen molecules, which have a very large diffusion coefficient, is a serious problem. The effect of oxygen varies between molecules but it is believed that the collision of the excited state singlet with the oxygen molecule which is a ground state triplet enhances singlet-triplet interconversion (intersystem crossing) and thus quenching of the fluorescence.

As has been already discussed in section 1.3.2, fluorescence only obeys a linear relationship with concentration at dilute sample concentrations. At higher concentrations, where the absorbance of the sample is greater than 0.05 Abs. units, light is not evenly distributed along its path in the cell. Therefore, the part of the sample nearest the light source absorbs much of the radiation so less is available for the rest. As a consequence, considerable excitation occurs at the front of the sample but less throughout the rest of the cell. As the majority of fluorescence is emitted away from the slit exit to the detector, a smaller proportion of fluorescence is measured. This fluorescence loss is called the Inner Filter Effect. It can be reduced by working in the absorbance range stated, opening the slits widths or using a special cell with thick walls.

Another important form of concentration quenching involves dimer or polymer formation called Excimer Quenching. In aqueous solution organic dyes and aromatic hydrocarbons form dimers and higher aggregates[56] due to their hydrophobicity and so become insoluble in water. Excimers (Excited state Dimer) are formed from the association of the ground state and excited state of the same species. The excimer has a different electron orientation and a longer emission wavelength than the monomer and so alters the fluorescence characteristics of the molecule[55]. Exciplex quenching (excited state complex) is formed by the association of two different species, one excited and the other in its ground state[57], again emission occurs at longer wavelengths.

Many analysts have become increasingly baffled with fluorescence due to the phenomenon of impurity quenching. Although fluorescence is supposedly a specific and sensitive technique, when impurities are high in concentration, quenching results. This quenching occurs during the lifetime of the excited state singlet and is probably due to exciplex formation, electron transfer, energy transfer, charge transfer or the heavy atom effect[55]. These interactions will cause various changes in the fluorescence of the molecules including changes in the fluorescence spectra, quantum yield, intensity, fluorescence polarization and decay time.

1.3.6 Photodecomposition Effects on Fluorescence.

Fluorimeters use intense light sources to produce high sensitivity and in some cases this light may be intense enough to cause photodecomposition of the sample. The decomposition only occurs at the point where the excitation beam is focused and its existence can be confirmed by blocking the incident radiation to prevent illumination of the sample. If the fluorescence intensity returns to its original value,

photodecomposition is probably taking place. This phenomenon is due to the diffusion of unaffected fresh molecules into the light path whilst the sample is unlit, with a subsequent renewal of fluorescence. Photodecomposition may be reduced by selecting a longer excitation wavelength (lower energy) or by reducing the source intensity by the use of narrower slit widths or a neutral density filter.

1.3.7 Light Scattering.

Light scattering is another of the major disadvantages associated with fluorescence, as it seriously limits the sensitivity of a fluorescence assay. Light scattering causes a background fluorescence and as one of the benefits of fluorescence is that it is supposedly measured over a zero background, the method loses its principle advantage over other techniques.

Rayleigh-Tyndall Scattering.

For Rayleigh scattering to occur the scattering molecules must have dimensions smaller than the wavelength of the incident radiation and be distributed in a medium of refractive index different to their own. Scattering occurs at the same wavelength as the incident radiation and in all directions with a symmetrical intensity distribution. The intensity of the scattering varies with the inverse fourth power of the wavelength of the incident radiation and so becomes increasingly significant at shorter wavelengths.

Light scattering may also occur from particles in a colloidal suspension (i.e. the particles have dimensions greater than a tenth and less than 1½ times the wavelength of the incident radiation.) This is called Tyndall scattering and in contrast to Rayleigh scattering, it occurs predominantly

in the forward direction. As fluorescence is mainly measured at right angles to the incident beam Tyndall Scattering is less influential than that of Rayleigh.

Generally, light scattering is, unlike fluorescence, non-specific and can be shown by cuvettes, optical surfaces, dust particles and solvent and solute particles. Rayleigh-Tyndall scattering is especially a problem when the excitation and emission wavelengths are close together and the fluorescence intensity is low. The effect can be reduced by using narrower slit widths, appropriate filters or monochromators in the fluorescence spectrometer. However the fact that Rayleigh Scattering is largest when an excitation wavelength of ca. 350nm is used[58] is highly disadvantageous. Unfortunately this wavelength region is one where many common fluorophores are excited, so the background effect can be serious despite all precautions.

Raman Scattering.

Raman scattering is an effect related to Rayleigh scattering and occurs from pure solvents. Raman bands are displaced by a fixed frequency from the incident radiation and occurs because during Rayleigh scattering some of the incident light is taken and converted into vibrational/rotational energy. The resulting energy scattered is therefore at lower energy and longer wavelength than the incident radiation.

Raman scattering is a much weaker effect than the Rayleigh effect but can be just as damaging. It is therefore beneficial to run a solvent blank prior to analysis to check where the Raman peaks occur (see Table 1.1).

Table 1.1: Raman bands for several solvents.

Solvent	Wavelength (nm) of Raman band produced by excitation at				
	248	313	365	405	436
Carbon Tetrachloride	-.-	320	375	418	450
Chloroform	-.-	346	410	461	502
Cyclohexane	267	344	408	458	499
Ethanol	267	344	409	459	500
Water	271	350	416	469	511

Thus when an excitation wavelength, for a sample in water, of ca. 350nm is used the water Raman signal (often used as a test of the sensitivity of a fluorescence spectrometer) occurs at ca. 397.5nm where it can interfere with the genuine fluorescence signals. When proteins containing tyrosine are excited in aqueous solution at the amino acids absorption maxima of 278nm, the water Raman band occurs at 307nm, almost exactly the same as the tyrosine emission maxima. So despite their relatively low intensities, solvent bands can effect many fluorescence results.

Raman scattered light, just like Rayleigh-Tyndall is strongly polarised and so insertion of an appropriate polarizer may help reduce its intensity[59].

1.3.8 Long Wavelength Fluorescence.

In the past fluorescence studies have involved the UV/visible region of the spectrum. The advantages involved with this technique (section 1.3.3) have enabled it to be used in many exceptional, elegant methods of

analysis. However, it is still associated with a number of drawbacks which have been discussed in the previous sections. For this reason many authors[58,60-63] have begun to look at long wavelength fluorescence to remove some of these experimental problems.

Long wavelength fluorescence can arbitarly be defined as including all fluorophores which emit above 600nm and up to 1000nm. So the region of interest spans the long wavelength end of the visible spectrum and the very near infra red region (Fig. 1.3).

The principle advantage of working in this region is that scattering problems (Section 1.3.7) are greatly diminished at higher wavelengths. Thus the intensity of Rayleigh Scattering using an excitation wavelength of 600nm is 10% of that at 340nm in comparable optical conditions. Excitation at 800nm reduces the background scattering by a further factor of three. The effects of Raman Scattering are also greatly diminished. Excitation of an aqueous sample at 600nm would produce a water Raman band at ca. 754nm of much lower intensity than a lower wavelength excitation. The Stokes' Shift obtained here of 154nm is much larger than most Stokes' Shifts associated with fluorescent molecules. This effect increases with wavelength, for example, an excitation of 830nm will produce a water Raman band at ca. 1150nm well beyond any capacity to interfere with any fluorescence excited at the same wavelength.

Another major advantage of working in the 600-1000nm region is that there are fewer intense fluorophores. Theory predicts that quantum efficiency decreases, as the energy difference between the ground and excited state decreases[58]. In practice what seems to happen is that there is a relevantly small number of bright fluorophores in this region and compared with the UV/vis region, a much larger number of non fluorescent or poorly fluorescent molecules. This is advantageous

because if there are a small number of fluorescent molecules in the wavelength region under examination, observations can be made against a very low background fluorescence, even when complex molecules are under study. Whereas, at lower wavelengths if say, a biological matrix was being monitored, many proteins and drugs fluoresce in this region and so the background will be high. The combination of this reduced endogeneous fluorescence with the reduced scattering effects, means that, a blood sample for example has a fluorescence background at ca. 700 nm an order of magnitude less than that at ca. 500 nm. This reduced background will allow an excellent limit of detection even for fluorophores with moderate molar absorptivites and quantum efficiencies. Also, as lower energy is involved at these higher wavelengths the problem of photodecomposition will be removed.

The availability of cheap solid state optical components on the long wavelength region is another major advantage. UV/vis measurements generally involve the use of expensive xenon light sources which require a large input and photomultiplier detectors which are large and delicate. By contrast excellent simple cheap long wavelength detectors can be constructed, from solid state light sources like light emitting diodes and diode lasers[61,64] to simple optical filters and solid state detectors dedicated to a specific function[63,65-69].

All the points discussed in this section emphasize why long wavelength measurements are the way forward in fluorescence and why in this project it has been examined as a technique for studying drug-protein interactions.

1.4 FLUORESCENCE METHODS FOR STUDYING DRUG-PROTEIN INTERACTIONS.

As previously mentioned the use of fluorescence for measurement of binding kinetics depends on a change in measurable parameter when a drug or a ligand binds to the protein. This parameter is normally, a shift in emission wavelength, fluorescence intensity, quantum efficiency, fluorescence lifetime or polarization. For the binding system to be suitable for fluorescence measurements the following conditions need to be satisfied:

1. The change in fluorescence must be very rapid, to ensure that the binding is the only time dependent process.

2. The association constant of the drug must be large enough for a readable fluorescence signal and also for the lowest concentration of protein solution.

3. The fluorescence changes incurred on binding must bear a quantitative relationship to the amount of binding.

There are three main areas in which fluorescence is used to monitor drug-protein interactions. These involve changes in fluorescence of the protein or drug and the use of covalent labels or non-covalent probes.

1.4.1 Changes in the Intrinsic Fluorescence of the Protein on Binding.

In most proteins the majority of light is absorbed by the aromatic amino acids tryptophan, tyrosine and phenylalanine and these three amino acids play an important role in fluorescence. In fact, it has been proven that the fluorescence of proteins is due entirely to these three proteins, especially tryptophan. For this reason as far as fluorescence is concerned proteins are often divided into two classes - class A and class B[70-72].

Class A Proteins.

This class includes all proteins which contain no tryptophan. The fluorescence of this class is due entirely to tyrosine residues and for this reason they have a tyrosine fluorescence maximum at 304nm which is not affected by conformational changes in the macromolecule.

Class B Proteins.

The main feature of the fluorescence of proteins in this class is that, despite containing three different fluorescent amino acids, generally only the tryptophan maximum appears in the florescence spectra[73]. This peak occurs between 320nm and 350nm depending on the conformational state of the tryptophan.

It is this protein fluorescence that is used in one technique in the determination of drug-protein binding interactions.

When a drug molecule binds to a protein, the protein fluorescence may be quenched. There are many examples of authors who have used this phenomena to study drug-protein and ligand-protein binding. These include: the interaction of chlorpromazine with cell lactins[74]; the binding of dicoumerol[75], warfarin[75], flufenamic acid[76], fatty acids[77] and bile salts[78] to serum albumin; the binding of phenothiazine neuroleptics to α_1-acid glycoprotein[79-80]; the binding of biotin to avidin[81]; the interaction of retinol with ß-lactoglobulin[82] and the addition of chlorpromazine to myosin[83]. It has been shown that the quenching is mainly caused by energy transfer from the protein to the bound drug[84].

This method can also be used for competitive interactions of many drugs to a protein provided the binding of only one drug quenches the protein fluorescence. However, there are some problems associated with this technique. Firstly, when a drug binds to the protein the change in fluorescence may not always be proportional to the number of drug

molecules that have bound. This is because unless each drug is binding to an identical subunit, the tryptophan and tyrosine residues may be rearranged and therefore quenched by different amounts. Even if each drug molecule is binding to identical subunits its quenching radius may reach into adjacent subunits making the environments of the two sites different. Another problem is that if a conformational change takes place on binding, previously identical environments may be changed by different amounts. However the major problem with this technique for long wavelength measurements is that the intrinsic fluorescence of proteins occurs at too low a wavelength where the background fluorescence is too high.

1.4.2 Changes in the Intrinsic Fluorescence of the Drug on Binding.

Sometimes the drug itself may undergo a change in fluorescence when it binds to the protein. This fluorescence change may be associated with energy transfer from the protein or even polarity effects of the hydrophobic protein binding sites. This technique has been used in the study of chlorpromazine binding to certain protein fractions[85]; the binding of warfarin to serum albumin[75] and the characterization of the binding site[86]; and the investigation of propranolol, adrenalin and isoprenaline binding to α_1 acid-glycoprotein[87].

An obvious disadvantage, again, of this technique is that fluorescence measurements will be at low wavelengths. However, fluorescent analogues of certain drugs have been made which make fluorescence measurements much easier and at higher wavelengths. This method will be discussed in the following section.

1.4.3 Fluorescent Labels and Probes for the Study of Drug-Protein Interactions.

If the drug or protein do not posses the necessary fluorescence, it may be necessary to attach a suitable fluorophore to the drug. A fluorescent molecule that is covalently bound to the protein and/or drug is known as fluorescent label, while a molecule non-covalently attached to the protein is a fluorescent probe. A fluorescently labelled drug/ligand is also classed as a probe as it binds to the protein non-covalently.

The use of fluorescent probes and labels are essential for long wavelength measurements, as drugs or proteins do not possess fluorescence in this region. The techniques can not only provide information on a drug-protein interaction like the association constant and the number of binding sites, but also knowledge of the conformation of the binding site and the amount of protein present in a sample. Both of which are necessary to fully understand drug-protein binding.

1.4.4 Fluorescent Covalent Labels.

The main source of fluorescent labels and probes are that of dyes. There are obviously many groups of dyes and not all are fluorescent, however for a dye to be suitable fluorescent label, the criteria is as follows[88]:

1. The dye should posses chemical groups which will form stable covalent bonds with protein molecules or be easily convertible to such a reactive form without destroying the fluorescent nature.

2. It should not posses any other groups which may react with the primary reactive group to give unwanted by-products.

3. The fluorescence efficiency of the dye must be high, so it has a high detection sensitivity.

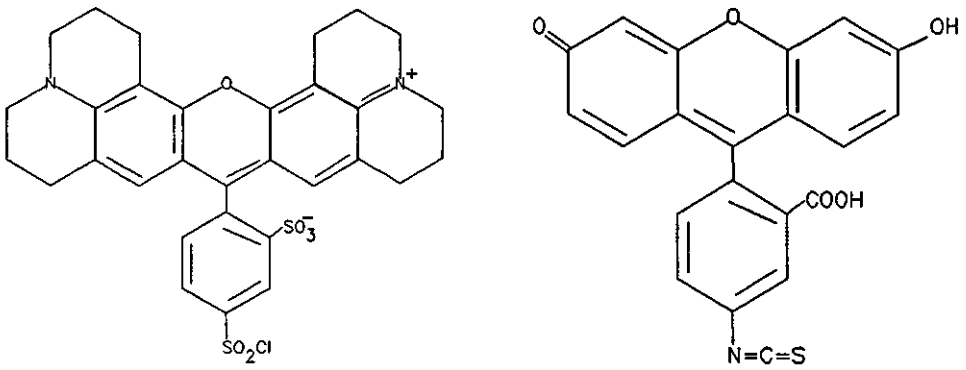
4. The conjugate should be stable under normal conditions.

5. The conjugation procedure should be as simple and as short as possible.

There are three main types of chemically reactive groups which have been used in the coupling of dyes to proteins: sulphonyl chlorides which are prepared from sulphonic acids; isocyanates (or isothiocyanates) and diazonium salts both of which are usually prepared from the corresponding amines.

These groups are attached to the dyes if they are not already present and can bind to several types of groups on the proteins. The obvious areas of attachment are the free amino acid and carboxyl groups at the ends of the protein. There are also many free amino groups in the lysine side chains and many carboxyl groups in aspartic acid and glutamic acid residues. The type of dye and the reaction conditions will determine where and what type of attachment occurs between the drug and protein.

The first group of dyes found to be adaptable as fluorescent labels was the xanthene type dyes. This group of dyes itself can be divided into two types, the rhodamines and the lower wavelength fluoresceins, which differ at the 2 and 7 positions in the xanthene ring by amino and hydroxyl groups respectively (Fig. 1.8).



Texas Red FITC

Fig. 1.8 The structures of the xanthene dyes Texas Red and FITC.

Fluorescein isothiocyanate was one of the original covalent labels and was first used in the early 1950's for the tracing of albumin in plasma and the determination of its renal clearance[89]. Since then, derivatives of both fluorescein and rhodamine have been used in a number of techniques including: measurement of the activity of the enzymes Na+-K+ ATPase[90-92], α-actin[93-94] and actin[95]; in a variety of conditions in cancer determination[96] and in many fluorimetric immunoassays[97].

The development of reactive dyes for textiles has also helped techniques involving the use of covalent labels. Reactive dyes contain one or two groups in their structure which can react directly with proteins and so need no functionalising to make covalent labels. Two common examples are (a) Lucifer Yellow VS and (b) Lucifer Yellow CH (Fig. 1.9).

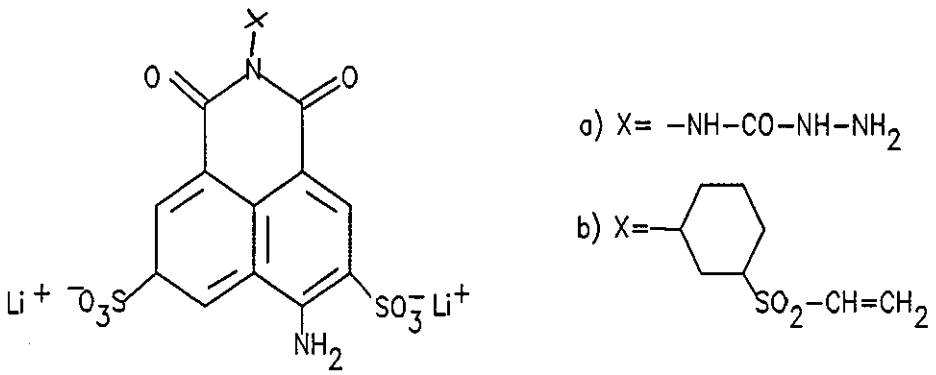


Fig 1.9: Different structures of Lucifer Yellow.

Lucifer yellow VS labels proteins rapidly under mild conditions. In fact, it is similar to FITC in its ease of attachment and has the advantage of having its fluorescence maxima at 530nm, approximately 30nm more to the red than FITC. It is also very stable with pH which has made it useful for monitoring pH induced conformational changes in proteins as well as immunoassay[98]. The most frequent application of Lucifer yellow CH is that of mapping the nervous system and to determine the branching pattern and cause of regenerating neurones.

The major drawback of all the dyes mentioned so far is that none are really long wavelength dyes, they all have emission maxima below 600nm. Recently, polymethine cyanine dyes have been studied for use as covalent labels. Many of the dyes fluoresce in the long wavelength region and they typically have large molar absorptivities and good fluorescence properties[52]. Recent work has involved the binding of iodoacetamide derivatives of cyanine dyes to sulphydryl residues in certain proteins[99]. More specifically, isothiocyanate functionalised forms of indocyanine green (Chapter 3), have been covalently bound to human serum albumin. Extensive work on the phthalocyanines has also been carried out including their use in the photodestructive therapy of tumours[100].

Phenoxazine dyes are another group of dyes that are being studied for fluorescent labelling. This group are the most stable type of dyes and can maintain their fluorescence efficiency in organic solvents for months at a time[52]. Both Nile Blue (Chapter 3) and Oxazine 750 (Fig. 1.10),

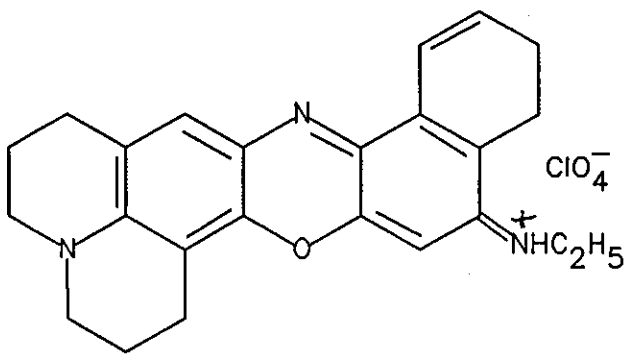


Fig. 1.10: Oxazine 750

have been attached to serum albumin[64] in the hope of developing an optical fibre immunological sensor. Current work by the authors' co-workers involves the development of Nile Blue as a covalent label in immunoassay.

Despite the advantages that arise with the use of long wavelength fluorophores, some disadvantages do have to be considered. The dyes involved, especially the cyanine dyes, are large highly conjugated molecules often with molecular weights of more than 1000. Even as a covalent label this size may result in a conformational change in the protein. The extensive conjugation in the label also increases its instability which can lead to photobleaching or a shortening of the shelf life of the label, both of which are serious problems. It is for these reasons that naturally occurring fluorophores like phycoerythrins are being studied and why compounds that only become fluorescent on irradiation after labelling are being developed[97].

1.4.5 Fluorescent Covalent Probes.

For the actual determination of drug-protein binding interactions, and for many other methods of analysis the use of non-covalently attached fluorescent probes is favoured. Most non polar groups in proteins are buried in the interior of the molecule. However, there are some found on the surface of proteins or in the pockets of the protein, others may become exposed as a result of a biologically significant conformational change in the protein. These so called sites form a point of hydrophobic interaction with drugs and this is where many of the probes bind. On binding, changes in fluorescent properties of the probe will result which can be assessed both quantitatively and qualitatively. However, in order to achieve the desired effects a fluorescent probe is expected to meet the following requirements:

1. Good affinity for the protein so that while the complex is fairly dissociable, a good percentage of binding is reached at a reasonable protein to probe concentration ratio.

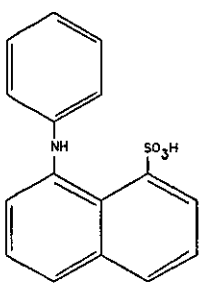
2. Specificity that allows measurements in the presence of other proteins.

3. As an assay will involve proteins a good solubility in aqueous media is required.

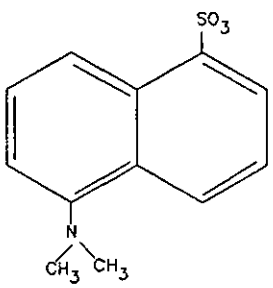
4. Stability both under measurement conditions and on storage so that long term assay procedures can be performed and standardised.

5. A good Stokes' Shift to prevent interference of Raman and Rayleigh scattering.

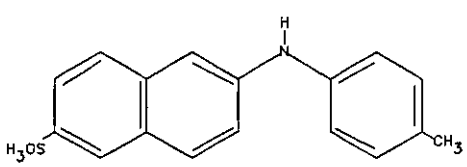
Until recently, most work involving fluorescent molecules as probes for proteins and drug-protein interactions has been at the visible end of the spectrum. The most common group of fluorescent compounds studied has been the anionic aminonaphthalenes (Fig 1.11) which are all environmentally sensitive, their



ANS



Dansyl



TNS

Fig. 1.11: Structures of some common fluorescent probes.

fluorescence increasing with hydrophobicity of their environment. The most frequently used aminonaphthalene is probably 1-aminonaphthalene-8-sulphonic acid (ANS) and its dimeric form bis-ANS. These have been used to detect and study serum albumin in both normal[78, 101-105] and nephrotic patients[24]; to monitor by changes in its hydrophobic environment the effects of lipids on gastric mucin[106]; the influence of the erythrocyte plasma membrane on the uptake of iron and tranferrin by reticulocytes[107]; the effect of iodination on thyroglobulin formation[108]; the assembly of tubulin into microtubules[109] and the influence of the concentrations of ATP and ADP on the activity of the chloroplast protein rubisioactivase[110].

Drug-protein interactions have also been studied using ANS and its displacement from albumin[111-113] and α_1 acid glycoprotein[87, 113-114] both statically and with the more novel rapid technique involving flow injection analysis[115-116]. Other aminonaphthalenes like 2-p-toluidnylnaphthalene-6-sulphonic acid (TNS) and 6-propyl-2(N,N-dimethyl)aminonaphthalene (PRODAN) have been used to study the conformation of the proteins chymotrypsin, lysozyme, ovalbumin and ß-lactoglobulin[117]; the polarity of the myoglobin haem pocket[118] and to follow the conversion of pepsinogen to pepsin[119].

Chignell[120] showed that 5-dimethylaminonaphthalene-1-sulphonyl (Dansyl) derivatives of amino acids were of great use as fluorescent probes for the hydrophobic regions of the proteins and that information could be obtained on the binding sites of certain drugs to albumin. Sudlow *et al.,*[112] continued this work and dansyl derivatives of amino acids helped him identify the two main binding areas in serum albumin. More recently dansyl derivatives have helped to identify the binding sites of tryptophan[121] and fatty acids[20] on serum albumin; to compare drug binding sites on different mammalian albumins[86]; to determine any differences in conformation of bovine and human albumin upon the N-B transformation[122] and to distinguish between the plasma protein binding of certain drugs in both normal patients and people with malaria[123]. Dansyl analogues of the drug propranolol have been used both statically and with flow injection analysis to study the areas of attachment for basic drugs in α_1-acid-glycoprotein[115, 124-125]. Dansyl derivatives of retinoic acid have helped in the study of the hydrophobic pockets in its receptors[126] while Steven *et al.,*[127] bound dansyl to guanidino, a ligand that binds to guanidinobenzoatase an enzyme present on the surface of tumour cells, to detect their presence.

The technique of making fluorescent derivatives of ligands to study their binding and effect on macromolecules has also been used with xanthene dyes. Fluorescein and rhodamine have been attached to ligands to study their binding to opioid receptors[128], dopamine receptors[129], acetylcholine receptors[130] and Na^+-K^+ ATPase[131]. However the major disadvantage of the production of fluorescent

derivatives or ligands is that the process could be complicated and the reaction conditions harsh. Using simple fluorescent probes and their displacement with drugs or ligands is much easier.

However, the aminonaphthalenes are not the only dyes used as fluorescence probes. The cationic probe auramine O has been successfully bound to α_1-acid-glycoprotein with displacement by certain basic drugs[132,133] and to liver alcohol dehydrogenase[134]. Acridine orange dodecyl bromide has been found to bind serum albumin and α_1-acid-glycoprotein[132]. 4'hydroxybenzene-2-carboxylic acid has been used for an estimation of serum albumin concentration and as an assay for avidin and biotin based on the binding of the dye to avidin[135]. N-methyl acridinium has been bound to the enzyme acetylcholenesterase and displaced with many drugs[136].

All the probes mentioned so far bind to the protein hydrophobically or electrostatically or a combination of these, their increase in fluorescence on binding is probably due to energy transfer from the fluorescent amino acids in the protein. The emission spectra of the protein overlapping with excitation spectra of the dye. However, other probes have different binding mechanisms, Hoeschst 33256 and ethidium bromide have been found to intercalate with DNA and RNA[137], while rhodamine 123 has been found to stain certain mitochondria probably as a result of high membrane potential across the membrane and has been used for the determination of multidrug resistance[137] and the diagnosis and treatment of carcinomas[136].

Another membrane probe is Merocyanine 540 (MC540, Chapter 3), which is also a member of the polymethine cyanine group of dyes. It was first developed as a sensitizing additive for photographic emulsions. Biomedical applications of merocyanine dyes began in the mid-seventies when they were used for the recording of trans-membrane potential changes in cells[138-141]. More recently MC540 has been bound to electrically excitable cells such as nerve or muscle cells, immature blood cells and leukaemia/lymphoma cells[142-145] with a higher affinity than it binds to non-excitable cells. Activated lymphocytes, monocytes and

platelets also bind MC540 with a greater affinity than their non-active counter parts[146]. The explanation of the binding of MC540 is still not completely understood, although many theories have been put forward[142, 147-148]. It is believed that the interaction of MC540 with the membrane is a hydrophobic interaction in the lipid bilayer and when electrically excited there is probably a change in conformation of the membrane which causes a change in orientation and affinity of the dye and hence a change in fluorescence. MC540 is a photosensitive dye and this fact has been utilised along with its preferential binding to leukaemic cells to help in the treatment of leukaemia. Excitation of the membrane bound dye at its excitation wavelength ca. 540nm appears to cause an increase in dye uptake, impairment of the membrane functions and eventually cell death[149]. This technique has also been used in viral diseases like herpes and has prospects for HIV[150]. Although no literature can be found on the binding of MC540 directly to proteins, work on other cyanine dyes and its properties suggest it may make a good probe in the determination of drug-protein interactions.

Perhaps the most promising group of dyes in terms of fluorescent probes is the phenoxazine/phenoxazone group, which have the advantage of over the probes previously discussed of being fluorescent in the long wavelength range. One of the most common phenoxazine probes is 9-diethylamino-5H-benzo[α]phenoxazine-5-one or Nile Red which was found as a contaminant of another phenoxazone Nile Blue[151].

Nile red is an uncharged, pH and photochemically stable dye[152]. The fact that the fluorescence of Nile Red increases with decreasing polarity[151] has resulted in its use as a hydrophobic stain for intracellular lipids[151-155] and the determination of lipid related diseases in the heart and liver[156]. More recently, Nile Red has been shown to bind to serum lipoproteins[157] and many other proteins[62,156,158-159], the fluorescence depending on the hydrophobic character of the protein itself. Sackett et al.,[62,156] have even used Nile Red to detect changes in hydrophobic character in proteins, caused by dimerisation, unfolding and ligand binding of tubulin, serum albumin and calmodulin respectively. Again, however, no literature can be found on drug displacement of the probe when attached to proteins.

Higher wavelength probes which are necessary if the new solid state light sources are to be used in this type of fluorescence assay generally come from the carbocyanine group of dyes, indocyanine green was first bound to serum albumin in 1974[160]. Later this property was utilised to develop an assay for albumin using semi-conductor laser fluorimetry[161]. Indocyanine green has also been bound to α_1-lipoprotein and γ-globulin and used in an enzyme immunoassay of insulin[68]. In recent work 3,3'-diethylthiatricarbocyanine iodide (DTTCI) and some of its analogues have provided information about the nature of the binding sites on albumin[162]. Kessler *et al.*[63,163] have devised a novel assay for low levels of albumin using derivatives of cyanine dyes and laser induced fluorimetric detection which will soon be on the market. It should be noted however that with larger, longer wavelength probes there will be an optimum size where the probe will be too large to fit in the binding site of the protein.

CHAPTER TWO

MATERIALS, INSTRUMENTS AND GENERAL PROCEDURES.

2.1 MATERIALS.

SUBSTANCE	SUPPLIER AND DESCRIPTION
Proteins.	
α_1-Acid Glycoprotein	Sigma Chemicals, bovine purified from Cohn fraction VI and serum, 99% pure.
Bovine Albumin	Sigma Chemicals, fatty acid and globulin free, 99% pure.
Human Serum Albumin	Sigma Chemicals, crystallised and lyophilized, fatty acid and globulin free.
Horse Albumin	Sigma Chemicals, fatty acid free.
Guinea Pig Albumin	Sigma Chemicals, crystallised and lyophilized.
Rabbit Albumin	Sigma Chemicals, crystallised and lyophilized.
Immunoglobulin G	Sigma (Immuno) chemicals, bovine, technical grade.
α-lactalbumin	Sigma Chemicals, Bovine Milk, 85% pure.
ß-Lactoglobulin	Sigma Chemicals, Bovine milk, 3x crystallised and lyophilized, containing ß-lactoglobulins A and B.

ß-Lactoglobulin A	Sigma Chemicals, Bovine milk.
ß-Lactoglobulin B	Sigma Chemicals, Bovine milk.
Ovomucoid	Sigma Chemicals, Chicken egg white, free from ovo-inhibitor.

Fluorescent Labels.

1-anilino naphthalene-8-sulphonic acid (ANS)	Sigma Chemicals
D,L-N-[2 hydroxy-3-(1-naphathyloxy)-propyl]-N-dansylethylenediamine. (DAPN)	Sigma Chemicals
3,3'-Diethylthiatricarbocyanine iodide. (DTTCI)	Eastman Kodak 99.9% purity
Indocyanine Green (IR-125)	Eastman Kodak
Merocyanine 540 (MC 540)	Eastman Kodak 95% purity
Nile Blue	Eastman Kodak 99.9% purity
Nile Red	Eastman Kodak 99.9% purity
Rhodamine 800	Lambda Physik

Drugs.

Amitriptyline	Sigma Chemicals
Diazepam	Sigma Chemicals
Digitoxin	Sigma Chemicals
± Ephedrine Hydrochloride	Sigma Chemicals
+ Ephedrine Hydrochloride	Sigma Chemicals

- Ephedrine Hydrochloride	Sigma Chemicals
Ethacrynic Acid	Sigma Chemicals
Flufenamic Acid	Sigma Chemicals
Phenylbutazone	Sigma Chemicals
D,L Propranolol Hydrochloride	Sigma Chemicals
S, Propranolol Hydrochloride	Sigma Chemicals
R, Propranolol Hydrochloride	Sigma Chemicals
Salicylic Acid	Fisons Scientific
Stearic Acid	Sigma Chemicals
Sulphadiazine	BDH Chemicals
Sulphamethoxazole	Sigma Chemicals
Warfarin	Sigma Chemicals

Buffer Reagents. (All Analytical Grade)

2-(N-morpholino) ethanesulphonic acid (Mes)	Sigma Chemicals
Ethylenedioxydiethylenedinitrotetracetic acid (EGTA)	Sigma Chemicals
$MgCl_2$	BDH Chemicals
Trizma/HCl	Sigma Chemicals
NaCl	BDH Chemicals
KH_2PO_4	BDH Chemicals
Na_2HPO_4	BDH Chemicals
KCl	BDH Chemicals
Sodium Azide	Fisons Scientific

Deionized, triply distilled water from a Liqui-Pure Modulab system was used to make all buffers. All solvents were of analytical-reagent grade and obtained from Fisons Scientific Equipment.

2.2 INSTRUMENTS.

Fluorescence spectra were obtained using a Perkin-Elmer MPF-44B and LS-50 spectrofluorimeters both of which were fitted with a R298 photomultiplier tube to enable spectra to be taken at the high wavelengths involved in this project.

The MPF-44B was attached to a Perkin-Elmer 056-1002 chart recorder and used in some of early preliminary work.

The LS-50 was interfaced to an Epson AX3 personal computer fitted with the Fluorescence Data Manager (FLDM) software package which controls all aspects of the fluorimeter. The instrument was used for all binding and displacement titrations as well as derivative spectra measurements.

All absorbance measurements were taken on the Uvikon 810 ultraviolet-visible spectrophotometer between the wavelengths 200-900 nm. The diluent buffer was always used as the reference.

Any pH adjustments were made with a calibrated Corning 140 pH meter.

2.3 GENERAL PROCEDURES.

2.3.1 Preparation of Buffers.

Mes Buffer.

For a litre of 0.1M Mes buffer:-

19.52g of Mes (0.1M)

0.38g of EGTA (1mM)

0.203g of $MgCl_2$ (1mM)

0.05% w/v of Sodium Azide.

The buffer was adjusted to a pH of 6.9 or 8.75 with the use of 5M NaOH and then stored at -4°C.

Tris Buffer.

For a litre of 0.1M Tris buffer:

15.76g Tris /HCl

0.05% w/v Sodium Azide.

The pH was adjusted to 7.4 with 5M NaOH and stored at -4 °C.

Phosphate Buffer Saline (P.B.S.).

For 1 litre of 0.15M P.B.S.:

8g NaCl (130mM)

0.2g KH_2PO_4 (1.4mM)

1.13g Na_2HPO_4 (8mM)

0.2g KCl (2.6mM)

0.05% w/v Sodium Azide.

The pH adjustment to 7.5 was with 5M NaOH and storage was at -4 °C.

To prevent bacterial growth 0.05% w/v sodium azide was added to the buffers and they were stored at -4 °C. At the first sign of contamination the buffers were discarded and fresh ones prepared.

All fluorescence measurements in aqueous media were carried out in all buffers to see if any difference was observed. All fluorescent measurements shown are in Mes buffer unless otherwise stated.

2.3.2 Preparation of Proteins.

All proteins were prepared in buffer at the required stock solution concentration, freshly each day. The stock solution was then aliquotted out to give the desired final concentrations. This was necessary to avoid any errors due to sticking of the protein to the glass or plastic walls of its container and to reduce the chances of contamination.

Purity of the proteins was examined using cellulose acetate electrophoresis. Single bands were observed for all the proteins tested indicating the absence of impurities.

2.3.3 Preparation of Drugs.

The drugs were made up as stock solutions and stored at -4°C for no longer than a week. However, some of the drugs like Flufenamic acid, sulphamethoxazole, sulphadiazine and warfarin were not soluble in the neutral buffer pH and required a slightly alkaline media which was obtained by adding a few drops of 0.88M ammonia to the solution. Other drugs like diazepam, digitoxin and stearic acid were insoluble in aqueous media of the required concentration and were therefore prepared in methanol. Drugs like salicylic acid and ephedrine which are light sensitive were always protected from light by storage in a darkened container. The structures and therapeutic use of all the drugs are shown in Appendix I.

2.3.4 Fluorescent Probe Preparation.

All the dyes were fairly insoluble in aqueous media and so were stored in various solvent systems (Chapter 3).

Purity of the dyes were tested using thin layer chromatography using silica plates with a mobile phase consisting of various solvent systems[62].

2.3.5 Sample Preparation and Measurement.

For static measurements all samples were prepared in 5 ml volumetric flasks. The required concentrations of protein, probe and drug were aliquotted into the flask, in that order and the sample volume made up to the mark with the buffer. As the probes were dissolved in organic solvents their effect on the fluorescence could have been a problem. However, this was overcome by adding a maximum of 25μl of the probe

solution to the sample, dilutions of the stock solution were made if necessary. In addition, the protein volume was always large enough to ensure that the probe volume was always less than 1% even before the final volume was reached. After each measurement, all glassware was washed out with dilute nitric acid, rinsed with distilled water and dried to remove any deposits of the proteins or other contaminants.

An incubation time for the sample was used if required and unless otherwise stated, readings were taken in 10mm acrylic cells at room temperature. All samples for binding and displacement titrations were kept at an absorbance of less than 0.05 absorbance units to avoid the inner filter effect. The excitation and emission slits were set at values which gave a large signal to noise ratio for the fluorescence spectra with the lowest amount of scattered light.

All parameters used were recorded with the spectra. For the MPF-44B all fluorescence intensities were adjusted to a range and gain of one for easy comparison, this was not necessary for the LS-50.

2.3.6 Quantitative estimation of drug-protein binding parameters.

The usual way of studying the interaction of a ligand with its binding site is to keep the total concentration of binding sites constant and vary the concentration of ligand. If some physical property of the bound ligand, in this case fluorescence, is being used to study binding, then the variation of this property with increasing ligand can be used to estimate various binding parameters.

A number of methods involving graphical and computer assisted analysis which include molecular models and curve fitting procedures have been developed. All procedures are generally devised from various linearised forms of the simple binding equation.

$$B + L \underset{K_2}{\overset{K_1}{\rightleftharpoons}} BL \qquad 2.1$$

where: B = unbound protein site,

L = free ligand,

BL= complex formed,

K_1, K_2= rate constants for the forward and reverse reaction respectively.

The most common graphical representations are probably the Scatchard plot[164,165], the Klotz plot[166,167], and the double reciprocal plot[168] and adaptations there of[12,169].

For example, the Scatchard plot which is probably the most common, is essentially based on the relationship between the fraction, R, of macromolecule sites occupied to the association constant K_a, and is defined as:

$$\frac{R}{C_L} = K_a(1-R) \qquad where\ R = \frac{C_{BL}}{C_B^o} \qquad 2.2$$

or

$$\frac{C_{BL}}{C_L} = K_a(C_B^o - C_{BL}) \qquad 2.3$$

where C_B° = concentration of potential binding sites, $C_B = C_B^\circ - C_{BL}$.

The derivation of which is based on equation 2.1. Various forms of the equation are derived depending on the experimental data available. Much literature has been published on the benefits and drawbacks of the plots and computer software packages available for the calculation of binding parameters. The papers by Weder[170], Klotz[171], Monot[172] and Panjehshahin[173] are just a few. However, although some software

packages were tried, for simplicity and speed, simple Scatchard plots were used.

Abdallahi[174] adapted the Scatchard plot for use in fluorescence measurements where only the total concentration of ligand is known. This could be used from the analysis of data obtained by direct titration where the concentration of protein is kept constant and the concentration of ligand is increased resulting in an enhancement of fluorescence intensity.

If n = number of binding sites

$$C_B^o = nC_p^o$$

C_p^o = total molar concentration of protein.
Similarly,

$$C_{BL} = nC_{pL}$$

and

$$C_L = C_L^o - nC_{pL}$$

C_L^o = total concentration of ligand added.

Assuming the n binding sites are independent and identical and R is the fraction of completely bound protein, then,

$$R = \frac{C_{pL}}{C_p^o}$$

equation 2.1 becomes,

$$K_a = \frac{nC_{pL}}{nC_pC_L} = \frac{C_{pL}}{(C_p^o - C_{pL})(C_L^o - nC_{pL})} \qquad 2.4$$

Substituting RC_p^o for C_{pL},

$$K_a = \frac{RC_p^o}{(C_p^o - RC_p^o)(C_L^o - nRC_p^o)}$$

$$\therefore \frac{1}{K_a} = C_L^o\left(\frac{1}{R} - 1\right) - nC_p^o(1 - R)$$

$$\frac{1}{1-R} \times \frac{1}{K_a} = \frac{C_L^o}{R} - nC_p^o \qquad 2.5$$

R is also F_e/F_e^o and obtainable from fluorescence measurements, where the unbound probe is assumed not to be fluorescent:

F_e^o = fluorescence intensity due to fully bound protein:probe complex
F_e = fluorescence intensity at any point in the titration.

A plot of $1/(1-R)$ versus C_L^o/R gives a straight line if K_a (the association constant) is constant. The intercept of the C_L^o/R axis is equal to nC_p^o. C_p^o is known therefore n is evaluated.

If curvature of the plot occurs, different classes of binding sites or co-operativity were assumed and the curve was analysed according to the methods of Rosentha[165] and treated as separate plots. Alternatively the Hill Plot could be used[12].

For displacement titrations, where competitive binding is observed and the fluorescence of the probe:protein complex is quenched, the data was analysed by using a simple equation based on the law of mass action:

$$\frac{r}{R} = K \frac{C_D}{C_L} \qquad 2.6$$

where r and R = fractions of binding sites occupied by the two competing ligands.

C_D and C_L = concentration of the free competing ligands in the binding system.

Adapting this for a displacement titration using fluorescence, where the protein:probe complex is kept constant and the concentration of the drug increases and the concentrations of the free drug and free probe are not known,

$$C_D = C_D^o - C_{BD}$$

and

$$C_L = C_L^o - C_{BL}$$

where C_D° = total concentration of added drug.

C_L° = total concentration of added probe.

C_{BL} and C_{BD} = the probe:protein and drug:protein complexes.

$$\therefore \frac{r}{R} = K \frac{C_D^o - C_{BD}}{C_L^o - C_{BL}} \qquad 2.7$$

also,

$$\therefore \frac{r}{R} = K \frac{(C_D^o/C_B^o) - (C_{BD}/C_B^o)}{(C_L^o/C_B^o) - (C_{BL}/C_B^o)}$$

$$\text{if } r = \frac{C_{BD}}{C_B^o} \quad \text{while } R = \frac{C_{BL}}{C_B^o}$$

$$\therefore \frac{r}{R} = K\left(\frac{(C_D^o/C_B^o) - r}{(C_L^o/C_B^o - R}\right) \qquad 2.8$$

$$\textit{where,} \quad R = \frac{F_e}{F_e^o} \qquad r = \frac{F_e^o - F_e}{F_e}$$

in this case F_e° corresponds to the value of fluorescence intensity when no drug is added, i.e., R = 1, r = 0.

A plot of r/R versus $[((C_D^\circ/C_B)-r)/((C_L^\circ/C_B^\circ)-R)]$ will give a straight line if binding is competitive for sites which are identical and independent. The slope K is the relative constant, i.e., the ratio of the association constant of the drug K_b to that of the probe K_a. K_a has been determined in the titration experiment hence K_b can be calculated.

For both the binding titrations and displacements, the fluorescence intensity measurements were always taken at a fixed wavelength normally the emission peak of the probe:protein complex. For Nile Red measurements a twenty minute incubation period was allowed. All binding titrations were repeated at least five times and the averages of each reading were used for any further analysis. A similar technique was carried out for displacement titrations. All data was converted to binding and displacement plots by using the spread sheet software package Quatro Pro and the necessary equations discussed previously.

CHAPTER THREE

THE INVESTIGATION OF LONG WAVELENGTH FLUORESCENT MOLECULES FOR PROBE PROPERTIES.

3.1 INTRODUCTION

Initially the aim of this project was to find a long wavelength fluorescent molecule with properties suitable for it to be used as a probe and eventually in drug-protein studies. Many compounds have been examined from those found in the literature (Section 1.4.5) to those provided by our organic section and externally, which are not yet commercially available and cannot be discussed. This chapter will contain a brief account of a series of dyes which were eventually investigated in protein binding experiments. These dyes consist of the polymethine dyes, DTTC, Indocyanine Green and Merocyanine 540; the xanthene and laser dye Rhodamine 800 and the phenoxazine and phenoxazone dyes, Nile Blue and Nile Red respectively. The reactive dyes were not favoured because of the added complication of direct covalent binding.

Experiments performed on the dyes were initially to find out if they could be dissolved in aqueous media, either directly or via an organic solvent, which is obviously an essential requirement for drug-protein studies. Next, the fluorescence and the stability of the dye in the aqueous media were examined and finally any effects on a change in polarity of the environment had on the fluorescence of the dye was investigated. The latter test being a good indication of possible protein binding of the dye. Most protein ligands undergo a polarity change from their initial polar water environment to the less polar, more hydrophobic binding sites. If the fluorescence of a dye is affected by a change in polarity of its surroundings it may therefore make a good hydrophobic fluorescence probe. The success of any protein binding will be discussed in later chapters.

3.2 EXPERIMENTAL.

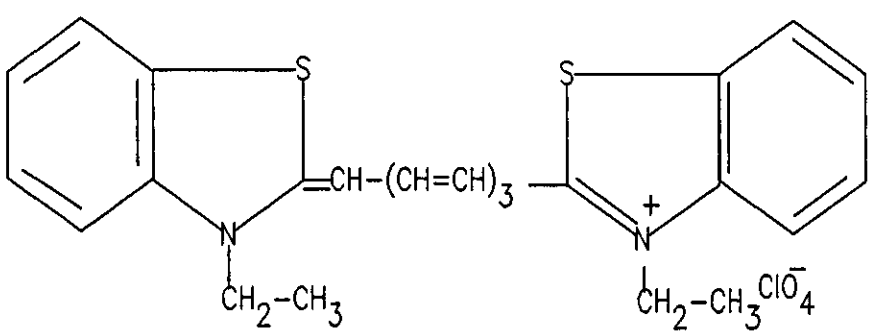
For excitation measurements the slit widths were set at 2.5 nm and for emission at 5 nm. When measuring fluorescence stability of a dye the sample was either illuminated at all times by the fluorimeter source or protected from light in a darkened container to test for photodegradation.

For polarity experiments the solvents used were water, 30% ethanol, 50% methanol, ethanol, methanol, acetonitrile, dimethyl sulphoxide and acetone. The percentage mixtures mentioned, are percentages by volume in water. Solvent polarity was measured using the E_T^n [175, 176] polarity scale, where water (the most polar solvent) has a value of one and tetramethylsilane (the least polar solvent) a value of zero.

All the spectra in this section were recorded on the LS50 fluorimeter with the same parameters and so the results for all the dyes can be compared.

3.3 RESULTS AND DISCUSSION.

3.3.1 DTTC.



DTTC

DTTC was found to be fairly soluble in aqueous media, however to aid solubility and stability it was prepared in a stock solution of methanol and protected from light and stored at -4 °C in accordance with other authors[52,162]. The stock solution was regularly checked for decomposition and it was found to be necessary to prepare fresh stock solution at the beginning of each series of experiments as the dye solution very quickly lost its colour and appeared to fall out of solution.

The excitation and emission spectra of DTTC in aqueous media are shown in Fig. 3.1. Two excitation peaks are shown at ca. 695 nm and ca. 735 nm and are indicative of a dimer-monomer equilibrium[52]. The larger intensity, higher monomer wavelength was used to produce the emission spectra with a maximum at ca. 775 nm. The Stokes' Shift produced in water of ca. 40 nm was fairly small and could be increased if necessary by exciting off peak or using the lower excitation wavelength.

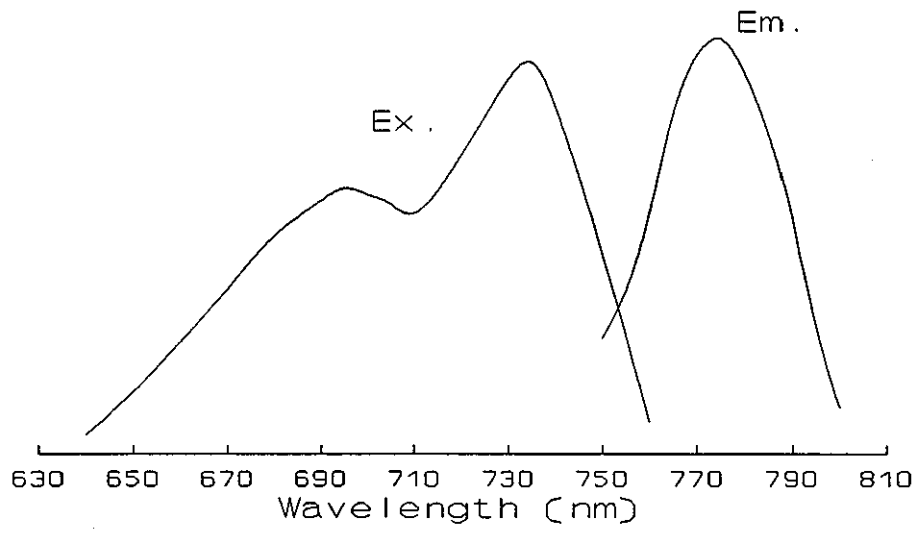


Fig 3.1: The excitation and emission of 1μM DTTC in aqueous media.

Table 3.1: Fluorescence and stability of 1μM DTTC in aqueous media.

	Initial Reading		20 mins.	
	Fluorescence Intensity	λ_{em} (nm)	% Decrease in F.I.	Shift in λ_{em} (nm)
LIGHT	16.1	774	53.3	0
NO LIGHT	16.0	774	54.5	0

The fluorescence of DTTC in aqueous media was found to be fairly small and its stability poor and independent of light (Table 3.1).

Fig. 3.2 shows that as the polarity of the environment increased both the excitation and emission maxima underwent a hypsochromic shift with

corresponding decrease in fluorescence intensity. This was probably caused by a decrease in the dipole moment of the ground state in the more solvating polar solvent[174].

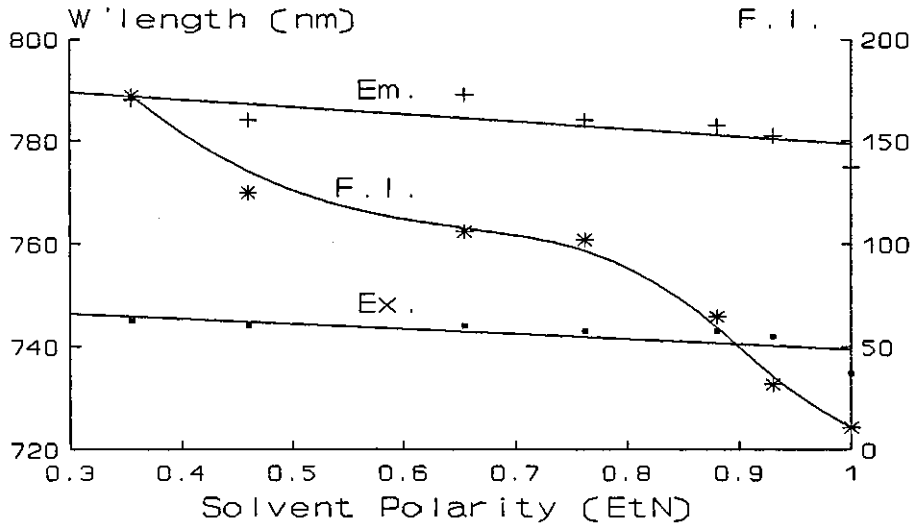
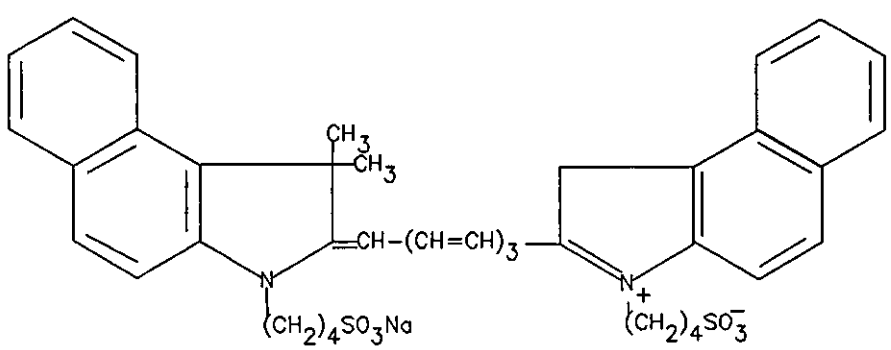


Fig. 3.2: The effect of solvent polarity on 1μM DTTC.

3.3.2 Indocyanine Green.



Indocyanine Green

The stock solution of indocyanine green or IR 125 was prepared in DMSO for solubility and stability purposes, which have been previously reported[52,177]. The stock solution was found to be stable over a period of months with no apparent visible degradation. In aqueous media indocyanine green produced an excitation maxima at ca. 780 nm and an

emission maxima of ca. 805 nm. The Stokes' Shift of ca. 25 nm was found to be too small as it was often difficult to distinguish the emission peak from the Rayleigh Scatter. For this reason an excitation wavelength of 760 nm was selected in aqueous media.

The fluorescence and stability of indocyanine green in aqueous media are shown in Table 3.2. The fluorescence of indocyanine green was of a similar order of magnitude to that of DTTC, however over a period of twenty minutes indocyanine green lost only 10% of its initial fluorescence with no change in emission wavelength. The effect being independent of whether the dye was or was not exposed to light throughout that time. It would therefore appear that indocyanine green is a great deal more stable than DTTC.

Table 3.2: Fluorescence and stability of 1μM Indocyanine Green in aqueous media.

	Initial Reading		20 mins.	
	Fluorescence Intensity	λ_{em} (nm)	% Decrease in F.I.	Shift in λ_{em} (nm)
LIGHT	10.4	806	8.7	0
NO LIGHT	10.2	806	7.4	0

Fig 3.3 shows that the general trend of indocyanine green, like DTTC was a shift to blue of excitation and emission wavelength with increasing polarity of its environment. The changes however are not as large as those of DTTC and the fluorescence of indocyanine green would not appear to as dependent as DTTC on polarity.

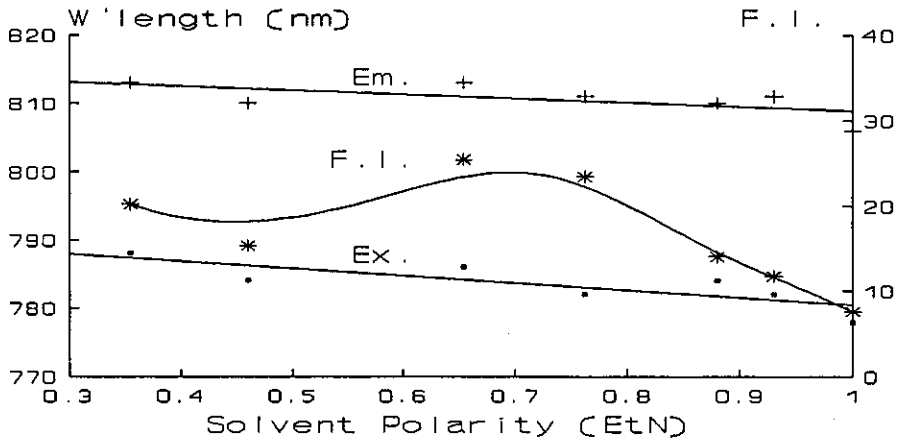
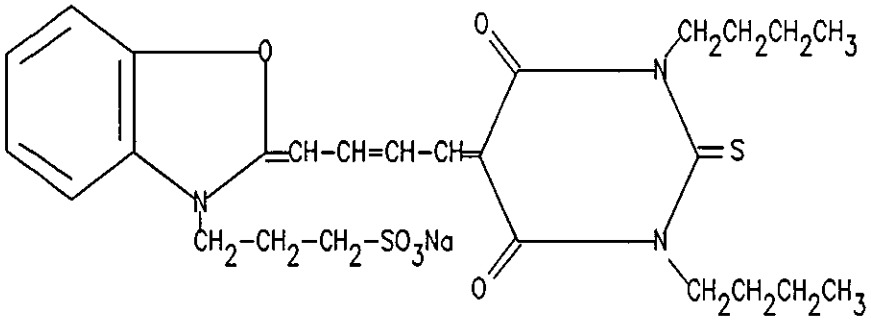


Fig 3.3: The effect of solvent polarity on 1μM MC 540.

3.3.3 Merocyanine 540.



Merocyanine 540

To increase its stability in stock solution Merocyanine 540 (MC 540) was prepared in 50% ethanol solution, protected from light and stored at -20°C. This was the recommended method for storage[178-180]. However, the dye was still seen to visibly degrade over a period of time and so the stock solutions were often prepared for the beginning of each experiment to ensure good reproducibility.

The excitation spectrum of 1μM MC 540 (Fig. 3.4) in aqueous media, consists of two peaks at ca. 495 nm and ca. 536 nm.

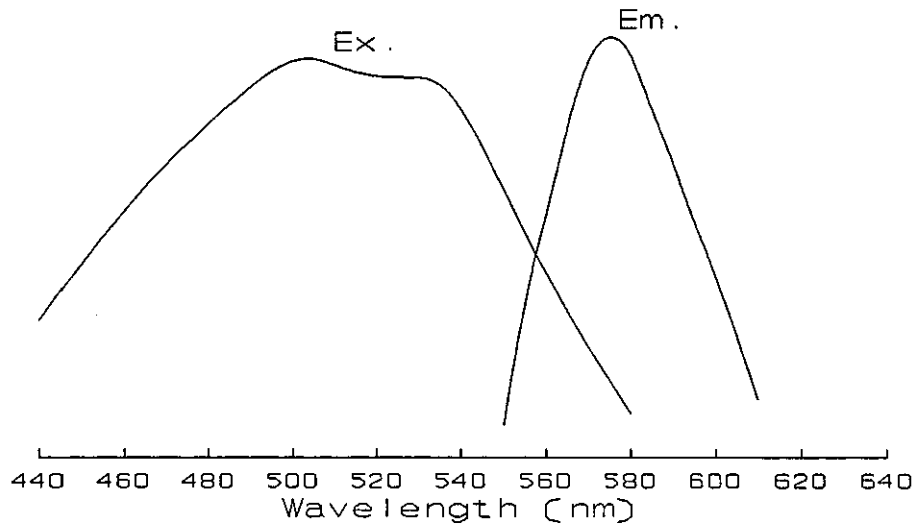


Fig 3.4: The excitation and emission of 1μM MC 540 in aqueous media.

The difference in intensity between the two peaks is fairly small and so an excitation wavelength mid way between of 515 nm was selected. The broad fluorescence peak at 575 nm provided a large Stokes' Shift of 60nm which encouraged the investigation of the dye even though it does not strictly fluoresce in the long wavelength region.

The stability of MC 540 in water, in or out of the light source is also very good (Table 3.3), the fluorescence of MC 540 dropping by only 4% over a twenty minute period. The intensity was also much higher than any of the previous polymethine dyes. However, a major drawback found with MC 540 during experimentation was that of sticking to all

Table 3.3: Fluorescence and stability of 1μM MC 540 in aqueous media.

	Initial Reading		20 mins.	
	Fluorescence Intensity	λ_{em} (nm)	% Decrease in F.I.	Shift in λ_{em} (nm)
LIGHT	150.1	575	3.2	0
NO LIGHT	149.3	577	3.5	0

glassware. It was even very difficult to aliquot out the dye accurately as it stuck to the pipette and this could be a major problem in terms of the accuracy and precision of later studies.

The solvent effects of MC 540 (Fig. 3.5) are similar to those of the other polymethine dyes in that a hypsochromic shift in excitation and emission occurs with a fluorescence decrease, with increasing polarity of the solvent.

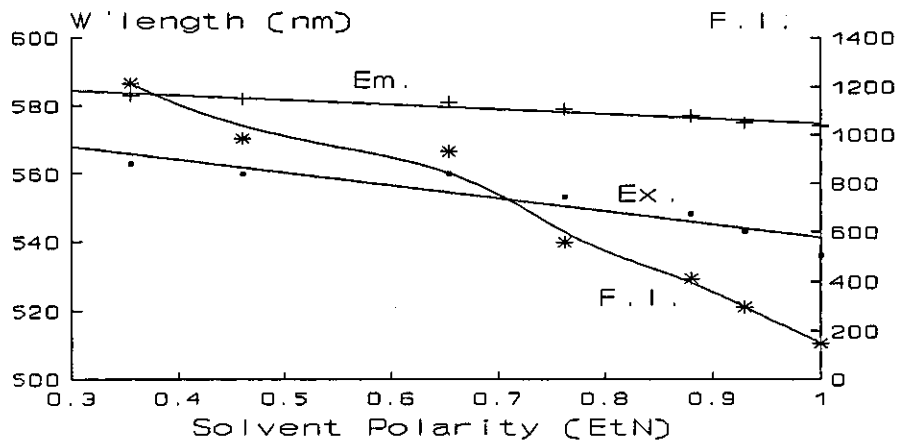
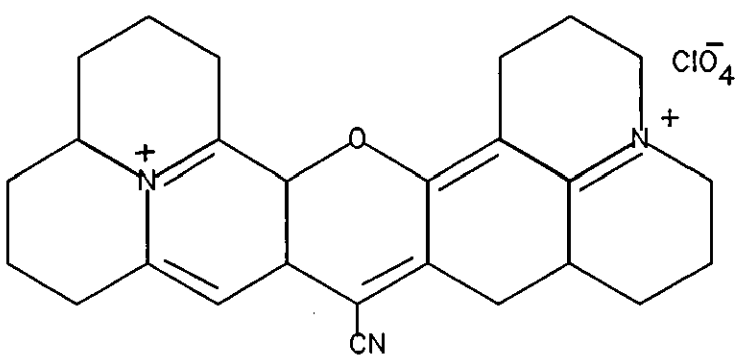


Fig. 3.5: The effect of solvent polarity on 1μM MC 540.

These changes are of a similar magnitude to those of DTTC but more distinguishable because of the larger fluorescence of MC 540.

3.3.4 Rhodamine 800.



Rhodamine 800

Little work has been previously reported on Rhodamine 800 (R800), however to aid its solubility and stability in stock solution it was stored in DMSO at -20°C. The excitation and emission profiles of 1μM Rhodamine 800 in aqueous media are shown in Fig. 5.6. The excitation spectra consists of two peaks at ca. 630 nm and ca. 680 nm which could correspond to different polymers of the dye. The emission maxima of 710 nm necessitated that for aqueous fluorescence studies an excitation wavelength of 660 nm was used to obtain a larger Stokes' Shift. Rhodamine 800 was found to be fluorescent in aqueous media, this fluorescence was also very stable (Table 3.4) and independent of light, which would be expected of a dye that can be excited by such an intense source of light such as a laser.

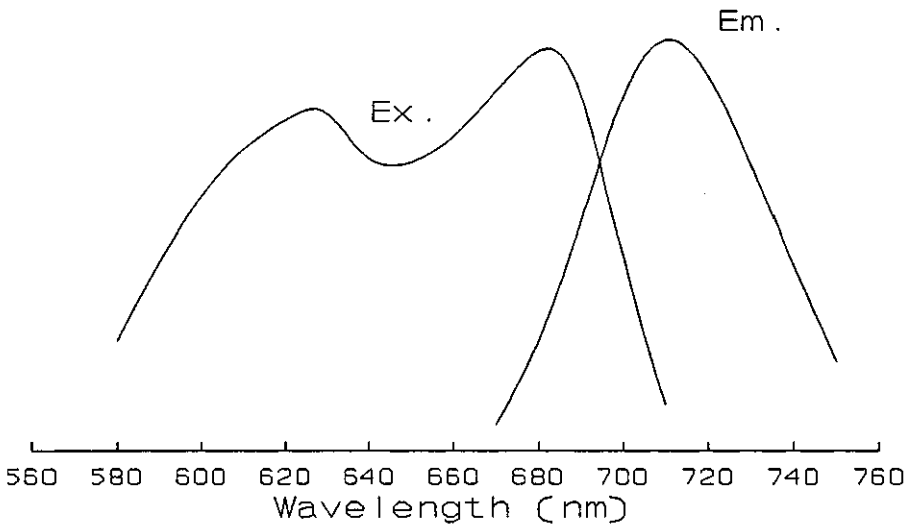


Fig 3.6: The excitation and emission of 1μM R800 in aqueous media.

Table 3.4: Fluorescence and stability of 1μM R800 in aqueous media.

	Initial Reading		20 mins.	
	Fluorescence Intensity	λ_{em} (nm)	% Decrease in F.I.	Shift in λ_{em} (nm)
LIGHT	82.3	710	10.2	0
NO LIGHT	83.9	710	10.4	0

The effects of varying solvents on Rhodamine 800 are shown in Fig 3.7. The results are not as obvious as with some of the other dyes, the changes in excitation and emission wavelengths being quite small. However, the general trend was the opposite in terms of wavelength to that found with polymethine dyes in that with increasing polarity there was a bathochromic shift in excitation and emission wavelengths with a prominent decrease in fluorescence intensity, the explanation of which will be discussed in section 3.3.6.

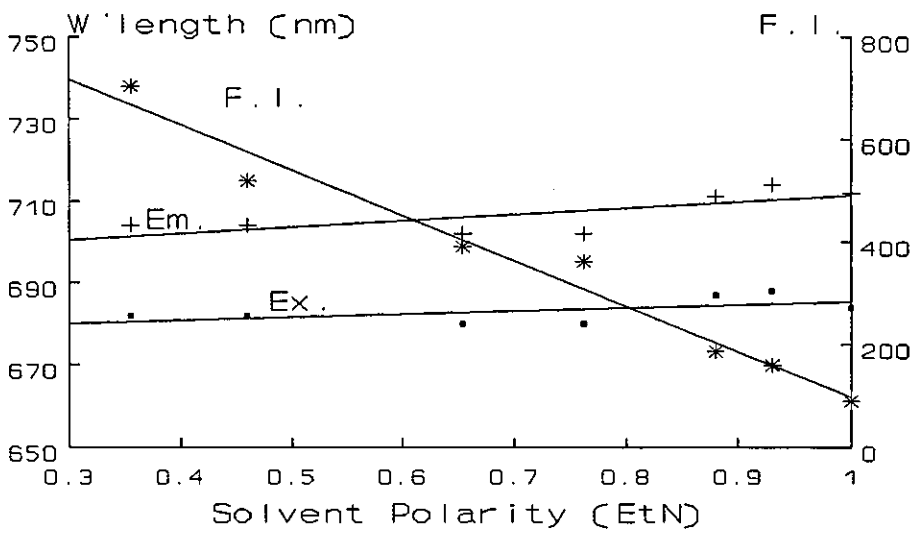
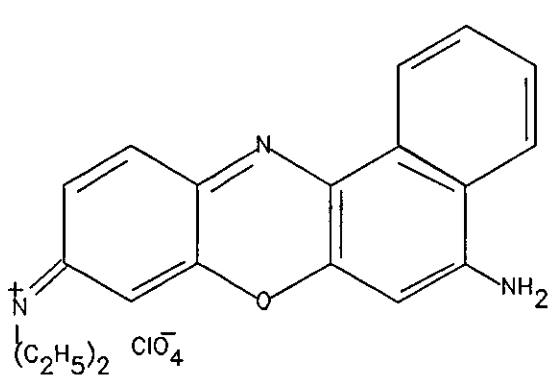


Fig 3.7: The effect of solvent polarity on 1μM R800.

3.3.5 Nile Blue.



Nile Blue

The solubility of Nile Blue in aqueous media was poor and DMSO was found to be the most suitable solvent for obtaining the concentrations required for the stock solution. The dye was stored at -20°C and found to be stable for several months. In aqueous media Nile Blue produced an excitation spectrum with one peak at ca. 638 nm and an emission spectrum with a peak at ca. 674 nm thus a Stokes' Shift of about 40 nm. Table 3.5 shows that the dye was fairly fluorescent in water and that this fluorescence is very stable and unaffected by light.

Table 3.5: Fluorescence and stability of 1μM Nile Blue in aqueous media.

	Initial Reading		20 mins.	
	Fluorescence Intensity	λ_{em} (nm)	% Decrease in F.I.	Shift in λ_{em} (nm)
LIGHT	39.4	674	2.5	0
NO LIGHT	38.9	674	5.8	0

The effect of solvent polarity on Nile Blue (Fig. 3.8), like Rhodamine 800 was fairly small and there were slight discrepancies. However, the general trend was a shift to red in excitation and emission wavelength and intensity quenching with increasing polarity of solvent. The results show that the excitation and emission of Nile Blue is not largely dependent on the polarity of its environment.

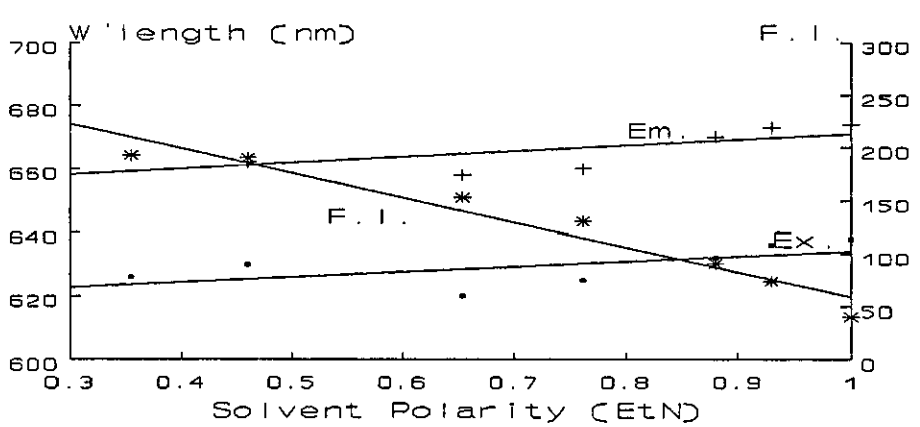
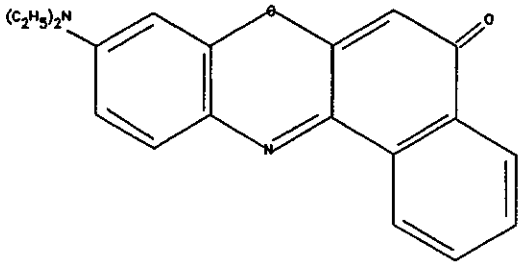


Fig. 3.8: The effect of solvent polarity on 1μM Nile Blue.

3.3.6 Nile Red



Nile Red

The solubility of Nile Red in aqueous media was the worst of all the dyes studied, for this reason Nile Red, as with Nile Blue was stored in DMSO at -20°C[62,154]. The dye was also found to be stable in the solvent for a period of several months.

The excitation and emission spectra of Nile Red in aqueous buffer are shown in Fig. 3.9.

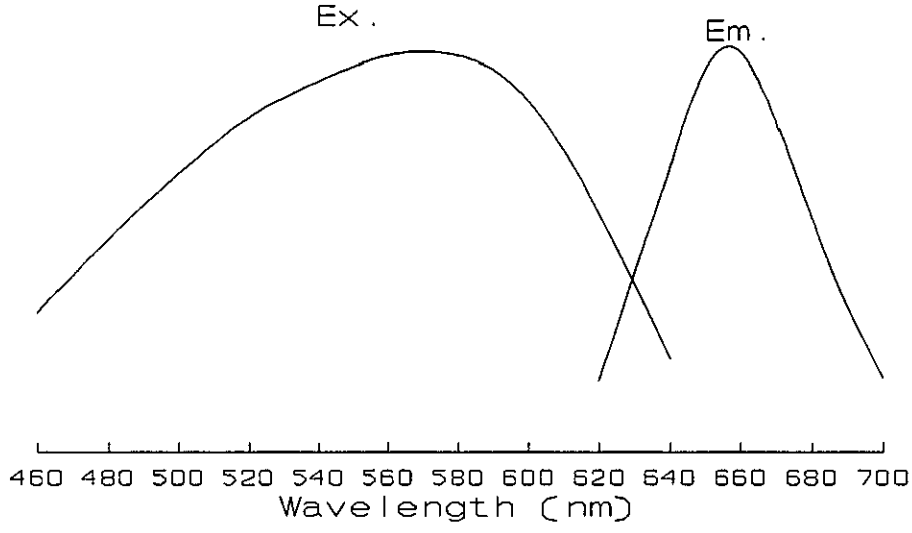


Fig 3.9: The excitation and emission of 1μM Nile Red in aqueous media.

The excitation spectrum shows a peak at ca. 554 nm and a shoulder at 524 nm, while the emission spectra shows a maxima at ca. 660 nm. The absorbances of different concentrations of Nile Red were investigated more closely (Table 3.6). As the concentration of Nile Red increases the

Table 3.6: The effect of Nile Red concentration on its absorbance.

[Nile Red]/ μM	Peak at 584nm		Peak at 520nm	
	Abs. units	% decay after 20 mins.	Abs. units	% decay after 20 mins.
1	0.068	25	0.059	28
5	0.010	38	0.09	43
10	0.22	40	0.25	50
25	0.43	45	0.58	55
50	0.81	50	1.11	55

shoulder at 524 nm becomes a more prominent peak and the peak at ca. 584 nm becomes a less noticeable shoulder. It is therefore probable that the lower wavelength absorbance is associated with the dimeric form of Nile Red and the higher wavelength the monomer. The absorbance seems to fall away quite substantially at either wavelength, the % decay, increasing with increasing Nile Red concentration to a maximum of about 50%.

The fluorescence of Nile Red also undergoes a similar decrease in intensity (Table 3.7), which appears to be independent of light and

Table 3.7: Fluorescence and stability of 1μM Nile Red in aqueous media.

	Initial Reading		20 mins.	
	Fluorescence Intensity	λ_{em} (nm)	% Decrease in F.I.	Shift in λ_{em} (nm)
LIGHT	61.9	660	70.2	-7
NO LIGHT	60.8	660	73.6	-7

any photodecomposition. Fig 3.10 shows this decay more closely, the fluorescence stabilising after about 20 minutes with a 70% drop in intensity. Associated with this intensity quenching is a shift to blue of the

emission maxima. This effect also occurs at higher Nile Red concentration with the rate of quenching and the shift to blue in

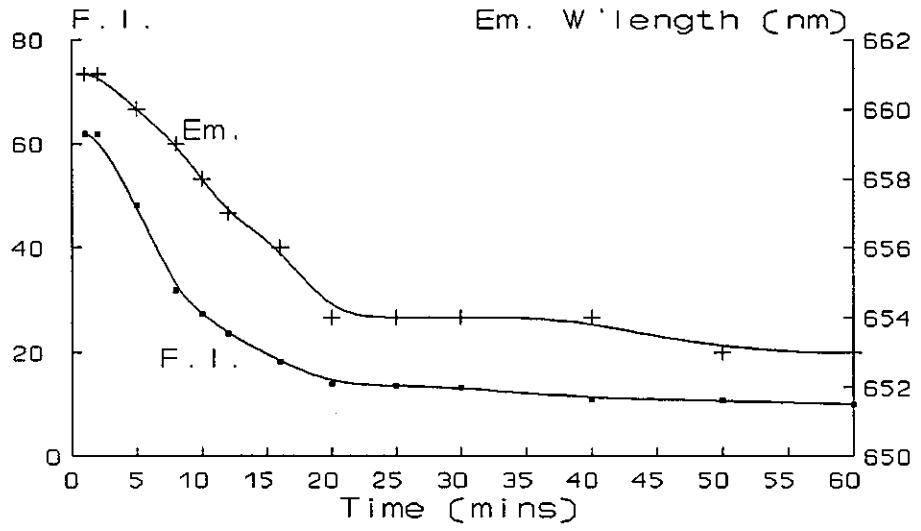


Fig. 3.10: Fluorescence decay of 1μM Nile Red in aqueous media.

wavelength increasing with increasing Nile Red concentrations (Table 3.8). These findings support that fact that Nile Red is forming dimers

Table 3.8: The effect of time on fluorescence decay of increasing concentrations of Nile Red in aqueous media.

[Nile Red]/ µM	20 mins.		60 mins.	
	% Decrease in F.I	Shift to blue (nm)	% Decrease in F.I	Shift to blue (nm)
1	70	7	78	7
2.5	80	7	80	8
5	82	8	85	9
7.5	82	8	86	9
10	83	9	86	10

and higher order polymers because of its insolubility in polar solvents like water[52]. The phenomena takes place over a period of time because

initially as the Nile Red is dissolved in DMSO, aggregation will not be favoured in this less polar environment. As the water molecules begin to replace the DMSO shell around the Nile Red, the dye molecules begin to aggregate in the more polar environment. It would therefore appear from these findings that Nile Red is a hydrophobic molecule. This is further supported by the effects of polarity on the excitation and emission of Nile Red (Fig. 3.11). Nile Red was the most solvatochromic

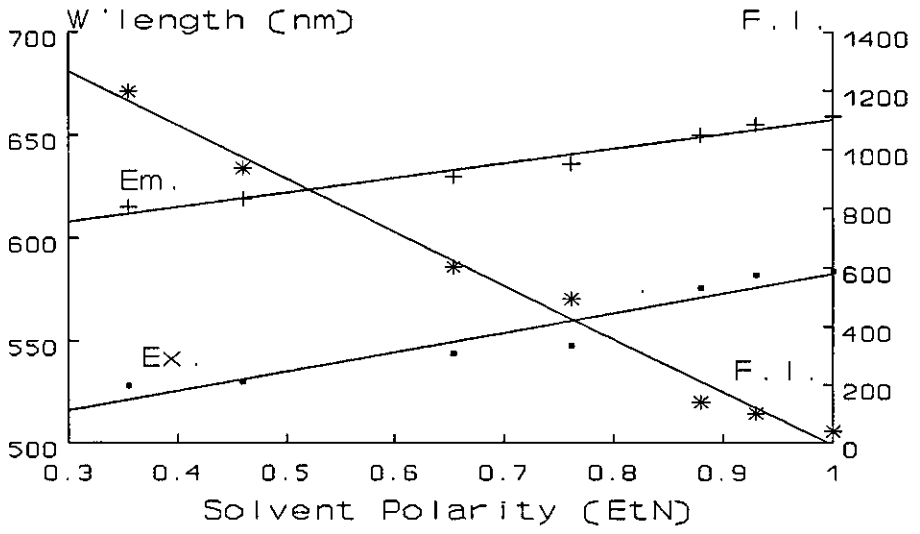


Fig 3.11: The effect of solvent polarity on 1μM Nile Red.

of all the dyes, it was extremely sensitive to changes of polarity in its environment. With increasing polarity, both the excitation and emission wavelength were largely shifted to red, with a corresponding decrease in fluorescence intensity. This is explained by the fact that molecules like Nile Red[62] (with a widely separated electron donor and acceptor group), Nile Blue and Rhodamine 800 have an excited state dipole moment greater than that of their ground state. With increasing polar solvents, solvent molecules can relax around the excited state prior to emission, which lowers the energy of the excited state resulting in a red shift in the emission wavelength[174,181].

The drop in fluorescence with increasing polarity of the environment of the dye is common with all the dyes tested. It can be explained by solvent collision, perhaps dye aggregation and again solvent relaxation. In polar solvents, solvent relaxation around the excited state decreases the energy gap between the first excited singlet state S_1 and the triplet state, T_1, thus facilitating intersystem crossing, thereby quenching fluorescence. For non-polar solvents this energy difference is much larger which favours fluorescence transitions rather than intersystem crossing[181].

It would appear then that Nile Red is hydrophobic in nature and its fluorescence favours less polar environments where aggregation does not occur. This theory was further supported by the fluorescence and stability of Nile Red in increasing percentages of methanol i.e., an increasingly less polar environment (Table 3.9).

Table 3.9: Effect of methanol on the fluorescence of 1μM Nile Red.

% Methanol	Initial Reading		After 20 mins	
	Fluorescence Intensity	λ_{em} (nm)	% Decrease in F.I.	Shift in λ_{em} (nm)
0	60.1	660	74.2	-7
5	61.2	660	36.0	-5
10	65.9	659	27.2	-4
15	80.6	659	26.5	-4
20	88.2	659	13.2	-2
30	103.5	657	0	0
40	133.9	654	0	0
50	197.6	651	0	0

Key: '-' indicates a shift to blue in emission wavelength.

The fluorescence and fluorescence stability of Nile Red increases with increasing hydrophobicity of its environment. Above 20% methanol the quenching effect caused by dimerisation of the dye was inhibited.

The non-ionic surfactant Triton X-100 (polyoxyethylene tert-octylphenol) was also used to test the hydrophobic character of Nile Red. Surfactants form micelles in solution, the hydrophilic heads of the surfactant face the aqueous phase while the hydrophobic tails project inwards forming a hydrophobic pocket. The effect of increasing % Triton on the fluorescence of Nile Red is shown in Table 3.10.

Table 3.10: The effect of Triton on 1μM Nile Red fluorescence.

% Triton	Fluorescence Intensity	λ_{em} (nm)	% Decrease in F.I. after 20 mins.	Shift in λ_{em} (nm)
0	12.6	656	70	-7
0.001	15.6	654	61	-7
0.005	23.2	646	42	-7
0.01	50.7	639	7.3	-6
0.05	377.0	634	0	0
0.1	508.8	634	0	0
0.5	536.3	634	0	0
1	501.4	634	0	0

Key: '-' indicates a shift to blue in emission wavelength.

Triton causes a massive enhancement and shift to blue of the emission maxima of Nile Red along with complete fluorescence stability. This effect could be due to the fact that the surfactant is increasing the solubility of the hydrophobic Nile Red. However, this is very unlikely judging by the massive changes in fluorescence found. It is more likely that the Nile Red is sitting in the hydrophobic pocket of the surfactant micelle, causing the enhancements associated with a hydrophobic

environment and also forming a protective shell around the Nile Red molecule inhibiting dimer formation and excluding quenchers.

A problem throughout the work involving Nile Red in aqueous media was the deposition of a layer of the dye on inner walls of vessels in which the dye solution was contained. Such a characteristic has obvious problems in the accuracy and precision of binding titrations and as a result all glassware was washed using the procedure listed in section 2.3.5. An experiment was performed to estimate the amount of Nile Red which had fallen out of solution and thus formed this layer. Initially, a series of 5 ml volumetric flasks were set up containing the same concentration of Nile Red in aqueous solution. At time intervals ranging from 2½ minutes to 120 minutes, the Nile Red solutions were removed, the volumetrics dried and then acetone added (5 ml) to remove the Nile Red layer. The fluorescence of the deposited Nile Red in acetone for both 5 and 10 μM solutions at each time interval were then measured. These results were then compared to a calibration plot of increasing Nile Red in acetone which was previously obtained (Fig. 3.12) and the

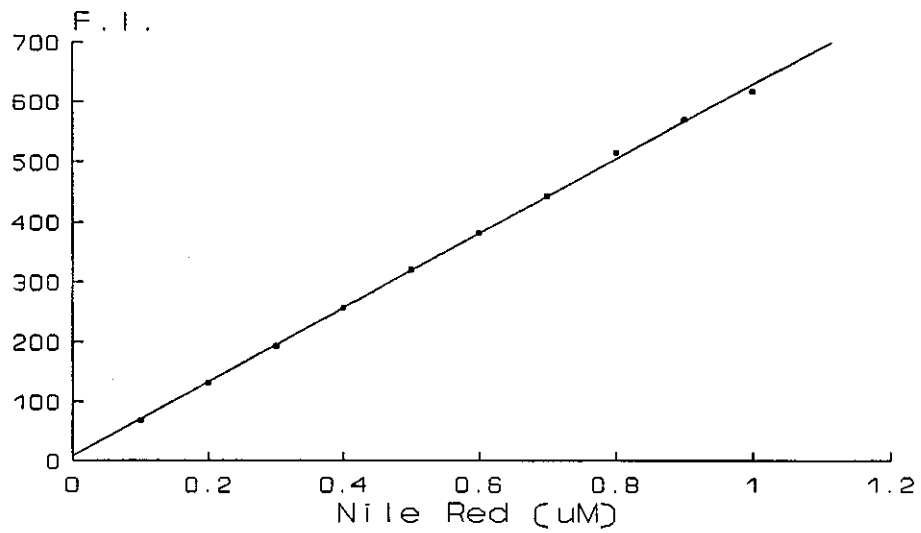


Fig. 3.12: Calibration plot of Nile Red in acetone.
(Emission maxima 611nm, correlation coefficient=0.996)

concentration of Nile Red interpolated. The amounts of Nile Red deposited are shown in Table 3.11, and indicate that approximately 8% of Nile Red falls out of solution. The fact that this Nile Red deposit is occurring along side the fluorescence quenching suggest that these effects are inter-related. This fall out, along with the aggregation and poor solubility of the dye in aqueous media could explain the drop in fluorescence.

Table 3.11: Amount of fall out in 5μM and 10μM Nile Red aqueous solutions after twenty minutes.

Inital [Nile Red] / μM	F.I. of reconstituted Nile Red in acetone	[Nile Red] reconstituted	% fall out
5	28.5	0.43	7.6
10	58.5	0.76	8.6

3.4 CONCLUSIONS.

This chapter contains a brief overview of some dyes tested for use as fluorescent probes, a more detailed report can be found in the thesis of Summmerfield[52] a fellow worker in the authors research group. The major disadvantages associated with cyanines are their poor stability both physically and chemically. Both MC 540 and DTTC visibly lost their colour in stock solution after a few hours. Miyazoe and Maeda[182] showed that polymethine dyes in solution could be preserved for about a month in a refrigerator and less than a week in the dark. Although these techniques were used, they were still far from ideal and the dyes often had to be prepared freshly. However, over a period of an hour indocyanine green and MC 540 were fairly stable and resistant to light unlike DTTC which degraded rapidly. All the polymethine dyes were affected by the polarity of their environment especially MC540 and DTTC which indicated that they may make good fluorescent probes.

No problems of stability were found with either Rhodamine 800 or Nile Blue and the fact that their fluorescence was affected by solvent polarity especially Rhodamine 800 encouraged their investigation. However, both dyes, like MC 540, have a fairly high fluorescence in aqueous media which is a disadvantage in terms of any future binding assays.

The dye that appears most suited to use as a fluorescent probe was Nile Red. The stability of Nile Red in organic solvent was excellent ensuring it could be stored for months as a stock solution. The fluorescence quenching in aqueous media, probably due to aggregation meant that if a period of 20 minutes incubation was allowed its fluorescence was low. Thus, in any future binding studies the background from any unbound Nile Red would be small which is essential in any binding titrations. The most encouraging aspects about Nile Red were its large Stokes' Shift compared to that of the polymethine and the fact that its fluorescence characteristics were profoundly affected by changes in polarity of its environment. The only drawbacks of Nile Red were the poor solubility in aqueous media which was partly overcome by dissolving it initially in DMSO and its leeching and sticking properties which could produce errors and meant all glassware had to be constantly cleaned.

CHAPTER FOUR

SERUM ALBUMIN

4.1 INTRODUCTION.

As the simplest plasma protein to prepare in a relatively pure form, albumin has been known for over a century. Its name derives from the early name for proteins albumen, derived from the latin word *albus* meaning white in this case the white of an egg. Albumins in serum were initially defined as those proteins soluble in water in the absence of salts, in contrast to the globulins which were insoluble in such conditions. With the advent of electrophoresis in the 1940's, it was found that one electrophoretic fraction produced a very prominent peak and accounted for at least 95% of the protein in the albumin fraction. The protein of this peak was renamed serum albumin (or plasma albumin), the globulin fraction turned out to be a highly complex mixture of proteins. A detailed historical perspective of the developments of methods for isolating and purifying serum is included in the review by Peters[183].

Serum albumin has been assigned numerous physiological roles. It serves as a depot and transport protein for numerous endogenous and exogenous compounds including fatty acids, thyroid hormones, steroid hormones, numerous pharmaceuticals and bilirubin. The solubility of the compounds increasing while the toxicity of the bilirubin is greatly reduced. The biosynthesis of albumin is closely dependent on the amino acid supply, hence albumin has a minor nutritive role, acting as a reservoir and contributing about 5% of the amino acids used by peripheral tissues. Through its osmotic effect, albumin is a major contributor to the maintenance of the circulating fluid within the vascular system. In fact, albumin itself is dripped into the veins of patients who have various degrees of circulatory collapse or metabolic depletion. Serum albumins are also implicated in the facilitated transfer of many ligands across organ circulatory interfaces such as in the liver, intestine, kidney and brain[184] and evidence suggests the existence of an albumin cell surface receptor[185]. In addition to blood plasma, serum albumins are also found in tissues and secretions throughout the body, extravascular protein comprises 60% of the total albumin.

4.2 STRUCTURE, SEQUENCE AND PHYSICO-CHEMICAL PROPERTIES.

Serum albumin is an example of a simple protein, in that it consists only amino acid residues. It alone among the major plasma proteins contains no carbohydrate, the absence of which is a classic criterion for the testing of the purity of albumin. The first clues to the primary structure of serum albumin came from the early work of Foster[186], he suggested that albumin was composed of four domains which could rearrange to generate a range of binding sites or cavities which were essential for the main binding function of albumin. Later in 1975, the groups of Meloun[187] and Brown[188] independently reported the complete amino acid sequence of human serum albumin (HSA, Fig. 4.1). Brown's group also reported the sequence of bovine serum albumin (BSA, Fig. 4.2). A comparison of the amino acid composition of the two serum albumins is given in Table 4.1, they are both quite similar in composition.

The amino acid sequences of both HSA and BSA contained evidence for Foster's domain hypothesis and revealed evidence of three similar homologous regions. On the basis of the internal sequence homology, Brown proposed a three domain structure for serum albumin. These domains having the following amino acids residues: 1-190 (No. 1), 191-382 (No. 2), 383-582 (No. 3). He then proposed that each domain consisted of two homologous sub domains[189]. It was assumed that ligands could not bind in the interfaces between the domains as suggested by Foster, but would bind in cavities within each domain. More recent work by He and Carter[190] on HSA support this theory. They define three structurally homologous domains (denoted I, II and III) each domain consisting of two smaller sub domains, A and B. The intra-domain connections between sub domains IA-IB, IIA-IIB and IIIA-IIIB consist of extended polypeptide from residues Lys 106 - Glu 119, Glu 292 - Val 315 and Glu 492 - Ala 511 in domains I, II and III respectively. The inter-domain connections represent a continuation from the C terminal portion of IB and IIB to the N terminals of IIA and IIIA.

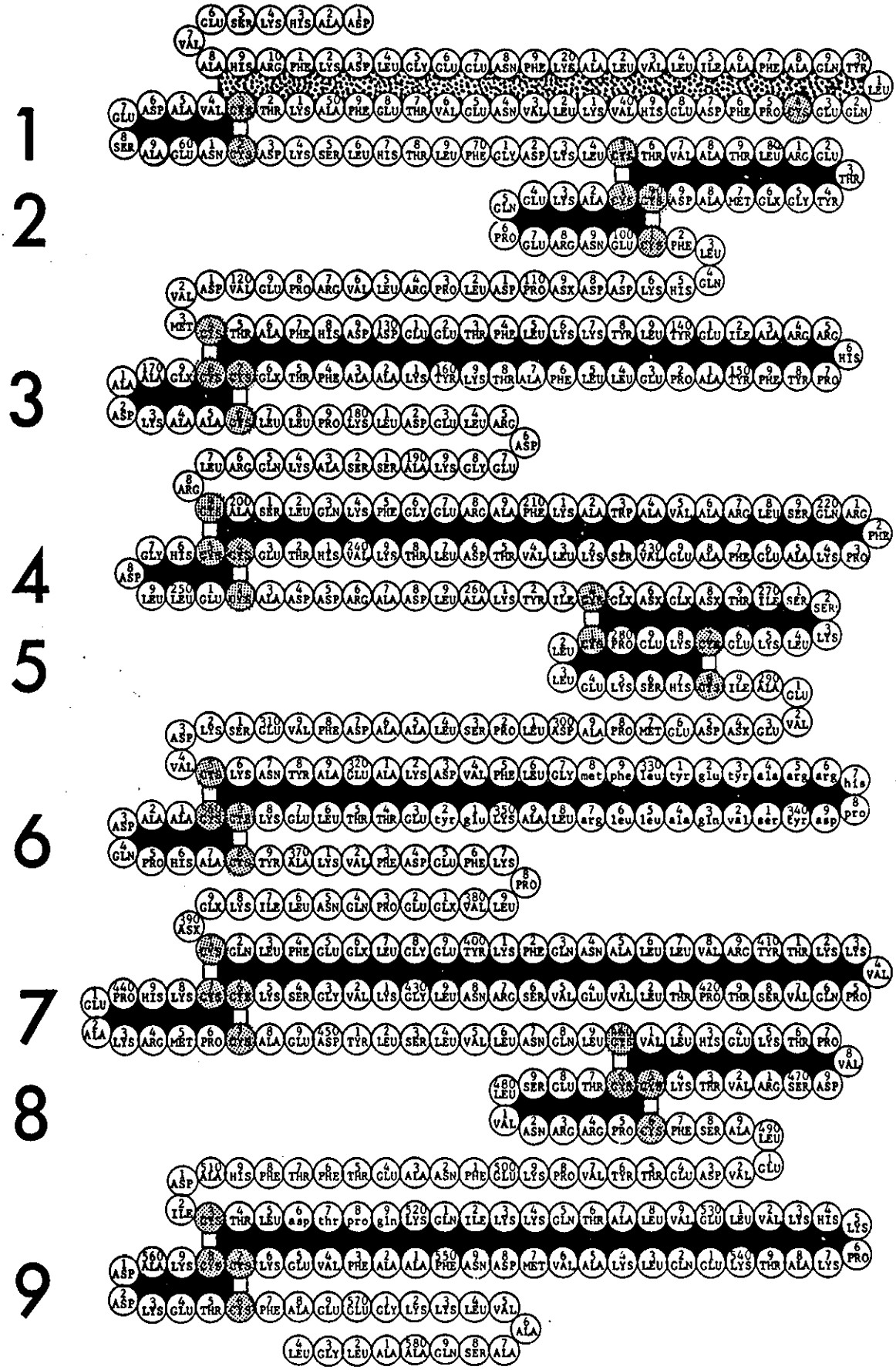


Fig 4.1: Amino acid sequence of HSA[188], showing cysteine linkages, dark areas show areas of similarity with BSA.

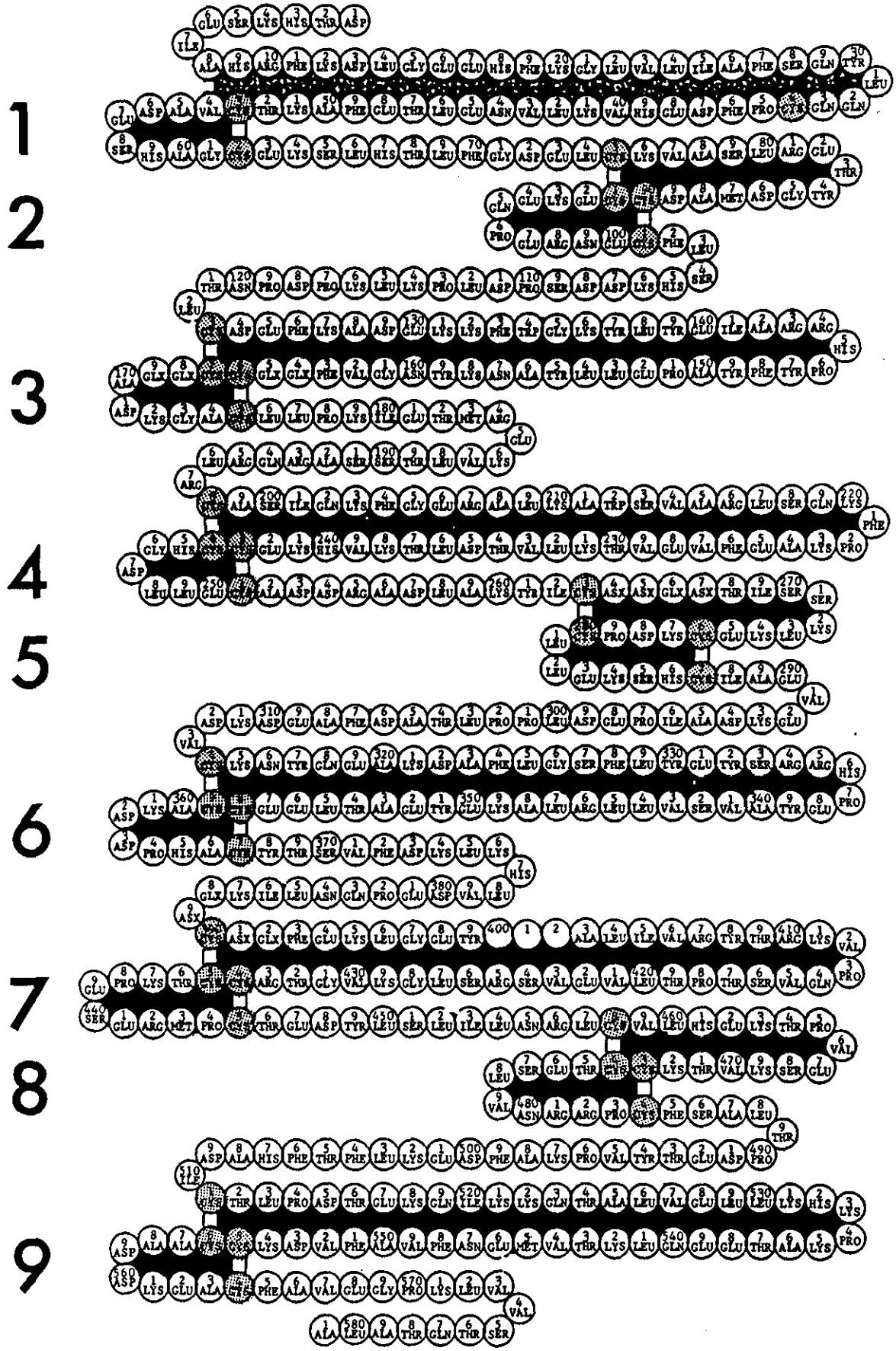


Fig. 4.2: Amino acid sequence of BSA[189], showing cysteine linkages, dark areas show areas of similarity with HSA.

Table 4.1: Comparison of amino acid residues in Human and Bovine Serum Albumin[183].

Amino acid	Human	Bovine
Aspartic acid	39	41
Asparagine	15	13
Threonine	30	34
Serine	22	28
Glutamic acid	60	59
Glutamine	23	20
Proline	25	28
Glycine	12	15
Alanine	63	46
Cysteine/2	34	34
Cysteine	1	1
Valine	39	36
Methionine	6	4
Isoleucine	8	14
Leucine	61	61
Tyrosine	18	19
Phenylalanine	30	27
Histidine	16	17
Lysine	58	59
Tryptophan	1	2
Arginine	23	23
Total	584	581
Molecular weight calculated	66,248.3	66,210.2

Many authors[183,186,189 and 191] have proposed theories of the three dimensional structure of serum albumin throughout the years, however it is only recently that the true structure of HSA has been solved and interpreted at atomic

resolution[190]. The six homologous sub domains assemble to form a heart shaped molecule in which the N and C terminal portions are nearer than originally believed. The main secondary structure present in HSA is the α-helix. Altogether 67% of HSA is helical with the remainder in turns and extended polypeptides. There are 28 helices which range in size from 5-31 amino acids in length and can be grouped into ten principle helices within each domain. The disulphide pairings in the primary structure of HSA occur as predicted by Brown. In the three dimensional structure the 17 disulphide linkages occur primarily between α-helices, often distorting the local helical conformation.

Other mammalian species have albumins with similar structures and properties to those of HSA especially BSA. The amino acid compositions of mammalian albumins exhibit many common features and these proteins tend to cross react serologically. Work on the phylogenetic origins of albumin has been reviewed by Peters[183]. It appears that the homology between the domains, sub domains and disulphide bridges has led to the view that both human and bovine albumin originated from a simple precursor consisting of 77-97 amino acids arranged as a peptide loop containing a disulphide bond. However, despite their similarities, different albumins have a tendency to show a marked difference in their capacities for binding drugs and other ligands and between their physical, chemical and spectroscopic properties[77,86]. It is possible to explain many of these differences by differing amino acid compositions. For instance, human albumin with one tryptophan has an absorptivity about 20% less than BSA which has two. If albumin is excited at a wavelength of between 295nm and 305nm to remove all fluorescence either directly or by energy transfer from tyrosine, the fluorescence of BSA will be three times that of HSA.

Albumin in general is characterized by its high solubility, total net negative charge, stability and flexibility and by its ability to bind a number of ligands. More than 200 positive and negative charges are distributed over an albumin

molecule giving it a hydrophillic character, thus contributing to its high solubility in aqueous media. The titration curve in the pH range 2-12 can be closely reconstructed as a composite of the ionisable groups of the constituent amino acids. At a plasma pH of 7.4 albumin has a net negative charge of 18 but it can nevertheless interact strongly at this pH with anions as well as cations.

Albumin is known to undergo many changes in conformation with changing pH. For instance on the more acidic side of neutrality at least two transitions are known to exist. In the region of pH from 5-7 the only transition is really the neutral-base, of N-B transformation which takes place between pH 7 and 8 and is characterised by a small net loss in α-helical structure[191]. Kramer *et al.*,[193] have described the effect of the N-B transition on the binding of warfarin to HSA while Seki and co-workers[122] showed that the N-B transition appeared to effect HSA more than BSA in terms of fluorescent probe binding. The second stage of the transition is the acid expansion of the albumin when it becomes larger and more asymmetrical, this occurs in the pH range of 2.6-3.8. Basically in high acidic media the albumin molecule expands by dissociation of the individual loops of the peptide chain into a long viscous molecule, which results in an exposure of hydrophobic regions, hence a decrease in solubility in aqueous media.

On the alkaline side of neutrality, an isomerization has been detected. It is known as the 'A'[194] or 'B'[192] form and is probably a rearrangement of the intramolecular disulphide bonds, catalysed by a single, free sulphydryl group of the protein. A more extensive unfolding of the albumin takes place in a narrower interval of pH between 11.2 and 12.0.

Unlike pH transitions, the thermal unfolding of albumin appears to involve broad continuous transitions with no detectable intermediate stages. Thermal transitions may represent the sum of simultaneous, but independent unfoldings of different regions of the molecule[192].

The structural and physico-chemical properties of serum albumin underline its unique ability to bind and transport a wide range of small molecules or ligands. Changes induced in these physical and chemical properties of serum albumin on the binding of ligands provide a way of studying ligand albumin interactions. Theories obtained from these studies on the location of the binding sites of certain ligands, especially drugs will be discussed in the following section.

4.3 DRUG AND OTHER LIGAND BINDING SITES ON SERUM ALBUMIN.

Literally hundreds of in vitro studies have supplied information on the binding of drugs to serum albumin especially HSA. Serum albumin associates with a number of drugs both charged and neutral. The majority of research involves the binding of acidic drugs to serum albumin of which it is known to have high affinity for, although there is evidence of basic drug binding.

Initial work by Weber and co-workers[101-103] involved the binding of fluorescent probes to proteins like serum albumin to study protein structure and how and where the hydrophobic probes bound to the protein. Later Chignell[76,195] and Meyer and Guttman[196] by using circular dichroism, equilibrium dialysis and dansyl derivatives of amino acids stated that there were only a small number of high affinity binding sites on HSA, that competitive displacement of drugs and fluorescent probes could be achieved and that the amino group of lysine was involved in the attachment of anionic drugs.

Two specific sites were characterised using fluorescent probe studies by Sudlow *et al.*,[112] and were designated sites (i) and (ii). Site (i) was the warfarin binding site. Warfarin and 5-dimethylaminonaphthalene-1-sulphonamide were used as selective probes for this binding site. Site (ii) was labelled with dansylsarcosine and ANS. By observing the competitive displacement of these fluorescent probes, a number of drugs were associated to either one or both of these specific sites on HSA. Sudlow like Chignell before concluded that the

binding sites probably involved both hydrophobic and electrostatic attachments.

In a similar way to the above Müller and Wollert[197] used tritiated L-tryptophan as a marker for a single binding site on HSA. While Mudge *et al.*,[198] used cholecstographic agents to identify the binding sites identified by Sudlow[112] and like his group found evidence of a third binding site. Sjöholm *et al.*,[199,200] in 1979 used warfarin and diazepam as markers for sites (i) and (ii) respectively and proposed that digitoxin and its analogues bind to a third high affinity site on albumin. It was also suggested that there may even be more than these three binding sites for drugs on albumin and that binding of these drugs may be both competitive or allosteric, as in some cases the binding of drugs increased the affinity of others.

Goya *et al.*,[201] synthesised a fluorescent probe from a coumarin derivative which was displaced from its primary binding site on HSA by digitoxin. Furthermore, Sudlows' site (i) and site (ii) drug did not significantly displace the fluorescent probe. The results clearly supported previous theories[199] that the digitoxin site was independent of sites (i) and (ii). However, studies involving the use of DNSA and dansylsarcosine to investigate the location of the L-tryptophan receptor site on HSA in relation to the drug sites (i) and (ii), have shown that distinction between the sites is not clear cut[121]. L-tryptophan with a single binding site on HSA displaced both probes in a competitive fashion suggesting that sites (i) and (ii) may be overlapping. It was suggested that site (ii) drugs are:

aromatic carboxylic acids which are largely ionized at physiological pH. The negative charge being located on the COO^- group away from the non-polar region.

while site (i) drugs are:
aromatic acids with a delocalised negative charge generally at the centre of a large non-polar region.

Wanwimolruk and Burnett[202], however, found that the N-B transition involved a change in conformation of site (i) resulting in increased binding of drugs and fluorescent probes. No corresponding effect was found with site (ii) suggesting the two sites are quite separate.

Fehske *et al.*,[203] also discovered that the warfarin binding site (site (i)) is not as easily distinguished as previously thought. It was found by the use of competitive displacement that site (i) consisted of two overlapping sub units for warfarin and azapropazone. Drugs being able to bind to neither, one or both of these binding sites.

Based on a comprehensive survey of competitive binding results available in the literature Kragh-Hansen[204] proposed that there are at least six binding regions on the albumin molecule. A summary of his findings are given in Table 4.2. It was suggested that binding regions number 1, 4 and 5 are probably very specific, whereas regions 2, 3 and 6 seem to be less specific and capable of high affinity interactions with several different ligands. Although only one of these regions was assigned as a drug binding site (region 6 which probably corresponds to Sudlows' site (i)), the author agreed that there was almost certainly other important drug binding areas. However, it was stated that caution must be shown with competitive binding results. For example, before two high affinity drugs or ligands are assigned to the same binding area it is essential to ensure that the decrease in binding of one of the ligands is caused by the addition of the other. It may be that any drop in binding could be due to changes in conformation of the protein on binding of the second ligand or in fluorimetric study by energy transfer from the first ligand to the second. Another important factor is that drugs/ligands may not only bind to one or two high affinity sites but also to a greater number of low affinity sites.

This theory was supported by Taira and Terada[205] who found that for certain drugs and ligands there are a definite number of specific high affinity sites and an unlimited number of low affinity sites. The latter being on the hydrophobic surface of serum albumin.

Table 4.2: A Scheme for Binding Regions Located on Serum Albumin[204].

Binding region or site	Ligand bound with high affinity
1	Long chain fatty acids
2	L-thyroxine
	L & D-tryptophan
	Octanoate
	Chloroazepate
	ß-iodobenzoate
	chloride ion
3	Bilirubin
	Certain dyes
	Iopanoate
4	Cu^{2+} and Ni^{2+} ions
5	Haemin
6	Salicylate
	Sulphaethidole
	Sulphathiazole
	Chloropropamide
	Tolbutamide
	Indomethacin

More recent research[206] has shown that drugs like benzodiazepenes do not bind to site (ii) only and it is not necessarily true and confusing to classify the binding of drugs to a certain number of binding areas or sites. Panjehshahin *et al.*,[86] also found that bovine, dog, horse and sheep albumin have binding sites for warfarin and dansylsarcosine with similar properties to the so called sites (i) and (ii) on human albumin, while the warfarin binding site and to a lesser extent the dansylsarcosine site of rat albumin have different characteristics from these sites on other albumins studied. It has also been found that the N-B transition of albumin affected drug binding to the protein. As the 'B' form becomes favoured with increasing ionic strength, the hydrophobic areas of serum albumin are exposed which encourages the binding of warfarin and phenylbutazone[207]. This effect being stronger in HSA than BSA[122].

It has become evident from binding studies and crystallography[208,209] that the principle binding regions in serum albumin are located in the sub domains IIA and IIIA. The binding area in IIA is believed to resemble Sudlows' site (i) as this is where warfarin binds. The amino acid residues Lys 199 and Trp 244 conserved in mammalian albumin, play an important structural role in this binding site, by helping to limit solvent accessibility and form an asymmetric hydrophobic pocket. The binding site in sub domain IIIA (Sudlows' site (ii)) involves the residue Tyr 41 and is another hydrophobic pocket formed by residues around this area. The authors report[208,209] that this binding area (IIIA) is the most accommodating and active on serum albumin and it could be possible that the so called Sudlow site (iii) could also be situated here. The absence of observed ligand binding by the homologous sub domain IA is explained by the structural differences between this and sub domains IIA and IIIA. In IA there is an extended region of polypeptide after Lys 62 which allows the helices involved to expand and thereby effectively eliminating the potential binding pocket in this region.

Acidic, anionic drugs are not the only ligands that bind to serum albumin. Fatty acids have been studied a great deal and it has been shown that albumin binds medium and long chain fatty acids at different sites[204]. Hsia[209] has shown by using a spin label probe for the bilirubin site on albumin that fatty acids of less than ten carbon atoms competitively displace the spin label while fatty acids of a longer chain length enhance the binding allosterically. It is believed that the binding site for ten or fewer carbon atom fatty acids is similar to that of the indole/benzodiazepene binding site[210]. The site for the medium chain fatty acids has been identified with the use of 5-(dimethylamino) naphthalene-1-sulphonic acid[20]. Evidence has also been found that the binding site for short chain fatty acids (7 or less carbon atoms) may lie in the same region as the warfarin binding area of HSA[211].

The binding of basic drugs to albumin has not been studied to anywhere near the extent of acidic drugs, probably because it is believed that the binding of such drugs is not critical. However, several authors have studied the binding of catecholamines[212], diazepenes[213], adrenergic beta receptors antagonists[31,214-218] and quinine[123] to serum albumin in conjunction with their studies involving α_1-acid glycoprotein. Serum albumin appears to bind these drugs but to a much lesser extent and lower affinity than α_1-acid glycoprotein.

In general, then it is believed that serum albumin has several areas of attachments for ligands, the binding being mainly hydrophobic but may also involve, electrostatic, Van der Waals and hydrogen bonds. These areas of attachment, it would appear, exhibit a certain degree of adaptability which allows some of them to bind a wide range of chemical compounds. However, the degree of adaptability is not unlimited and in some cases a tiny change in conformation of the drug/ligand can largely affect its binding characteristics. For example, iopanoate and iophenoxate (which differ by an amino group and a hydroxy group respectively), are found to bind to different binding sites on HSA[198]. Drug binding to serum albumin in some cases can also be stereospecific D-tryptophan binds to HSA with a binding constant one

hundred fold lower than L-tryptophan[219], S(-) phenprocouman has a two fold higher association constant than the R-(+) form. On the other hand the affinities of D- and L-warfarin[220] are very much alike. Narayanan[221] has recently reviewed the use of serum albumin as a chromatographic support for chiral resolution.

It is obvious that the binding of drugs or ligands to serum albumins is not as clear cut as some early researchers would wish to believe and that many factors must be considered when studying drug/ligand serum albumin interactions[196].

4.4 CHANGES IN SERUM ALBUMIN CONCENTRATION IN THE BODY.

A decrease in albumin concentration or hypoalbuminemia in the body is caused by many diseases (Section 1.1.8) as a result of a decrease in its synthesis or an increase in its removal. Determination of albumin in urine has gained in importance because many diseases have been associated with an increased albumin excretion rate in the urine[62]. For example, an increased albumin excretion rate is recognised as an early indicator of diabetic nephropathy and occurs in ca. 45% of people with insulin dependent diabetes[222]. As diabetic nephropathy is treatable at an early stage, its detection, monitoring and treatment are valuable for prevention of a disease in glomerular function. Also, diabetic nephropathy in its later stages is connected to renal failure and so screening for increased albumin concentrations or microalbuminurea (20-300 mg/l of HSA in urine) is an essential part of routine diabetic care[223]. In fact, it would appear that testing for microalbuminurea would be an essential part of routine health care, especially in early pregnancy where microalbuminurea appears approximately one and half weeks before pre-eclampsia. Screening can also help as an indicator for acute or chronic renal diseases, surgical trauma, arterial hypertension and renal and postural haematuria[163].

Although a few methods for monitoring an increase in albumin concentration in the urine are commonly used, in routine clinical analysis they lack sensitivity and selectivity or they are based on the use of expensive antibodies to albumin. Several dye binding assays are known for the quantitative determination of albumin in various biological fluids[21,62,163,224-226]. Generally, as with the other techniques, these methods are not sensitive enough for the concentrations necessary for screening microalbuminurea. Also, it is highly likely that the dyes are binding to the total protein rather than just albumin. Further investigation in such areas is therefore necessary.

4.5 EXPERIMENTAL.

All procedures given in chapter two were followed. All fluorescence measurements shown were in mes buffer pH 6.9 and recorded on both the MF44B and LS50 fluorimeters. Thus the results obtained can only be compared where stated and when shown on the same graph or table.

All excitation wavelengths used were taken from excitation scans of the sample. An excitation wavelength of 550 nm was used for Nile Red in all binding and titration experiments and double probe experiments. An excitation wavelength of 370nm was used for ANS. In the double probe experiments each probe was excited and measured independently. Nile Red was always measured first after twenty minutes followed by ANS whose fluorescence was not so time dependent.

For FIA experiments time scans were taken using fixed excitation and emission wavelengths. Slit widths were normally set at 10 nm.

Structures of all displacing ligands are shown in Appendix I.

4.6 RESULTS AND DISCUSSION (STATIC MEASUREMENTS).

4.6.1 Preliminary Investigation of the Binding of the dyes to Serum Albumin.

DTTC

From Fig. 4.3 it can be seen that when DTTC was added to BSA and HSA there was a change in fluorescence properties of the dye which would suggest that the dye is perhaps binding to both serum albumins. The approximate doubling of fluorescence intensity and 2 nm shift to red in emission wavelength of the fluorescence signal for DTTC when added to albumin is a similar change which was observed in the less polar solvents (Section 3.1) and suggests that DTTC may well be sitting in a less polar (hydrophobic) environment and undergoing a similar mechanism. However, the changes observed are very poor compared with the lower wavelength probes found in the literature and suggest along with the poor stability of the complex that DTTC is a very poor fluorescent probe for binding to albumin.

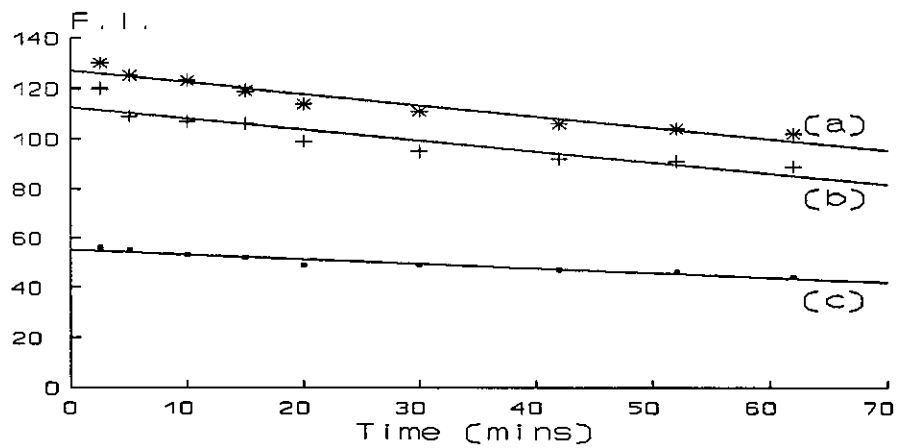


Fig. 4.3: Fluorescence changes of DTTC in serum albumin.

Key: (a) 5μM DTTC:10μM HSA, Em. W'length 776nm.
(b) 5μM DTTC:10μM BSA, Em. W'length 776nm.
(c) 5μM DTTC only, Em. W'length 778nm.

Merocyanine 540

When MC 540 was added to BSA and HSA (Fig. 4.4), there was a small shift in emission to red of ca. 7 nm and an approximate doubling of

fluorescence with the complex remaining very stable. This suggests that as with DTTC, when comparing these results with those of solvent polarity effect, MC 540 when bound is in a less polar environment.

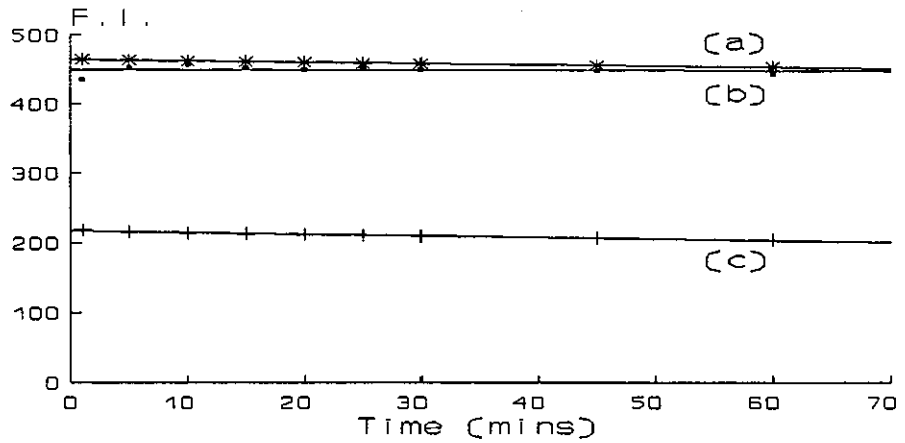


Fig. 4.4: Fluorescence changes of MC 540 in serum albumin.

Key: (a) 3.5μM MC 540:3μM HSA, Em. W'length 581nm.
(b) 3.5μM MC 540:3μM BSA, Em. W'length 581nm.
(c) 3.5μM MC 540 only, Em. W'length 574nm.

When these results were looked at more closely for BSA (Fig. 4.5), although the shift in emission remained constant, above a dye:BSA ratio of 5μM:1μM the intensity of bound MC 540 fell away and reverted back to its unbound form. It is probable that this may be due to MC 540 binding to itself at these higher concentrations rather than to the protein, i.e., dye aggregation may be occurring. It could even be that the dye is aggregating or sticking because all of its sites on the protein are saturated. However, as with DTTC the changes in fluorescence of the dye when bound are not large enough to facilitate its use as a probe for albumin.

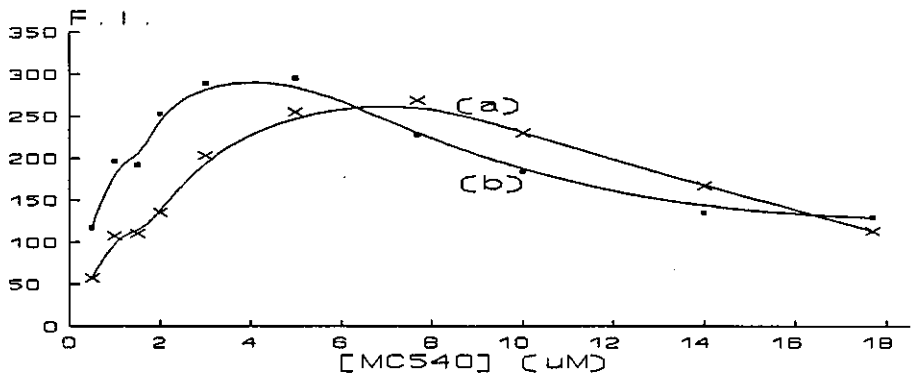


Fig. 4.5: Fluorescence of increasing concentration of MC 540 with 1μM BSA.

Key: (a) buffer only, (b) 1μM BSA.

Indocyanine Green

The results for the addition of indocyanine green to BSA and HSA are shown in Fig. 4.6. The results are much the same for both of the serum albumins and suggests that indocyanine green is in fact binding to the proteins. The resultant changes in fluorescence are similar to those achieved when the dye was placed in a less polar environment and indicate that the attachment between the dye and protein is of a similar mechanism. The stability of the complexes are also fairly good. However, as with the other cyanine dyes indocyanine green would not appear to make a good fluorescent probe for two main reasons. Firstly, the unbound fluorescence of the dye cannot really be considered negligible when compared with the bound fluorescence which was not even double that of the unbound. Secondly, the shift in emission to red of 5 nm was very small and a much more substantial difference would be expected for a good fluorescence probe such as those in the lower wavelength region.

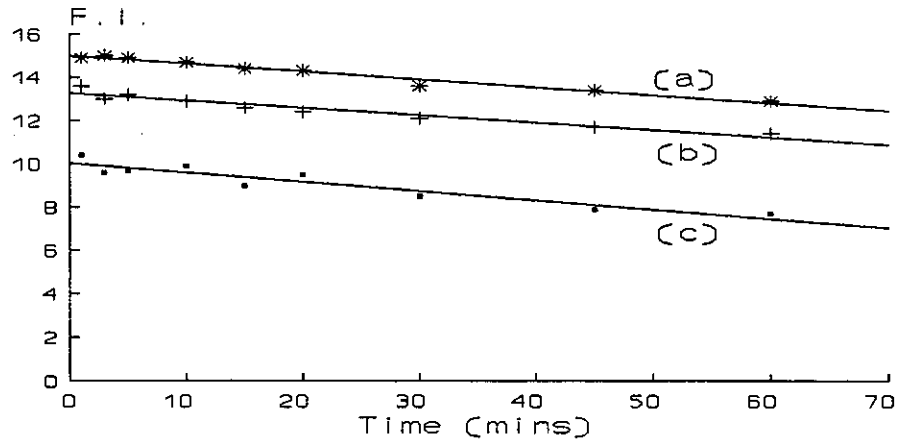


Fig. 4.6: Fluorescence changes of indocyanine green in serum albumin.

Key: (a) 1μM ICG:10μM HSA, Em. W'length 811nm.
(b) 1μM ICG:10μM BSA, Em. W'length 811nm.
(c) 1μM ICG only, Em. W'length 806nm.

Rhodamine 800

Fig. 4.7 shows the results when Rhodamine 800 was added to BSA and HSA. There was no change in emission wavelength although there was an enhancement in fluorescence intensity which remained stable throughout the thirty minutes the complex was studied. This enhancement would suggest that a certain amount of binding was occurring, however, the fluorescence changes observed are very poor and Rhodamine 800 would not appear to make a good fluorescent probe for binding to serum albumin.

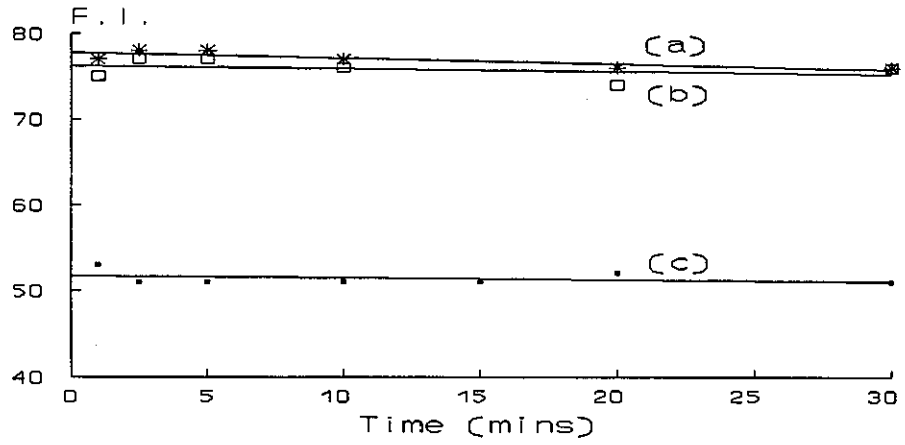


Fig. 4.7: Fluorescence changes of R800 in serum albumin.

Key: (a) 10μM R800:15μM HSA, Em. W'length 710nm.
(b) 10μM R800:15μM BSA, Em. W'length 710nm.
(c) 10μM R800 only, Em. W'length 710nm.

Nile Blue

With the addition of Nile Blue to BSA and HSA (Fig. 4.8) both an enhancement of fluorescence intensity and a shift in emission to lower wavelength was observed. These effects both of which were fairly stable over sixty minutes, follow the trends seen with the changes in fluorescence of Nile Blue with decreasing solvent polarity and thus suggest that Nile Blue may be binding to a non-polar binding site on BSA and HSA. The site on HSA being more hydrophobic than BSA. However, again the enhancement in fluorescence and shift in emission wavelength are very small and these factors along with the very high fluorescence of unbound Nile Blue means that it is far from being an ideal fluorescent probe.

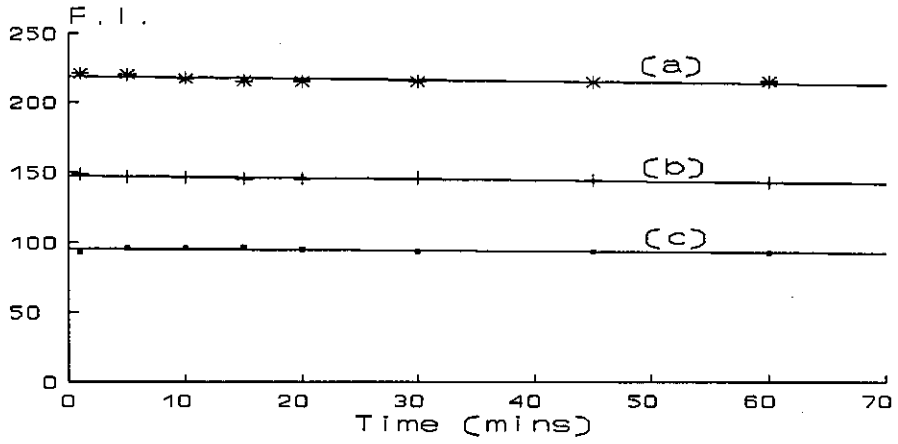


Fig. 4.8: Fluorescence changes of Nile Blue in serum albumin.

Key: (a) 5μM Nile Blue:10μM HSA, Em. W'length 667nm.
(b) 5μM Nile Blue:10μM BSA, Em. W'length 668nm.
(c) 5μM Nile Blue only, Em. W'length 674nm.

Nile Red

The initial results of Nile Red added to BSA and HSA are shown in Fig. 4.9. When Nile Red was added to albumin there was a large enhancement in fluorescence intensity and shift to blue in emission, far greater than previously seen. These changes were again similar to those found when the polarity of the solvent environment was decreased and indicate that Nile Red is hydrophobically binding to the serum albumins. The fact that the fluorescence properties of Nile Red in HSA and BSA are different could suggest as with Nile Blue that the hydrophobicity of the binding sites on each of the proteins are different. However, although HSA had the largest fluorescence intensity enhancement, the largest shift in emission wavelengths were associated with BSA. This would infer that it is perhaps not only the mechanisms explained in section 3.3.6 governing solvent relaxations that are involved. This is further supported by the fact that although the serum proteins are fairly similar in structure they are not identical especially around the tryptophan areas of the proteins[77].

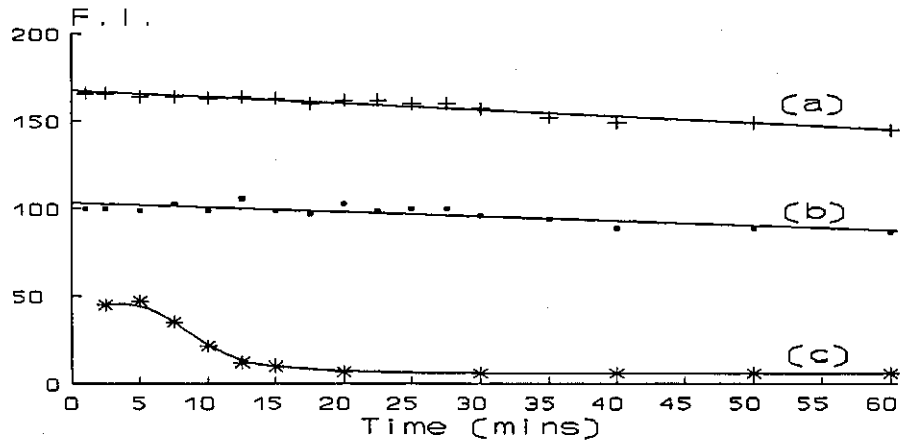


Fig. 4.9: Fluorescence changes of Nile Red in serum albumin.

Key: (a) 10μM Nile Red:15μM HSA, Em. W'length 635nm.
(b) 10μM Nile Red:15μM BSA, Em. W'length 630nm.
(c) 10μM Nile Red only, Em. W'length 658nm.

The stability of the Nile Red serum albumin complexes are also fairly good, the fluorescence not falling away to the extent of the unbound Nile Red, supporting the theory that Nile Red is in an more hydrophobic environment and resisting aggregation. Fig. 4.9 also shows that if a twenty minute incubation is allowed, the maximum difference in bound and unbound fluorescence will be obtained.

The concentrations used in Fig. 4.9 result in absorbance of greater than 0.05 Abs. units, and much lower concentrations of Nile Red were investigated to avoid problems of the inner filter effect. With both albumins the changes in fluorescence of Nile Red increased with increasing concentration of the protein along with an increase in stability of the complex (Figs. 4.10 and 4.11). This may be because at higher protein concentrations the probability of the dye molecule finding a protein molecule will improve and thus binding will increase. Also as Nile Red is thought to aggregate, a much higher ratio of protein to dye will mean that Nile Red is more likely to bind to the protein than aggregate to itself. This would also explain why even at a ratio of 10μM:1μM (protein:Nile Red) the binding as still increasing.

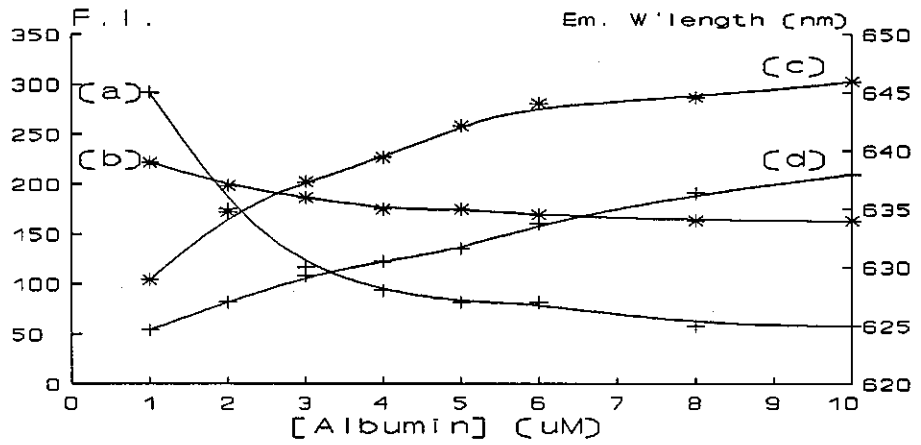


Fig. 4.10: Fluorescence change of 1μM Nile Red with increasing concentrations of the serum albumins.

Key: (a) BSA Em. W'length (c) HSA F.I.
(b) HSA Em. W'length (d) BSA F.I.

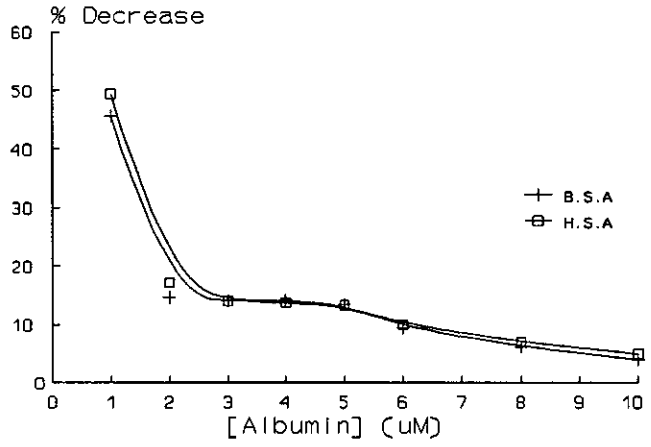


Fig. 4.11: Percentage decrease in fluorescence of 1μM Nile Red and serum albumin complexes after twenty minutes.

Obviously, the binding characteristics of Nile Red need to be studied further. However, from the results obtained for all the dyes Nile Red undergoes the largest intensity enhancement and shift in emission by far. In fact, the changes in fluorescence associated with Nile Red binding to serum albumin are comparable to those found with a common UV/ visible probe like ANS (Fig. 4.12) and indicate that Nile Red will make a good long wavelength fluorescent probe for serum albumins. In terms

of serum albumin the other dyes do not fit any of the criteria necessary to act a good fluorescence probe.

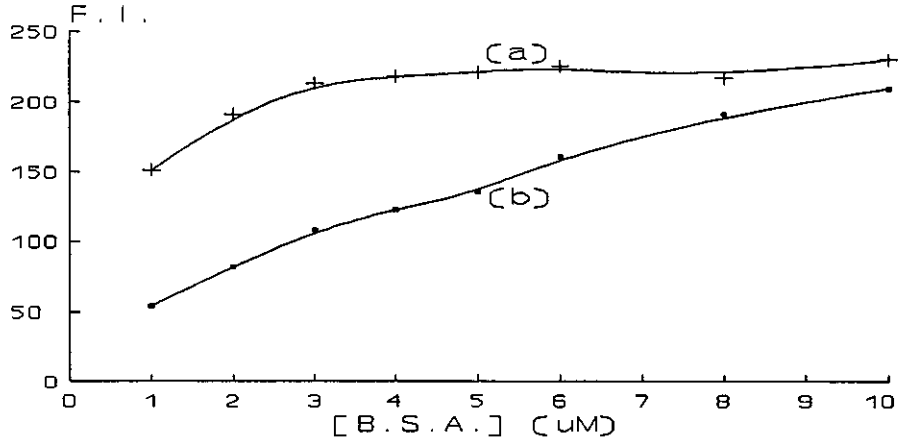


Fig. 4.12: Comparison of ANS (1μM) and Nile Red (1μM) binding to BSA.

Key: (a) 1μM ANS, Em. W'length shift to blue 60nm.
(b) 1μM Nile Red, Em. W'length shift see Fig. 4.10.

4.6.2 Nile Red Binding to Serum Albumin.

The changes in fluorescence found when Nile Red (1μM) was added to increasing concentrations of bovine, human, horse, guinea pig and rabbit albumin are shown in Figs. 4.13 and 4.14. Nile Red would appear to have hydrophobic sites on horse, guinea pig and rabbit judging by the enhancement in intensity and shift to blue in emission wavelength when it binds to the proteins. Again, the protein associated with the largest fluorescence intensity increase, in this case horse albumin, did not have the largest shift in emission wavelength (BSA) which indicates that the fluorescence changes may not be entirely due to a polarity effect of the binding site. However, it does show that the fluorescence of Nile Red can be used to distinguish the many types of serum albumin just by a slight difference in where it is attached.

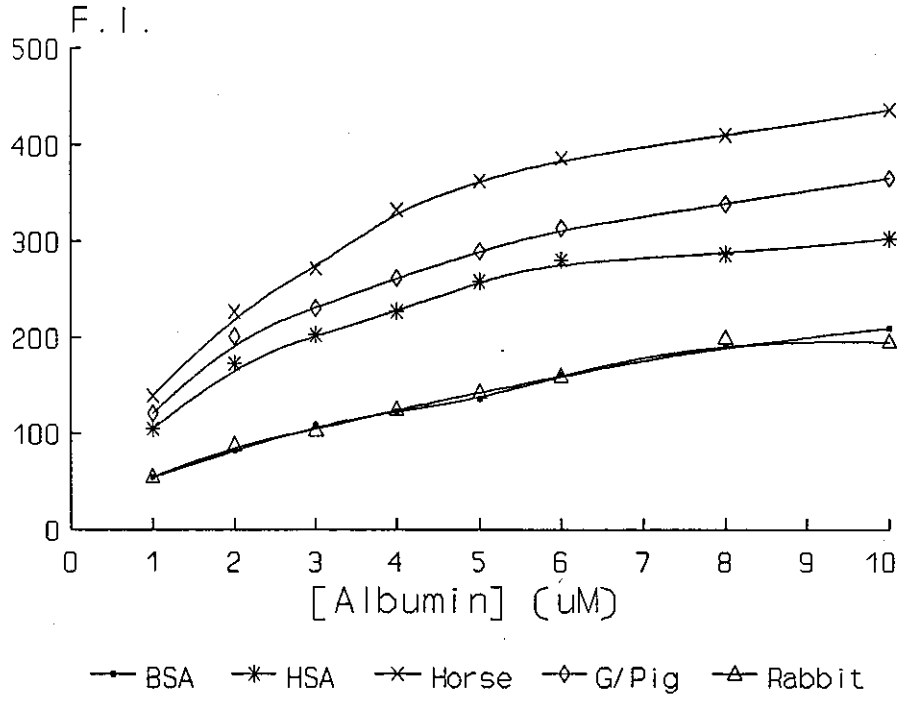


Fig. 4.13: Fluorescence intensity changes of 1μM Nile Red with different serum albumins.

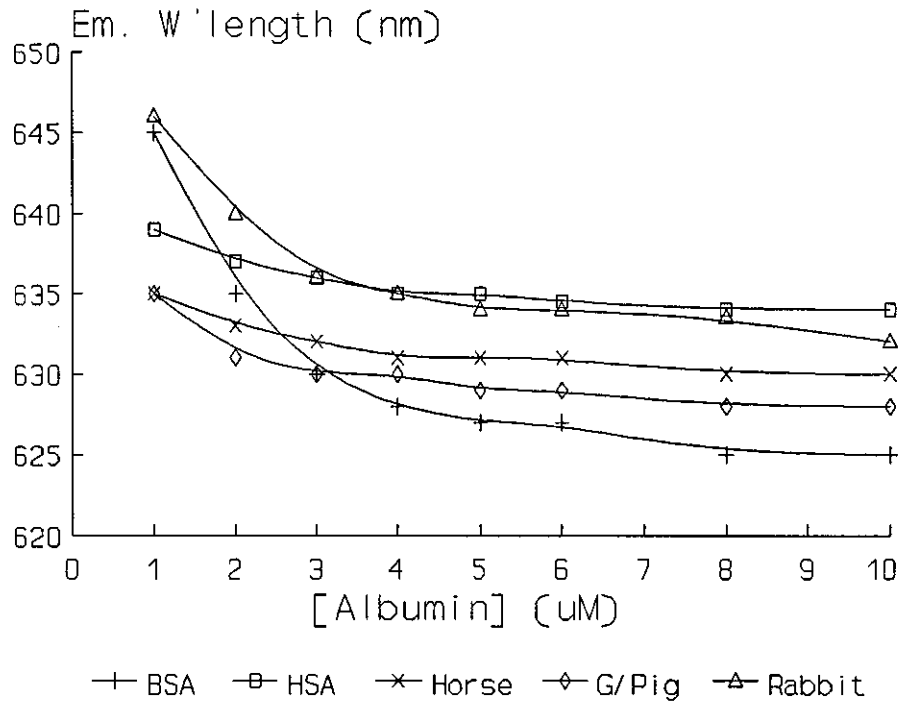


Fig. 4.14: Fluorescence emission changes of 1μM Nile Red with different serum albumins.

Examples of binding plots where increasing concentrations of Nile Red were added to 1μM of HSA and BSA are shown in Fig. 4.15. It would appear that the binding sites for Nile Red on BSA and HSA are saturated as a maximum fluorescence was reached. After saturation the fluorescence would normally plateau or in other words remain constant.

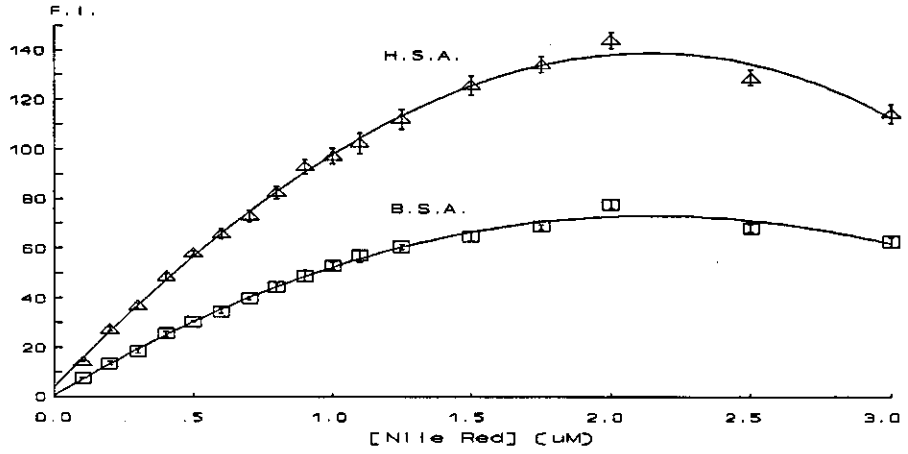


Fig. 4.15: Nile Red binding plot for 1μM BSA and HSA.

However for Nile Red the fluorescence slightly decreased probably because the free molecules aggregated, resulting in a similar effect to that associated with unbound Nile Red. The adapted Scatchard plots for both BSA and HSA (Figs. 4.16 and 4.17) do not show straight lines so it can be assumed that any binding sites present are not independent and identical. In fact, the data produces a curve in both plots which can be divided up into two straight lines, indicating that perhaps there are two types of binding site for Nile Red. The association constant and number of binding sites associated with each type of binding site are shown in Table 4.3.

Table 4.3: Binding parameters of Nile Red in BSA and HSA.

	Binding site A			Binding site B		
	K_a, M^{-1}	n	Correlation coefficient	K_a, M^{-1}	n	Correlation coefficient
BSA	1848000	0.44	0.913	3361000	1.16	0.995
HSA	3361000	0.71	0.906	23641000	0.74	0.971

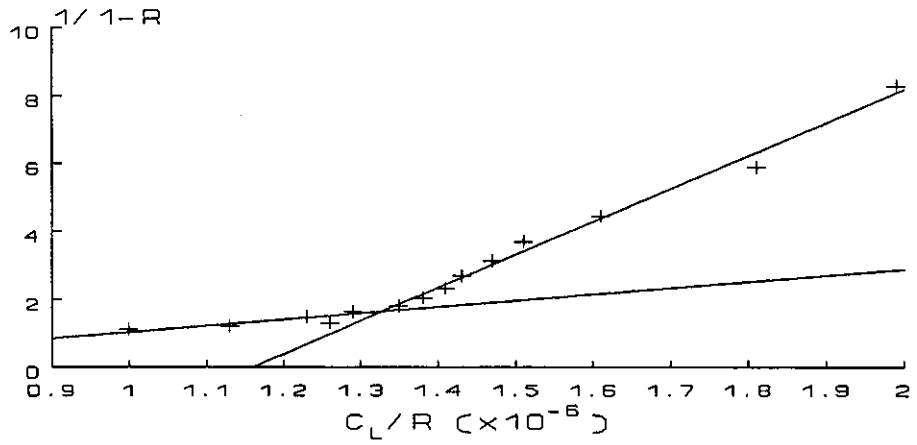


Fig. 4.16: Scatchard plot for Nile Red and 1μM BSA.

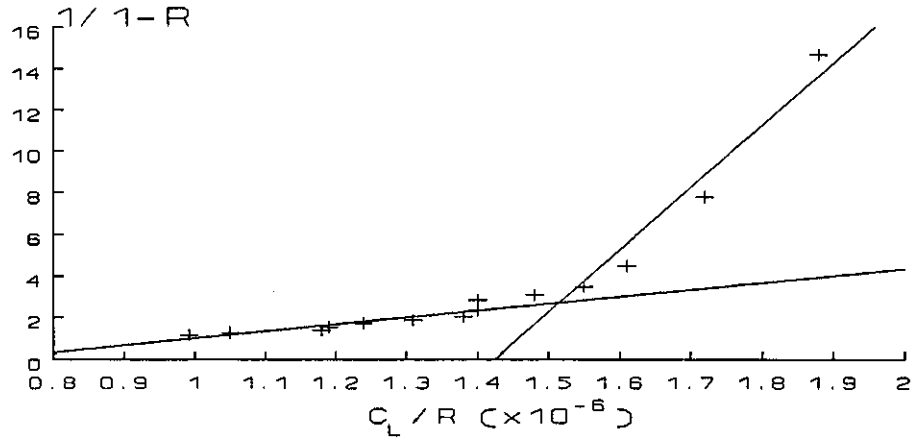


Fig 4.17: Scatchard plot for Nile Red and 1μM HSA.

Both BSA and HSA would appear to have primary and secondary binding sites for Nile Red, the affinity of the probe being greater for HSA than BSA in either type of site.

4.6.3 Displacement of Nile Red from Serum Albumin.

A number of basic and acidic drugs were originally studied to see whether displacement of Nile Red from its binding sites on serum albumin could be achieved. Displacement of Nile Red would be recognizable when the fluorescence of Nile Red was quenched in terms

of intensity and shifted to red in emission wavelength. In other words the fluorescence of Nile Red reverts back to that found with the unbound form in aqueous media. However, it must be noted that any drop in fluorescence may not be only due to a displacement of the probe by a drug or other ligand. For example, the possibility exists that a drug may cause a change in the spectroscopic properties of the probe, by modifying the protein conformation and hence the environment of the fluorescent probe, rather than by displacing the molecule from a specific class of binding site. Obviously, it is difficult to distinguish between these two effects when a drop in fluorescence is observed, but when an increase occurs the effect can provide additional information about the binding of drugs and probes. In terms of the acidic drugs tested, the only drug that caused displacement of Nile Red from BSA and HSA was flufenamic acid (Figs. 4.18 and 4.19). For BSA, with increasing concentrations of the drug, the fluorescence intensity was quenched and the emission wavelength shifts back to ca. 660 nm i.e., the wavelength of unbound Nile Red. However, with HSA, the effect was different, at the initial low flufenamic acid concentrations, the Nile Red fluorescence actually increased, displacement was only achieved at the higher drug concentrations. This suggests that initially with HSA, the drug molecules were binding to binding sites different to that of Nile Red, the binding perhaps causing a change in conformation in the protein and increasing Nile Red binding. At higher drug concentrations, the drug was probably binding to other less specific binding sites which was where the Nile Red was binding thus causing Nile Red displacement. The final displacement was not as large as that with BSA (Table 4.4) but it was still obvious. The affinity constants for the drug are shown in section 4.6.5.

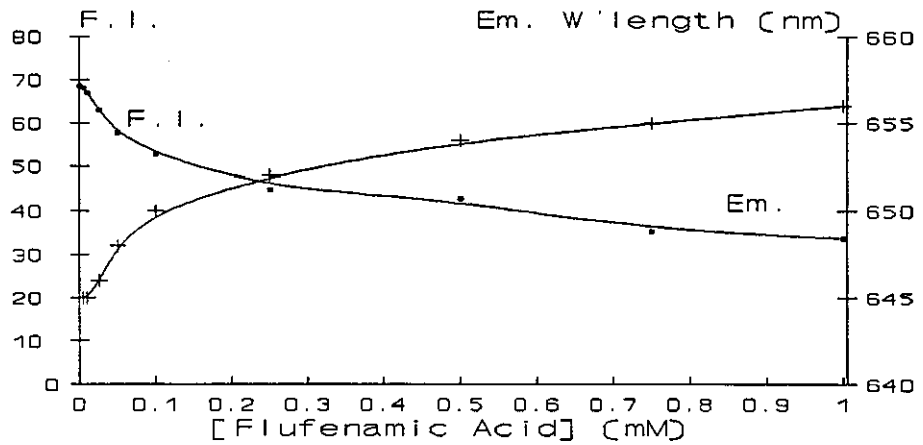


Fig 4.18: Displacement of 1μM Nile Red from 1μM BSA with Flufenamic acid.

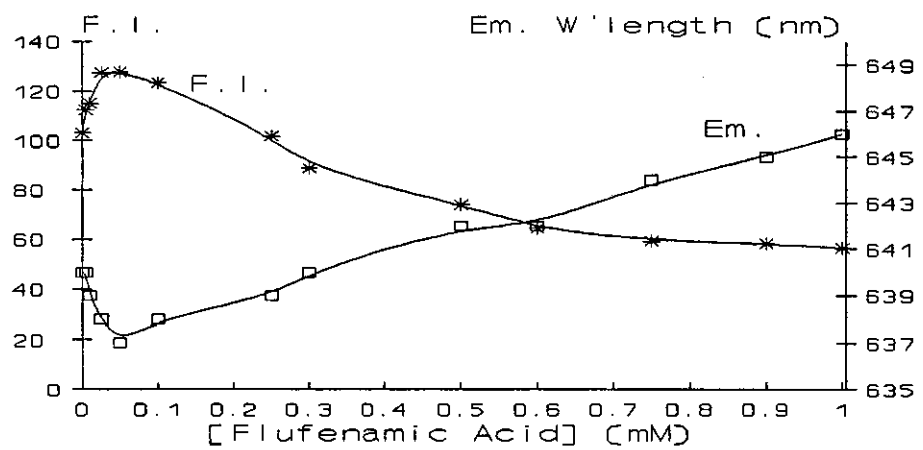


Fig. 4.19: Displacement of 1μM Nile Red from 1μM HSA with Flufenamic acid.

The affinity of the drug for the Nile Red binding sites on HSA and BSA would further support the fact that the Nile Red binding sites on HSA and BSA are not identical and are probably in the areas where the amino acid residues are not the same.

Table 4.4 Comparison of Nile Red (1μM) Displacement from HSA (1μM) and BSA (1μM).

	BSA	HSA
% decrease in fluorescence intensity of 1mM Flufenamic acid	65.4%	35.2%
Shift to red in emission wavelength with 1mM flufenamic acid	10 nm	5 nm

With all the basic drugs tested, independent of the serum albumin the results were quite similar in that no change in the emission wavelength was observed no matter what concentration was used. At the higher drug concentrations (>1 mM) the fluorescence intensity did decrease by between 12-15% however this was more likely to be due to an inner filter effect rather than any displacement. So, in summary although basic drugs have been reported to bind to the serum albumins (Section 4.3) they are not binding at the Nile Red binding sites and so could not be investigated in this study. Further investigation of ligand displacement of Nile Red will be shown in a following section.

4.6.4 Double Probe Binding to Serum Albumin.

To further investigate the binding of Nile Red to serum albumins, a novel method involving the binding of a second fluorescent probe to the proteins was used. The second probe used was ANS which is known to bind to Sudlows' site (ii) on serum albumin. If ANS could displace Nile Red or altered its fluorescent characteristics in any way, valuable information on Nile Red binding to albumin would be gained.

The results of ANS binding to HSA and BSA are shown in Fig. 4.20. Fluorescence of unbound ANS is almost zero but has an approximate emission wavelength of 525nm. ANS bound to BSA and HSA had

emission wavelengths at 470 and 475nm respectively. The fluorescence of ANS being larger when bound to BSA compared to HSA. The shift to blue and intensity enhancement of ANS bound to serum albumin is because ANS, like Nile Red, is a hydrophobic probe. It is situated in a hydrophobic area on the protein with limited access of solvent to the binding site and therefore causing the resultant changes in fluorescence[113].

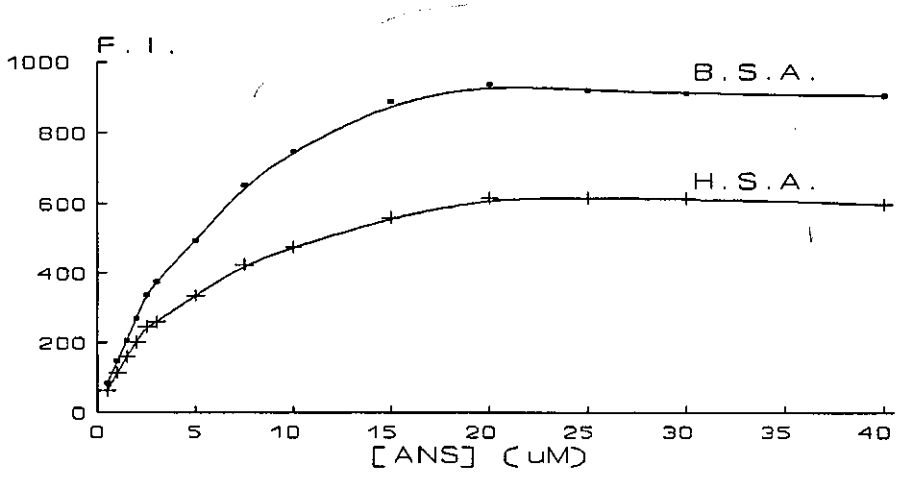


Fig. 4.20: ANS binding plot on HSA and BSA.

The difference in fluorescence intensity and emission wavelengths between the serum albumins could be explained by a difference in hydrophobicity in the binding site, however, it may also be explained by energy transfer from the tryptophan residues[102]. As BSA has two tryptophans compared to the one of HSA the fluorescence of ANS bound to BSA will be larger.

The association constants and number of binding sites for ANS on the different serum albumins are shown in Table 4.5 and show similarity to those values given in the literature[112,113,227].

Table 4.5: ANS binding parameters on BSA and HSA.

	K_a M^{-1}	n	Correlation coefficient
BSA	1008000	4.14	0.972
HSA	868000	4.01	0.957

The effects of Nile Red on the binding of ANS to BSA and HSA are shown in Figs. 4.21 and 4.22.

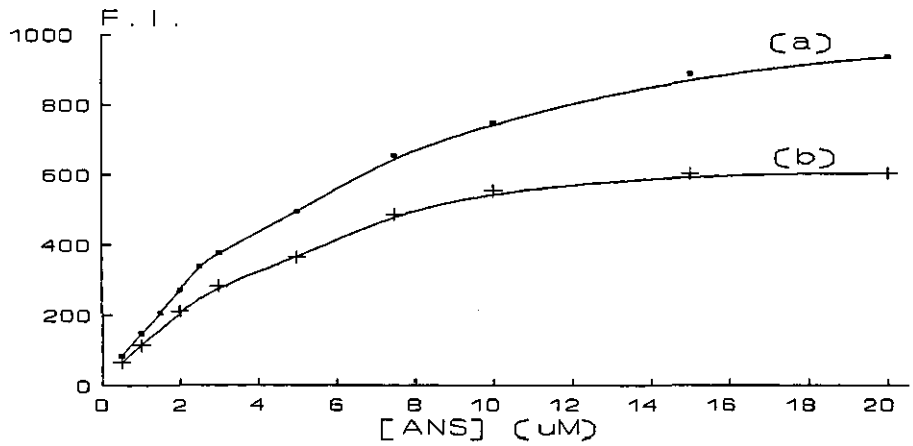


Fig. 4.21: ANS binding to BSA (a) without or (b) with the presence of Nile Red.

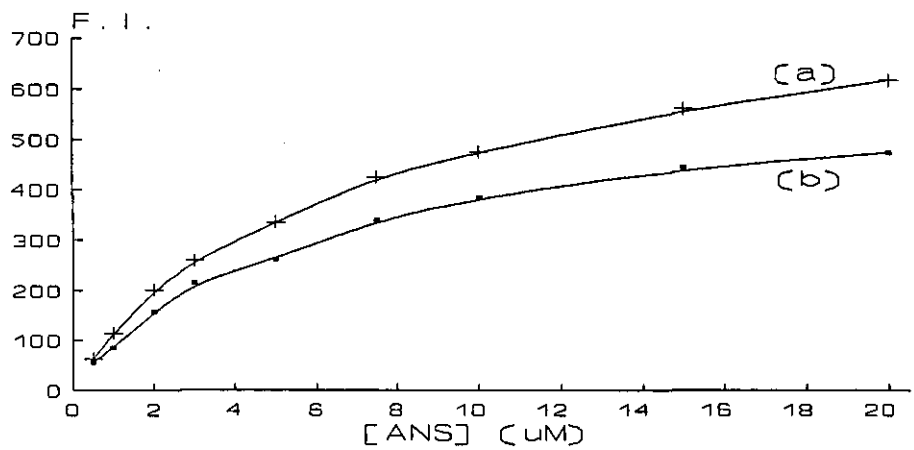


Fig. 4.22: ANS binding to HSA (a) without or (b) with the presence of Nile Red.

The presence of Nile Red decreases the fluorescence intensity of ANS when bound to both HSA and BSA without change in the emission wavelength. ANS, however did not affect the binding of Nile Red by any significant degree. It would appear that Nile Red was either affecting the binding of ANS to the serum albumins or altering its fluorescence when bound. The emission spectrum of the bound ANS and the excitation spectrum of the Nile Red in fact overlap, Fig. 4.23, and as discussed in section 1.3.3, this suggests that an energy transfer or absorption re-emission mechanism could be occurring. This would explain the drop in fluorescence of ANS with Nile Red present as the emission from ANS is used as the excitation energy for Nile Red and re-emitted, and is not seen as ANS fluorescence. This theory is supported by the fact

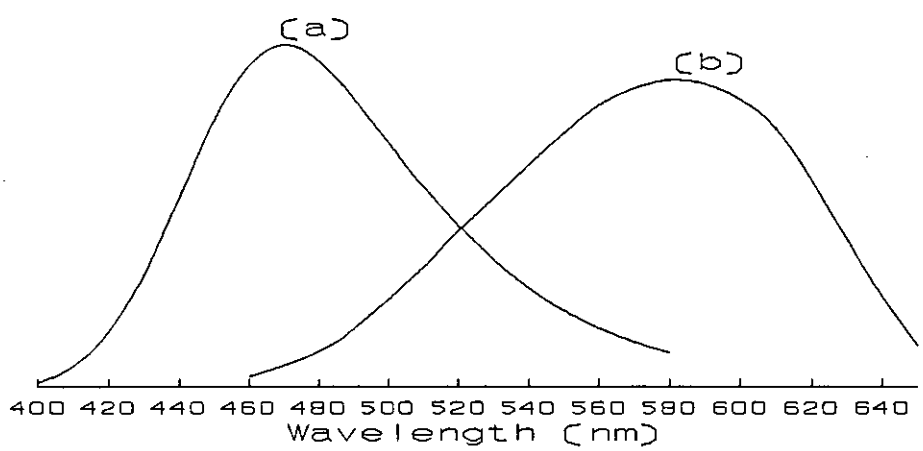


Fig. 4.23: Overlap of (a) emission of ANS with (b) excitation of Nile Red.

that at an excitation wavelength associated with ANS (370 nm) the fluorescence of Nile Red in the presence of ANS was larger in terms of intensity than with Nile Red alone. In fact, the intensity of fluorescence of Nile Red in the presence of ANS excited at 370 nm was nearly as high as that of Nile Red bound to HSA alone excited at 550 nm (Fig. 4.24).

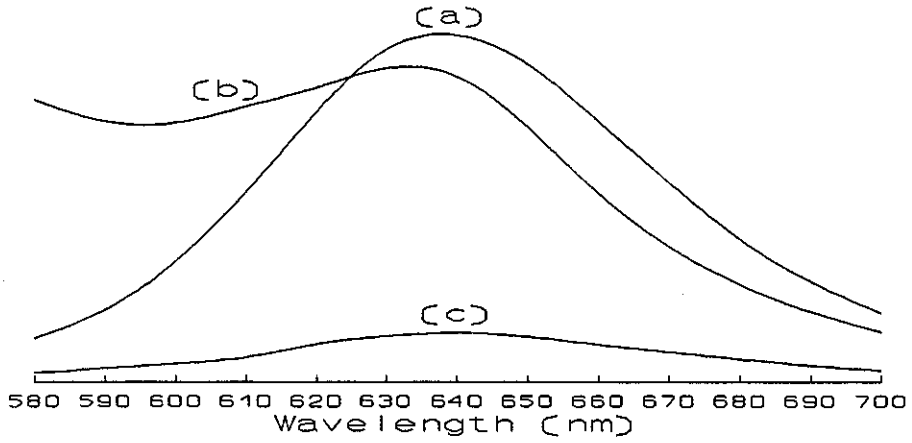


Fig. 4.24: Emission spectra of 1μM Nile Red: 1μM HSA.

Key (a) excited at 550 nm alone.
(b) excited at 370 nm in the presence of ANS.
(c) excited at 370 nm alone.

The difference in fluorescence of ANS bound to serum albumin in the presence of Nile Red disappeared upon dilution (Fig. 4.25). This indicates that the mechanism involved is not an energy transfer involving Nile Red and ANS on the same protein molecule. The two probes must be bound too far apart for this to happen on the protein i.e., >80Å[40] and instead the explanation is a simple excitation and re-emission involving the two probes on different albumin molecules. However, the result shows that Nile Red is not displacing ANS from its binding site.

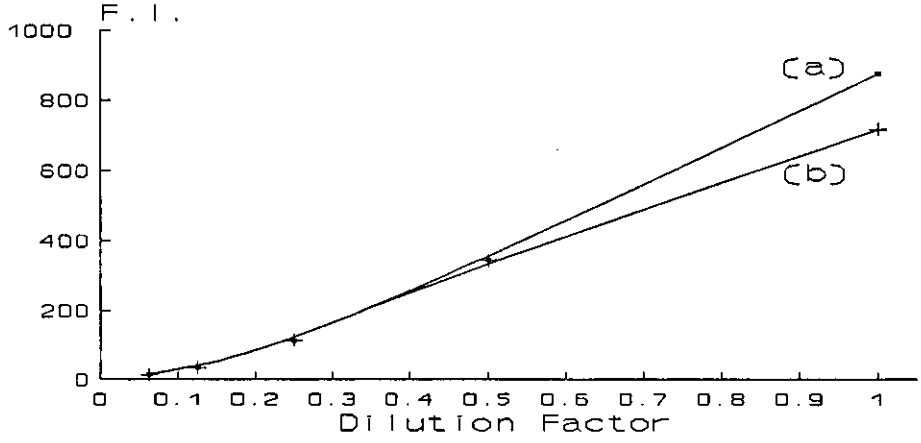


Fig. 4.25: Effect of dilution on fluorescence of ANS:HSA (2μM:1μM) complex, (a) without and (b) with the presence of Nile Red.

The stabilities of Nile Red, ANS and Nile Red and ANS bound to BSA and HSA are shown in Table 4.6. The stabilities of the complexes seem to be independent of the presence of the other probes and to the order of which they are added to the protein, apart from the fluorescence stability of Nile Red bound to BSA. Here the presence of ANS seems to increase the stability of the Nile Red:BSA complex approximately two fold, indicating a difference between Nile Red binding on BSA to that on HSA. No differences in emission wavelengths were observed between samples.

Table 4.6: Fluorescence stability of probes bound to serum albumin (1μM Nile Red:10μM ANS:1μM serum albumin).

	% decrease in fluorescence over 60 minutes	
	BSA	HSA
ANS		
ANS only	6.2 ±0.21	4.5 ±0.19
Nile Red added first	7.5 ±0.24	5.1 ±0.21
Nile Red added last	6.1 ±0.26	4.1 ±0.22
Nile Red		
Nile Red only	35.9 ±1.31	32.5 ±1.18
ANS added first	15.9 ±1.03	34.4 ±1.28
ANS added last	16.0 ±0.97	33.2 ±1.24

4.6.5 Drug Displacement of Nile Red and ANS from HSA and BSA.

The displacement of Nile Red and ANS by both acidic and basic drugs and other ligands from serum albumin are shown in Table 4.7 and 4.8. The problems associated with the assumptions made in drug displacement of probes have already been discussed in section 4.6.3. An extra complication when using two probes, especially when one is of a low wavelength is that energy transfer and quenching may cause changes

in fluorescence. This possibility however, was removed by the use of controls and using excitation wavelengths where possible suitable enough to remove the chances of energy transfer.

The first conclusion to be drawn from the resultant data is that the displacement of ANS is similar in both HSA and BSA supporting previous work that the site (ii) binding areas, like the site (i) binding areas on BSA and HSA are quite similar[86]. However the effects of the drugs on Nile Red are very different between HSA and BSA indicating that the binding sites of Nile Red on BSA and HSA are different and probably not of the site (i) or site (ii) variety.

Table 4.7: Effects of 250 μM drug on Nile Red:ANS:BSA complex(1:2:1μM).

	% Change in Fluorescence Intensity		Emission wavelength Change	
	Nile Red	ANS	Nile Red	ANS
Drugs				
Flufenamic acid	-50.2	-92	-8	-5
Ethacrynic acid	-1.2	-63.8	0	0
Salicylic acid	-4.4	-38.2	0	0
Warfarin	-11.7	-58.3	0	0
Phenylbutazone	-14.8	-63.9	0	0
Sulphamethaoxazole	-4.5	-20.7	0	0
Sulphadiazine	-2.1	-3.6	0	0
Digitoxin	-1.0	-15.8	0	0
Diazepam	+15.5	-11.4	+5	0
Ligands				
D-Tryptophan	-4.4	-2.2	0	0
L-Tryptophan	-3.1	-3.6	0	0
Stearic acid*	-62.7	-6.6	-11	0

Key for Table 4.7.

-,+ values for % change in fluorescence intensity indicates a drop and rise respectively.

-,+ values for emission wavelength change indicate a rise or fall, in wavelength. Maximum rise for Nile Red in BSA is -13 i.e., when Nile Red is completely displaced. For ANS full displacement in BSA is ca. -45nm.

* [Stearic acid] for measured displacement was 100 μM.

Table 4.8: Effects of 250 μM drug on Nile Red:ANS:HSA complex (1:2:1μM).

	% Change in Fluorescence Intensity		Emission wavelength Change	
	Nile Red	ANS	Nile Red	ANS
Drugs				
Flufenamic acid	-1.3	-94.6	0	-4
Ethacrynic acid	+28.3	-59.8	+3	0
Salicylic acid	-3.1	-29.2	0	0
Warfarin	+25.6	-52.8	+4	0
Phenylbutazone	+17.4	-45.0	+2	0
Sulphamethaoxazole	-3.2	-4.9	0	0
Sulphadiazine	-5.2	-2.2	0	0
Digitoxin	-37.4	-5.6	0	0
Diazepam	-0.9	-13.9	0	0
Ligands				
D-Tryptophan	-3.6	+3.6	0	0
L-Tryptophan	-4.4	+1.5	0	0
Stearic acid	-29.2	+12.0	-3	0

Key for Table 4.8.

-,+ values for % change in fluorescence intensity indicates a drop and rise respectively.

-,+ values for emission wavelength change indicate a rise or fall, in wavelength. Maximum rise for Nile Red in HSA is -18 i.e., when Nile Red is completely displaced. For ANS full displacement in HSA is ca. -45nm.

* [Stearic acid] for measured displacement was 100 μM.

The largest significant % decreases of ANS when bound to HSA and BSA was found with flufenamic acid, ethacrynic acid, warfarin, phenylbutazone and salicylic acid. The effects of increasing concentrations of the drugs are shown in Figs. 4.26 and 4.27 with their affinity constants in Table 4.9. Flufenamic acid, ethacrynic acid and salicylic acid are known to bind to sites (i) and (ii) but preferentially bind to site (ii)[112,198]. The results indicate that of the three drugs, flufenamic acid has the greatest affinity for this binding site not only because of the difference in K_a but also because it achieves near complete displacement in terms of fluorescence intensity and emission wavelength of ANS. The fact that the other ligands like D- and L-tryptophan and diazepam that bind to site (ii) do not displace ANS can be explained by two reasons. Either the concentration of the competing ligand was not high enough to cause complete displacement of ANS or that the site (ii) type binding is not the only area on albumin the ligands have to bind . In fact tryptophan and diazepam have been reported to possibly have more than one binding site[190,206].

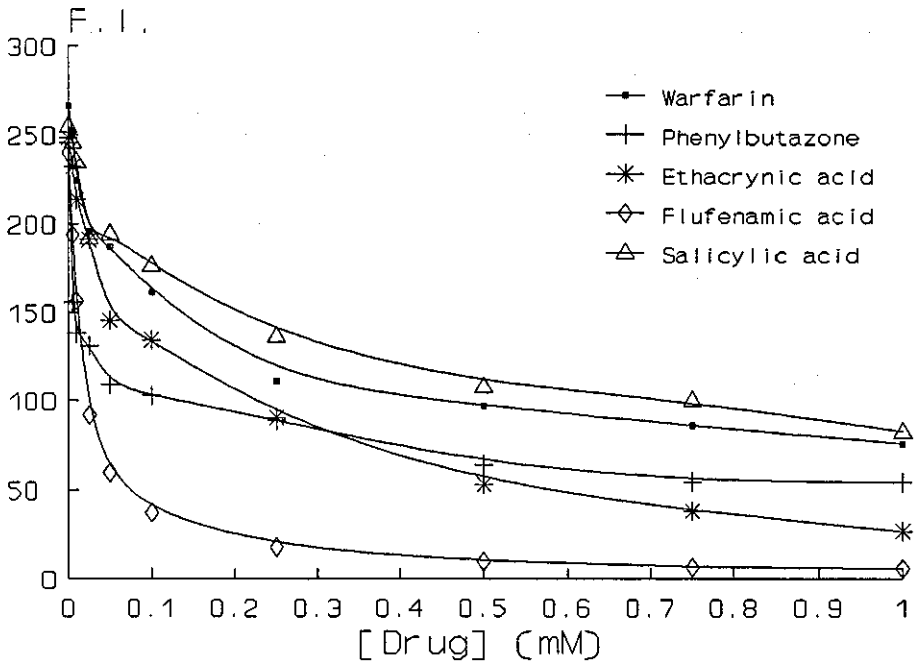


Fig. 4.26: Drug displacement of ANS from Nile Red:ANS:BSA (1μM:2μM:1μM) complex.

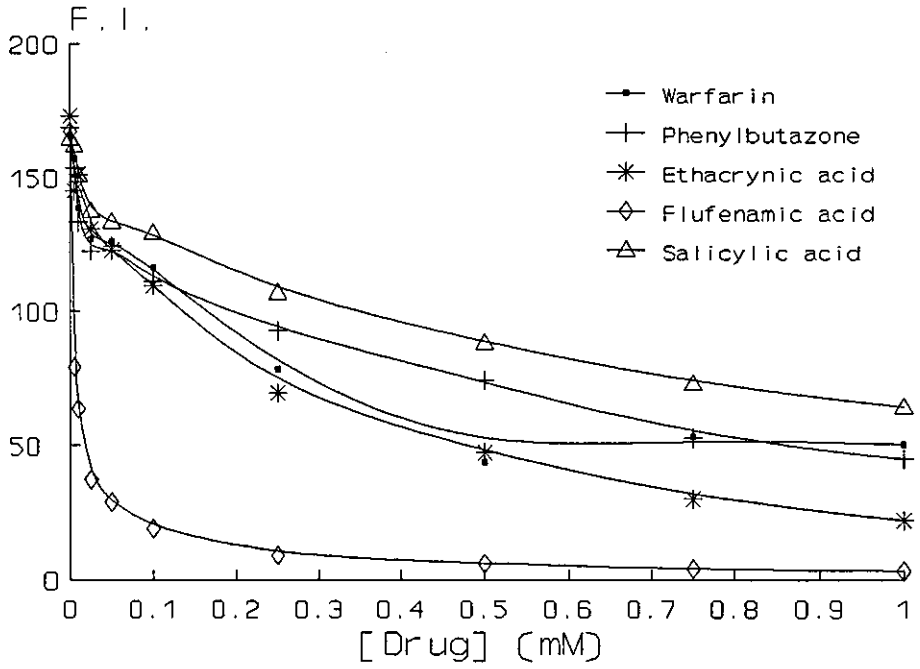


Fig. 4.27: Drug displacement of ANS from Nile Red:ANS:HSA (1μM:2μM:1μM) complex.

Table 4.9: Affinity constants for drugs that displace ANS from Nile Red:ANS:Serum albumin (1μM:2μM:1μM) complex.

	BSA		HSA	
	K_a M^{-1}	Correlation coefficient	K_a M^{-1}	Correlation coefficient
Flufenamic acid	95659	0.995	75169	0.996
Ethacrynic acid	15019	0.994	10312	0.990
Warfarin	4169	0.971	3506	0.971
Phenylbutazone	5553	0.970	3880	0.990
Salicylic acid	2958	0.991	2096	0.997

Warfarin and phenylbutazone were originally used as the early markers for site (i) binding[112], however, more recent studies by Taira and Terada on warfarin[205] and by Sturley on phenylbutazone[35] have indicated that these drugs have also more than one binding site. It is therefore possible that ANS could be competing with the drugs for a particular binding site or that the binding of the drugs are affecting the ANS binding sites. The fact that the sulphonamides, sulphadiazine and sulphamethaoxazole cause no real displacement of ANS from HSA does not contribute to the argument as although these are site (i) type binders their affinity for the site is small when compared to warfarin and phenylbutazone.

As already stated the effects of the drugs on the fluorescence of Nile Red are different in the bovine and human serum albumins. With BSA there is only one drug, flufenamic acid, which causes a drop in fluorescence intensity and shift to red in emission wavelength of Nile Red indicating displacement. The displacement of Nile Red by flufenamic acid in BSA has been discussed in section 4.6.3 and the presence of ANS did not seem to change its displacement. The only other significant displacement was caused by stearic acid (Fig. 4.28). The affinity constants for flufenamic acid (with and without ANS) and stearic acid are given in Table 4.10. The only other ligand that affected the

fluorescence of Nile Red bound to BSA was diazepam, which in fact, increased the fluorescence intensity and shifted the emission 5 nm to blue. An explanation for this is that the binding of diazepam may be causing a conformational change in the BSA structure resulting in an increases in hydrophobicity of the Nile Red binding site.

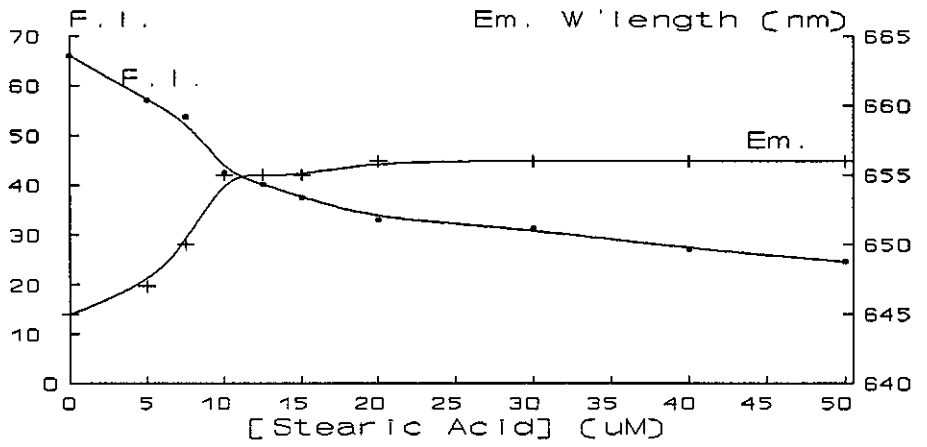


Fig. 4.28: Stearic acid displacement of Nile Red from Nile Red:ANS:Serum albumin (1μM:2μM:1μM) complex.

Table 4.10: Affinity constants for ligands that displace Nile Red (1μM) from BSA (1μM).

	Without ANS		With ANS	
	K_a M^{-1}	Correlation coefficient	K_a M^{-1}	Correlation coefficient
Stearic acid	-.-	-.-	91083	0.983
Flufenamic acid	1845	0.956	1694	0.984

Affinity constant for displacing ligand calculated from highest value K_a obtained for Nile Red.

For HSA, a decrease in Nile Red fluorescence occurs with digitoxin (Table 4.8) and stearic acid. Digitoxin is used as a definite marker for site (ii) type binding[199], stearic acid like other long chain fatty acids has a number of binding sites[204] the primary one being of the type IIA binding site on sub domain IIIA[190]. It is possible that digitoxin is

displacing Nile Red and although a displacement by stearic acid may also be occurring, there may be a slight chance, as previously reported by Hsia and Kwan[209], that the stearic acid is allosterically affecting the binding of Nile Red as it has done with other ligands.

Flufenamic acid is another drug that eventually causes a decrease in fluorescence of bound Nile Red to HSA, along with a shift in emission wavelength at high concentrations. However, as in section 4.6.3 an increase of the fluorescence, associated with an increase in binding of Nile Red was initially observed. As with BSA the effect of flufenamic acid on HSA would appear to be independent of the presence of ANS. The initial increase of the fluorescence makes the calculation of K_a difficult however, using the data when only displacement occurs, a value of K_a can be found (Table 4.11).

Table 4.11: Affinity constants for ligands that displace Nile Red (1μM) from HSA (1μM).

	Without ANS		With ANS	
	K_a M^{-1}	Correlation coefficient	K_a M^{-1}	Correlation coefficient
Digitoxin	-.-	-.-	10000	0.949
Flufenamic acid	18759	0.914	17959	0.916

Flufenamic acid is reported to bind to both Sudlows sites (i) and (ii) and has at least three high affinity sites on HSA[76]. It is possible that Nile Red is binding to one of the lower affinity sites of flufenamic acid on HSA.

Other increases in the binding of Nile Red to HSA were caused by ethacrynic acid, warfarin and phenylbutazone therefore the possibility of

Nile Red binding to site (i) in sub domain (ii)A can be ruled out as all these bind to this site.

The evidence found in this study for the binding of Nile Red to HSA is quite confusing and fairly contradictory, as is some of the literature that exists on the binding of certain ligands to serum albumin. However, it would appear likely that Nile Red is not binding to the site (i) type of binding area and that it could be binding to either the site (ii) or site (iii) types. The fact that neither ANS or diazepam displaces Nile Red but digitoxin does would favour the site (iii) binding site. However, diazepam did not displace ANS either of the reasons for which have been explained previously. He and Carter[190] have recently reported that the so called sites (ii) and (iii) are probably both found in the sub domain IIIA area of HSA. It is therefore possible that this is the area where Nile Red is binding and it is probable that the binding site of Nile Red on HSA is at site (iii).

4.7 FLOW INJECTION ANALYSIS.

4.7.1 Introduction.

The term flow injection analysis (FIA) was first used by Ruzicka and Hansen[228] in 1975 to describe the use of sample injection into an unsegmented flowing stream for rapid continuous flow analysis. FIA is now well established in analytical chemistry because of the advantages it has which include: its simplicity; the inexpensive equipment involved;the easy operation and its capacity for achieving results that are excellent in view of the rapidity, accuracy and precision with which they are obtained. The theory and applications of FIA are covered by many review articles[229-235] and books[236,237].

FIA is based on four main principles:

a) unsegmented flow

b) direct injection

c) controlled partial dispersion

d) reproducible operational times.

A simple scheme for an FIA system is shown in Fig. 4.29. It consists of the following components:

a) A propelling unit which should produce a steady pulse-less flow of one or more solutions. The solutions may be of dissolved reagents or merely a carrier for the sample plug. The most common method of propulsion is a peristaltic pump.

b) An injection system which allows the reproducible introduction of sample solution into the flow without stopping it.

c) A length of tubing along which the transport operation takes place, this is referred to as the reactor. Dispersion of the sample plug into the carrier or reagent takes place as the plug passes along the tube. There may also be chemical reactions occurring.

d) A flow cell, accommodated in a detector which transduces some property of the analyte into a continuous signal to a recorder or computer.

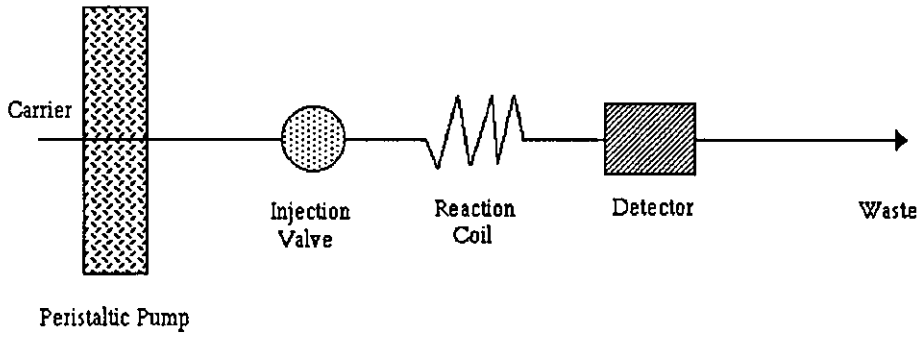


Fig. 4.29: Simple flow injection analysis manifold.

The signal obtained from FIA is transient. A typical peak is shown in Fig. 4.30.

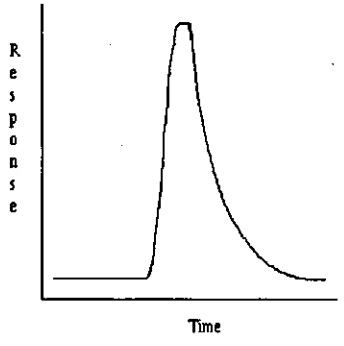


Fig. 4.30: Typical FIA peak.

The parameters effecting the peak shape are:

a) the flow rate of the carrier.

b) the volume injected along with the length and internal bore of the sample loop.

c) the length and bore of the manifold.

d) the viscosity of the sample and carrier.

e) the detector and recording time constant.

In FIA a sample injected into a carrier stream flowing through a narrow bore straight section of tube initially exists as a well defined plug. As the

plug travels down stream it disperses and mixes with the carrier stream and a well defined concentration gradient is formed. Two mechanisms contribute to this mechanism:

a) convective transport, which occurs under laminar flow conditions and yields a parabolic velocity profile, with the plug molecules at the tube walls having zero linear velocity and those at the centre having twice the average velocity.

b) Diffusional transport which consists of molecular diffusion in the axial direction (parallel to the direction of flow), and molecular diffusion in the radial direction (perpendicular to the direction of flow).

Axial diffusion is small compared with dispersion due to the flow velocity and can be ignored under most experimental condition. Whereas radial diffusion makes a large contribution to the overall dispersion molecules in the centre move to the edges and those at the edges move to the centre.

The dispersion of dilution of a sample in FIA is given by the following equation,

$$D = \frac{C_o}{C}$$

where D = dispersion.

C_o = the concentration of the sample.

C = the concentration of sample at the peak maximum

calculated from the peak response of the detector due to the analyte.

The degree of dispersion of the sample zone depends on the tube length and radius, the flow rate, the sample volume injected and the molecular diffusion coefficients of the species concerned. By changing these parameters, the dispersion can be easily manipulated to suit the requirements of a particular analytical procedure to produce an optimum response in minimum time and reagent expense.

The dispersion types fall into three categories: limited, medium and large. The peak shape varies from sharp and asymmetric for limited dispersion, Gaussian for medium dispersion and broad with exponential peak shape for large dispersion.

4.7.2 The Development of FIA for the Quantitative Study of Drug-Protein Interactions.

The study of drug-protein interactions involving static testing is normally slow, tedious and expensive and consequently flow injection analysis has been examined as a possibility for automating the process.

In the past, in the authors laboratory, homogeneous flow injection methods have been extended to the study of protein binding of both acidic and basic drugs[35,124,238]. Convential FIA manifolds with or without merging zone techniques[236,237] were used with fluorescent probes like DAPN and ANS for the study of basic drug binding to α_1-acid glycoprotein and acidic drug binding to serum albumin respectively. These studies used medium dispersion FIA, the required range of drug to protein concentrations being obtained by separate injection of drug and protein:probe solutions at different concentrations.

Applications of high dispersion have also received close scrutiny[115,125]. Such methods allow the formation of large concentration gradients in the flowing system, with the possibility that a complete drug-binding titration may be achieved in a single rapid experiment. Several distinct

experiments were used. In the first, a drug concentration gradient, produced in a mixing chamber constructed from glass, was subsequently merged at a Y-junction with a pre-formed protein-probe complex. Mixing of the solutions thus occurred in the tubing between the junction and the detector. Secondly, in a single channel method, the mixing chamber was equilibrated with one solution and the second solution was then injected into the chamber as a concentration step. The concentrations of both solutions thus varied simultaneously and mixing occurred throughout the FIA manifold. The third technique was a variant of the second, in which a large drug solution was injected into a mixing chamber made of coiled tubing containing the protein-probe complex. Mixing of the drug with the complex thus occurred at both ends of the plug although the trailing end phenomena was used in this work. If further mixing was necessary for any of the techniques stopped flow FIA[236,237,239] was used where the flow of all streams was stopped to allow more time for mixing. The resultant titration curve was used to determine K_a and the number of binding sites on the protein of the drug by combining the equations describing mixing chamber behaviour[240-242] with those described for ligand protein interactions in section 2.1.

4.7.3 Development of FIA Procedure for the Study of Nile Red Binding to BSA and its Displacement by Flufenamic Acid.

It was decided that the study of Nile Red attachment to BSA and the displacement with flufenamic acid would be best studied with the use of gradient titration to enable the production of all binding data as quickly as possible. The preliminary manifold was as shown in Fig. 4.31.

The buffer and Nile Red:BSA streams were propelled by a LKB peristaltic pump. Different flow rates were obtained by changing the diameter of the pump tubing or by increasing the speed of the pump heads by means of a switch. A Rheodyne 5020 injection valve with a 22

μl loop volume was used to introduce the drug plug into the buffer carrier stream. Pulse dampers were employed after the pump on both streams to minimize any pulsing of the flow. For speed a dispersion coil rather than a mixing chamber was used to achieve the large dispersion of the drug plug necessary for gradient titration. The coil consisted of a 0.5 or 1 metre length of tubing of 0.5 or 0.8 mm internal diameter wrapped around a cylinder of 1.5 cm diameter. The two streams merged at a Y-shaped switching valve, which was connected to the flow cell by a 50 cm length of tubing. All transmission tubing was made of polytetrafluoroethylene and had an internal diameter of 0.5-0.8 mm.

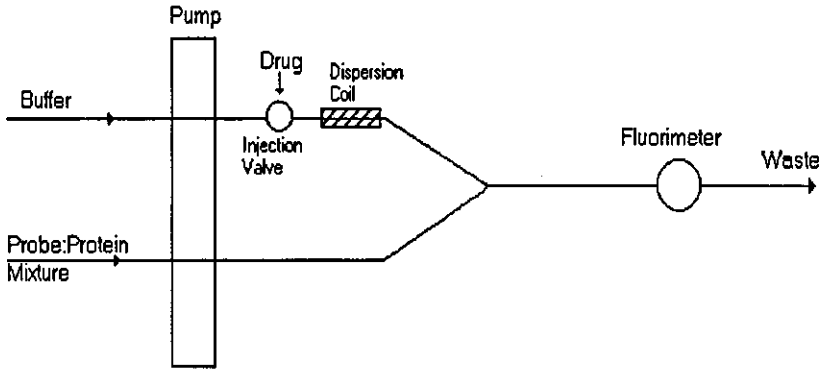


Fig. 4.31: Manifold for FIA gradient titrations.

The manifold shown in Fig. 4.31 was necessary because of the fluorescence properties of Nile Red in aqueous media. The dye loses its fluorescence in water and the more time it spends in water the less enhancement is achieved when it binds to proteins such as BSA. For this reason Nile Red was bound to BSA immediately and then ran through the system to obtain the maximum fluorescence intensity enhancement and so any displacement by the drug would be more easily seen.

Fig. 4.32 shows the displacement achieved with 2mM flufenamic acid in a 1:6 μM (Nile Red:BSA) complex with a one metre coil. The displacement trough is really an inverted version of Fig. 4.30. However,

it can be seen that the system works, the tail of the trough being ideal for a gradient titration. The next step was to optimise the system in terms of flow rates and materials while still following all the requirements for a gradient titration.

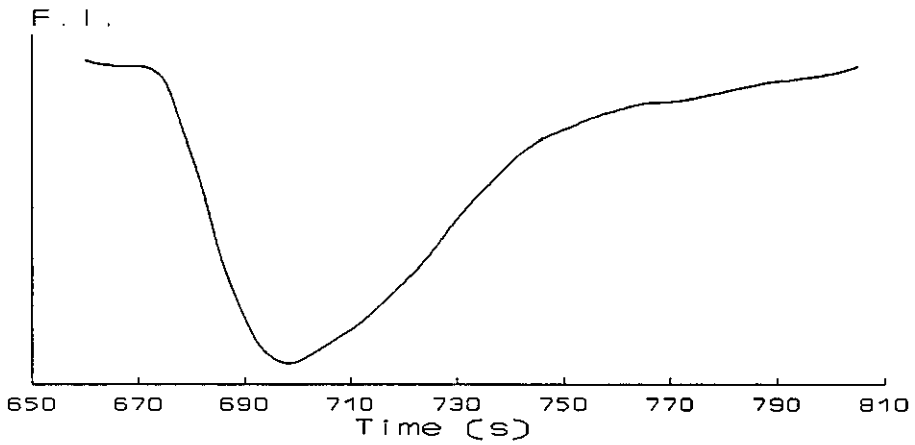


Fig. 4.32: Displacement trough.

As discussed previously large dispersion is necessary for gradient titration, which is a value of above ten using the equation given previously. The dispersion was measured using 1 ppm quinine sulphate (excitation wavelength = 350nm, emission wavelength = 450nm) the fluorescence of a constant stream of 1ppm quinine sulphate corresponding to Co and the fluorescence peak of an injected plug corresponding to C. The results are shown in Table 4.12, and indicate that the 1m coil achieved the required dispersion with the shortest length without affecting the displacement by any significant amount. The flow rate and bore of the transmission tubing were also examined to look at their effect on the dispersion of the system. To achieve the best gradient titration trough a flow rate in each stream of 0.475 ml/min was used with all transmission tubing having an internal diameter of 0.5mm.

Table 4.12: Effect of length of coil on dispersion and % displacement.

Length of coil (m)	Dispersion	% displacement of 6:1 with 2mM Flufenamic acid
0.5	9.0 ±0.34	39.55 ±0.94
1	11.7 ±0.36	37.2 ±0.58
3	13.4 ±0.39	35.3 ±0.79

In terms of material BSA was the ideal protein to test and optimise the system with, as it was the least expensive of the proteins tested which bound to Nile Red and showed displacement properties of Nile Red with a drug. As shown in section 4.6.2, the fluorescence of Nile Red increases with increasing BSA concentration. Fig. 4.33 shows the displacement of 1μM Nile Red by 2mM flufenamic acid bound to increasing concentrations of BSA. As would be expected as the protein concentration increases so does the amount of displacement. The effect of increasing the concentration of injected drug is shown in Fig. 4.34 and with conventional FIA this type of data would be used to calculate the binding parameters. As found with static experiments a concentration of drug is found where no more displacement is achieved.

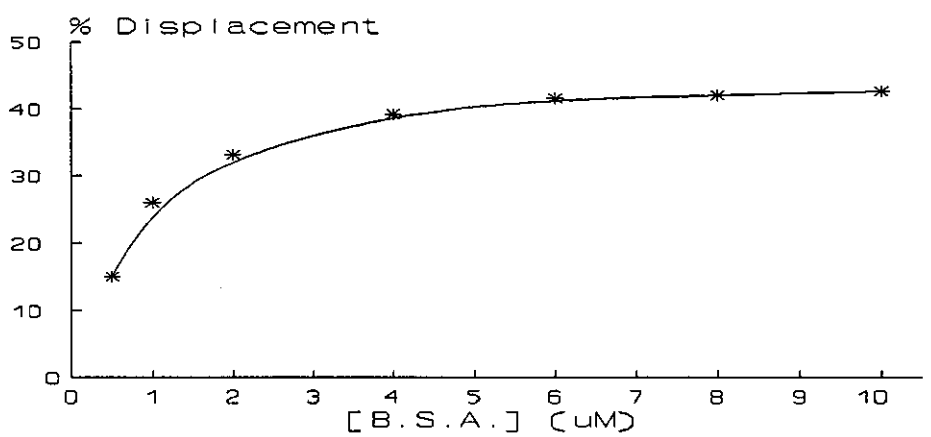


Fig. 4.33: Displacement of 1μM Nile Red by 2mM flufenamic acid from increasing concentrations of BSA.

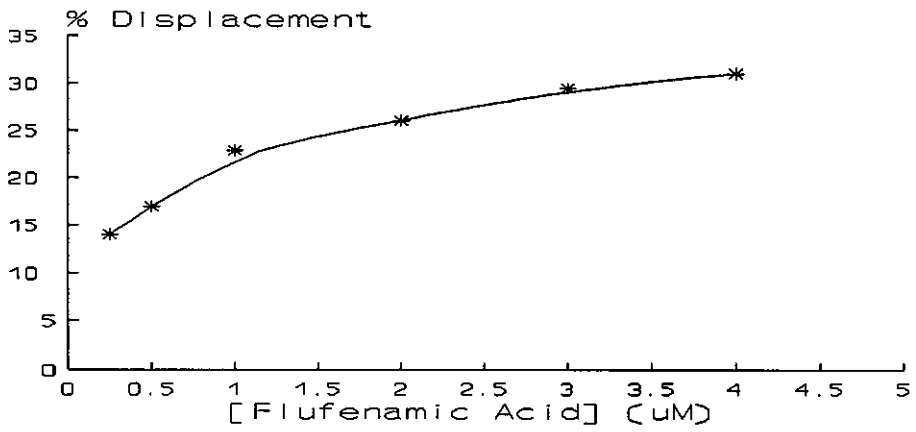


Fig. 4.34: Displacement of 1μM Nile Red from 1μM BSA by increasing concentrations of flufenamic acid.

During the optimization of the system it was noted that any change in the manifold that resulted in any increases in time for mixing after the two streams had merged produced an increase in displacement. For this reason stopped flow FIA was investigated[228,229]. Originally stopped flow FIA was used to examine the kinetics of a reaction in the detector. However, it can be used to increases the time for mixing to allow greater displacement[35]. After merging, the pump was turned off for a period of time, the results are shown in Fig. 4.35, and shows that ten minutes achieves the maximum displacement using this method. Although, this increases the time of the procedure, it would still be much quicker than any other conventional FIA measurement.

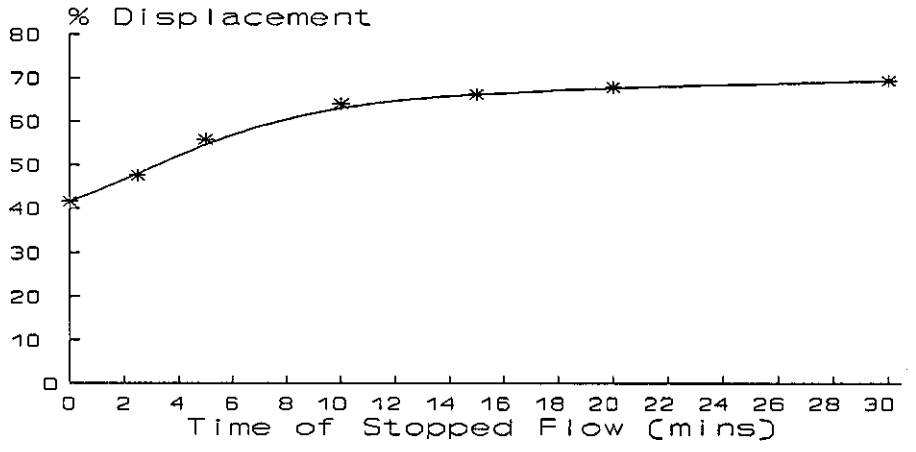


Fig. 4.35: Effect of stopped flow on displacement of 1μM Nile Red from 6μM BSA by 2mM flufenamic acid.

With the system optimized the next step was to calibrate the dispersion coil to calculate the concentration of drug at certain points of the dispersed plug and to use these values in a suitable binding equation to obtain a titration plot for each displacement trough. The calibration procedure involves 1ppm quinine sulphate in 0.05M sulphuric acid (using wavelengths given previously) and the equation for the tail end of the peak $C_{max} \rightarrow C_o$ [115]

$$C_t = C_{max} \exp [-kt]$$

where $k = u/V$

C_t = concentration of quinine emerging from dispersion coil at time t.

C_{max} = maximum concentration of quinine i.e., top of peak.

u = flow rate.

V = volume of mixing chamber.

Since the fluorescence (F) is proportional to the concentration of quinine sulphate,

$$F_t = F_{max} \exp [-kt]$$

$$or \quad \ln \left(\frac{F_{max}}{F_t} \right) = kt$$

A plot of ln (F_{max}/F_t) vs t should give a straight line with gradient k. From k the theoretical volume of the dispersion coil can be calculated and compared to the actual volume of the coil. If the two results compare, the calibration can be accepted and the results for the titration calculated.

However, with the manifold shown in Fig. 4.31 using the dispersion coil, calibration could not be achieved. Although the results obtained produced a straight line of correlation coefficient 0.99 (Fig. 4.36) the values obtained were different to the actual volumes. The volumes of the dispersion coils used in the manifold were 0.098 ml for a 0.5m coil and 0.196 ml for a 1m coil and were much smaller than volumes used in previous literature of 0.4-7.0[115]. It would appear that large volumes of the dispersion coil were necessary and although these were considered, the speed of the assay was lost. Further investigation of the manifold and procedure is therefore needed as it would appear that Nile Red could be used for the study of drug-protein interactions using the convenient, speedy and economic gradient FIA method.

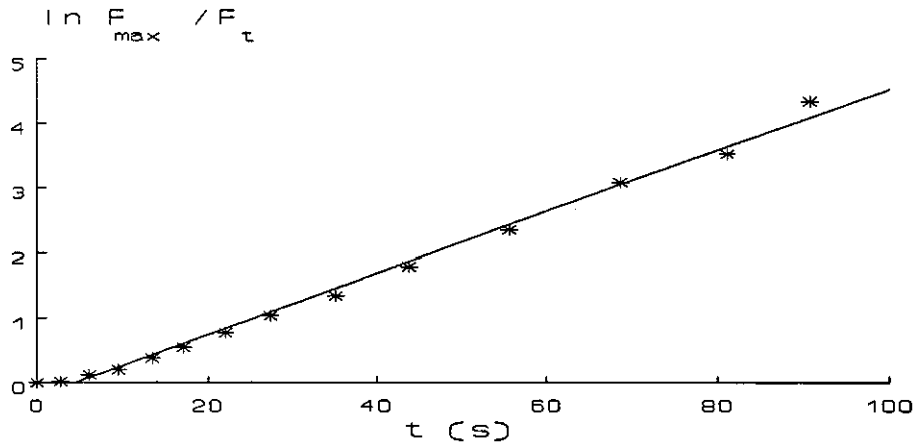


Fig. 4.36: Calibration plot of dispersion coil.

4.8 CONCLUSIONS.

The results in this chapter show that of all the dyes studied Nile Red was the best suited to act as long wavelength fluorescent probe for the study of serum albumins and their binding of certain drugs and other ligands.

Nile Red acted as a hydrophobic probe when binding to the albumins and enabled the production of accurate and repeatable binding titrations where either the probe or protein were kept constant and the other increased. These titrations showed that Nile Red had two binding sites of low and high affinity

on HSA and BSA. It was also possible to use the probe to distinguish between many other types of serum albumins just by the difference in fluorescence produced when bound to the varying hydrophobic sites.

When initially studying drug displacement flufenamic acid was the only drug that caused a drop in fluorescence intensity and shift back to red in emission wavelength of Nile Red which could be associated with its displacement. The difference in displacement of Nile Red from BSA and HSA showing the two binding sites are not necessarily the same.

The use of an independently excited second probe provided valuable additional information not only on the binding sites of Nile Red but also on those of other ligands and showed that this method could be very useful in future fluorescence studies. The fact that Nile Red was not displaced by many ligands supports the theory that ligand/drug binding to serum albumin is more electrostatic than hydrophobic in nature.

The preliminary investigation using Nile Red (or any other suitable probe) in gradient titration FIA showed that with further investigation and a calibrated system this technique could be an elegant way of rapidly and economically, quantitatively assessing ligand-protein interactions.

CHAPTER FIVE

α_1-ACID GLYCOPROTEIN

5.1 INTRODUCTION.

α_1-acid glycoprotein or orosomucoid (OMD) was first described in 1950 by two separate research groups of Weiner[243] and Schmid[244]. Since then a significant amount of research has been carried out on the protein and today it is one of the best characterised serum glycoproteins. Much is known about the genetic regulation of the synthesis of OMD and its polymeric forms. Its primary structure has been known for many years and various new data has been presented in respect to its secondary and tertiary structure.

Although the exact physiological function of OMD is not completely understood, it has demonstratable activity in a number of important physiological systems, and interacts with a variety of ligands. Thus it may inhibit or promote platelet aggregation[245], it has been reported to be immunosuppressive[246], and it binds steroids and both basic and acidic drugs. With over a hundred basic drugs identified as interacting with OMD it has become recognized as probably the only high affinity carrier for most of these in serum and therefore knowledge of the mechanism of binding is important, not only in its clinical uses of the drugs but also in the analogous descriptions of their physiological receptors.

5.2 STRUCTURE AND PHYSICO-CHEMICAL PROPERTIES OF OMD.

The structure and properties of OMD have been reviewed by Schmid[247-249] and Kremer[250] in many papers. The highly negatively charged protein consists of a single polypeptide chain, originally believed to consist of 181 amino acids (Fig. 5.1) although more recently 183 residues have been identified with five carbohydrate moieties[251]. The polypeptide chain has an amino terminal of pyroglutamic acid and C terminal serin residue, contains two disulphide bonds between the cysteine residues 5 to 147 and 72 to 84 and has multiple amino acids substituents on 21 of the residues which make OMD homologous with about 80% of the immunoglobulins. In addition, OMD also possess a high

Fig. 5.1: The amino acid sequence of α_1-acid glycoprotein with its multiple amino acid substitutions[249].

degree of sequence homology with the variable region of a human L- and a human H-chain, while another region was found to be related to the constant regions of a human L- chain, a mouse L-chain and a rabbit H-chain. Furthermore, OMD is related to the epidermal growth factor[252] which in turn is related to oncogenes.

The five carbohydrate moieties of OMD are located in the first half of the peptide chain and are in fact N asparginyl-linked glycol chains, (the points of attachment shown as * on Fig. 5.1) and consist of about 11% sialic acid, 14% neutral hexoses, 14% hexosamine and 1% fructose[250] and explains why OMD is such a hydrophilic molecule and the unusually low isoionic point of 3.4. The

glycan structures, however are not all the same, and it is clear that in OMD an individual polypeptide carries several types of glycan structure namely bi-, tri- and tetra-antennary chains[253].

The molecular weights reported for OMD range from 37,000 to 54,000[250]. These values depend upon the methods of determination, the isolation procedures, whether the OMD is native or desialyted and on the origin of the OMD samples (i.e., plasma, urine or membranes of normal patients). The molecular weight generally assumed for OMD is 40, 000, which is about the mean value of the molecular weights reported for native OMD isolated from plasma.

Assessment of the secondary conformation of OMD has been obtained from optical rotatory dispersion and circular dichroism. It has been shown that while the content of α-helices, is negligible, a considerable portion (approx. 70%) of the polypeptide chain of native OMD assumed a ß-conformation[254]. More recently, Aubert and Louchoux-Lefebvre[255] reported that OMD contains 21% α-helix, 21% ß-sheet, 8 reverse ß turns and 40% unordered structure. They observed that, of the five glycans four are linked to asparagine residues which are situated either in the reverse ß turn or in regions where charged and polar residues are numerous, that is, on the outside of the protein. They also reported that the glycans do not provide any perturbation of the protein conformation.

α_1-acid glycoprotein then is distinguishable from the other plasma proteins by:

1. A high carbohydrate content at ca. 40% the highest of the serum proteins. It makes up about 10% of the protein bound carbohydrate of normal plasma.

2. A large number of sialic acid residues about 16 residues per molecule (10-14% by weight)[80].

3. A very acidic isoelectric point (about 2.5) which is the lowest of serum proteins. This is due to the sialic acid residues, removal of which from the protein was found to raise the isoelectric point to pH 5.4[256].

4. The protein is very soluble in water and certain polar organic solvents even when it is grossly denatured.

5. OMD is stable even in boiling water. At a pH below 4 release of sialic acid residues takes place but no precipitation of the protein occurs.

6. A large number of amino acid substitutions.

7. A significant degree of homology with the immunoglobulins.

Most procedures for the isolation and purification of OMD are based on two of its fundamental properties:

(1) The electrostatic net charge which at pH values above 4, is always highly negative.

(2) The solubility, which largely as a consequence of the electrostatic properties is extremely high near neutrality.

Thus OMD can be relatively easily separated from other plasma proteins by electrophoretic and ion-exchange chromatographic methods or by solubility procedures.

5.3 THE BIOLOGICAL ROLE OF OMD.

Since the sixties it has become clear that the levels of OMD vary considerably in the plasma during several physiological and pathological conditions. Kremer

et al.[250], have published a comprehensive review on the effect of these conditions. They found that OMD levels are dependent on the severity of the diseased states. Whereas for healthy people plasma levels are reported to range between about 40 and 100 mg/ml (with an average of 73 mg/100 ml) which is 1% of the total serum protein and 60 times less than that of albumin. In disease states values of up to 300 mg/100 ml have been reported. It is for this reason that OMD has been classified among the acute phase proteins and its levels in the plasma can be used as a diagnostic and prognostic aid during the treatment of several diseases.

Increased levels of OMD have been reported with myocardial infection[257], surgical trauma[258], inflammation[259], chronic pain[260], rheumatoid arthritus[261], hepatic diseases[262], multiple sclerosis[263], renal dysfunction[264], malaria[123] and during certain infections[265]. Several forms of cancer[266-268] also cause large increases in the levels of OMD in the blood. Low OMD levels, however, have been found during pregnancy[269], in the serum of newborns[29], and in the serum of patients with liver cirrhosis[270] and thyroid disease[25]. The use of contraceptive steroids also appears to decrease OMD levels and must be related to the effect that occurs with pregnancy[271].

Much research has been carried out to discover the reasons for the changing levels of OMD. Winzler and Bocci[272] reviewed the turnover of the major plasma glycoproteins. They reported that most of the circulating plasma glycoproteins like OMD are synthesised in the liver probably in the form of an intrahepatic precursor. Sarcioni[273] demonstrated that tissues with a high cell proliferation release a factor into the circulation which effects an increased synthesis of OMD in the liver. Gamberg and Andersson[274] reported the presence of a membrane bound form of OMD with a molecular weight of ca. 52,000 on normal human lymphocytes, granulocytes and monocytes. They demonstrated that this protein was synthesized by lymphocytes and subsequently cleaved and released as the soluble normal form of OMD into the serum. They concluded that this mechanism may particulary explain the

increases in OMD levels in the serum in any disorders involving leucocyte proliferation. More recently, it has been shown that an increase in plasma concentration of OMD was due to a hepatic accumulation of the OMD-mRNA[275]. It was further concluded that different mechanisms and/or pathways are probably involved in regulating the synthesis of OMD under various stimuli. This is also the opinion of many authors however, the exact mechanisms/pathways and stimuli still remain to be completely understood.

Many other biological properties of OMD have been discussed in the literature. These include its immunological response behaviour; its inhibition of platelet aggregation[276]; its interaction with collagen[277], phospholipid membranes[278] and vitamin B_{12}[279]; its growth promoting effect for hela and H-6 cells[280]; its inhibition of phagocytosis[281]; its prolongation of the survival of skin grafts[282] and its histamine binding capacity[283].

The most studied of these effects is the role of OMD in immunosuppression. During an allergic reaction, a variety of cells are triggered by mediators such as the platelet-activating factor (PAF), to release vasoactive amines that contribute to the allergic inflammatory response after initial IgE-mediated mast cell activation. Bennett and Schmid[246] found that OMD was capable of suppressing lymphocyte proliferation and a variety of other immune functions. The modulation of activity was shown to be due to a decrease in the overall bi-antennary content of the molecule and it has been suggested that a particular glycan content on OMD may be responsible for mediating specific biological activities. OMD may control the duration of the allergenic response by down regulating the sensitization stage or by preventing the mediators PAF[249], histamine[283] and serotonin from causing acute or chronic inflammation. For these reasons much work is being carried out on the glycan patterns and the glycosylation sites in allergic reaction patients[284]. However, probably the most important role of OMD is its interaction with ketosteroids and with both acidic and basic drugs.

5.4 DRUG/LIGAND BINDING TO OMD.

Most literature published on the binding of drugs to OMD concerns basic drugs, as the protein is the most important basic drug binder of all serum proteins. Much of this literature has been reviewed by Kremer *et al.*[250] Table 5.1 shows some of the basic drugs and ligands studied. With the use of competitive displacement of drug markers which were known to bind OMD like propranolol[214,300] and imipramine[307] or fluorescent probes such as auramine O[137] and ANS[87,113-114], it was determined that the binding of these basic drugs to OMD involves one high affinity binding site, the area of which was common to all basic drugs.

Many steroids including progesterone, cortisone, corticosone and oestradiol have been reported by Westphal and his co-workers[309,310] to also bind to a single binding site on OMD with a K_a of approximately 10^6 M^{-1}. As indicated by displacement studies[313,314] and fluorescence investigations[80,114] the binding sites of both basic drugs and steroids show a considerable overlap indicating the presence of two different binding sites as part of one large binding area. The situation is probably similar to that of the warfarin-azapropazone binding area that exists on the so called site (i) on HSA[203]. It has been suggested that the binding to OMD is dominated by hydrophobic and Van der Waals interactions[30,215]. For this reason, there is no explanation why acidic (anionic) drugs should not bind to OMD. Urien *et al.*,[285-286] reported, using equilibrium dialysis, that clofibric, fenofibric, salicylic and valproic acids do not bind to OMD, whereas benoxaprofen, indomethacin and itanoxone bind at about 10% levels. In contrast, the percentages of bound warfarin, acenocoumarol and phenylbutazone were noticeably higher in the 90-26% range. They also suggested that basic drugs like propranolol displaced these acidic drugs indicating that there was only one binding site. These findings were more recently supported by Muruyama *et al.*,[133], who separately used basic probes like auramine O and acidic probes like dansyl D,L norleucine and measured the changes in their fluorescence on the addition of basic and acidic drugs. The basic drugs displaced both the basic and acidic probes, while the acidic

Table 5.1: Various ligands studied for their binding to OMD.

Drug/Ligand	References
Acidic Drugs	285,286
Alfuzosin	287
Antiarrythmic drugs	288
Atropine	289
ß-blockers	87,277,278
Catecholamines	212,218
Diazepines	213
Dipyridamole	290
Fluorescent Probes	87,113,114,132,133
Lidocaine	291
Medetomidine	292
Methadone	293
Metoprolol	294
Phenothiazines	79
Propranolol	31,214,295-297
Racemic Propranolol	298-306
Psychotropic	307
Quinidine	308
Steroids	309,310
Tricyclic Antidepressants	311
Various	29,30,113,133,250

drugs only displaced the acidic probes. Their work suggested that OMD possesses one wide and flexible high affinity drug or ligand binding area, which contains at least two binding sites which are not completely separated, significantly overlapped and are influenced by each other. The fact that certain acidic drugs bind to OMD and that certain acidic probes like ANS and auramine O undergo changes in fluorescence associated with a change in

polarity also supports the theory that hydrophobic binding is one of the major forces involved in drug/ligand attachment to OMD.

In the case of HSA, drug binding can be affected by various endogenous binding inhibitors especially fatty acids and bilirubin. Work involving these substances and their displacement of chlorpromazine[315] and disopyramide[316] - has indicated that only the non-saturated oleic acid and possibly the saturated palmitic acid of the fatty acids tested bind to the single high affinity site on OMD. Bilirubin showed no binding characteristics at all.

The presence of one common high affinity site for practically all drugs and ligands investigated does not mean that there are no secondary binding sites. Evidence of low affinity sites was probably first discussed by Kerkay and Westphal[309] who found that although most steroids had one common high affinity binding site, oestradiol had up to seven other sites depending on temperature. El-Gamel[290] discovered at least one low affinity site for the binding of the vasoactive dipyridamole while Shami *et al.*,[317] found two classes of binding sites for mianserin on OMD and in total seven binding sites, two of which had high affinity. Later, Gillis *et al.*,[288] found two classes of binding sites for many antiarrythmic drugs while Sager[212] discovered that the cathecholamines had one high affinity binding site and approximately ten low affinity sites. More recent work on ß-blockers has shown that propranolol[31,113] and its racemers[301,319] have two classes of binding sites on OMD. Johanssen *et al.*,[87] found evidence for two distinct classes of binding sites for ANS on OMD.

5.5 STEREOESELECTIVTY OF THE BINDING SITE.

Stereoeselectivity of drug is of obvious importance therapeutically and pharmacokinetically, an important example being thalidomide where only one enantiomeric form is a teratogen. Although originally it was thought that binding to OMD was non-stereospecific[249] recent work has suggested the contrary. The stereospecific binding of propranolol and its racemers has

probably been one of the most studied attachments to OMD because the (-) form is about 100 times more therapeutically active as a ß-blocker than (+) racemic form[318]. Experimentation has shown greater binding of the (-) enantiomer form to human[299,300,306] and dog[302,303,319] OMD while in the rat OMD the reverse is true[306]. Orovcova *et al.,*[301] discovered that the differences in binding to human OMD may be due to the fact that the (+) form of propranolol showed no evidence of saturation at the low affinity sites while the (-) form did. Other examples of drugs of which the stereoselective binding to OMD has been studied are pinidol, oxprenolol[319], isoproteronol[218], disopyramide[320], disocainol and tilidine[300]. The binding of the acidic warfarin to OMD also shows stereoselectivity in that the (-)(S) isomer binds more strongly than the (+) (R) form[300].

The stereoselectivty of OMD along with its tendency not to degrade or denature have allowed the use of a chiral OMD column for the separation of enantiomers of various drugs and ligands. These drugs and ligands include metetomidine[247], atropine[289], bupivacain, phenyramidol, chlorpheniramine, tropicamide, promethazine[312], alfuzosin[287], metoprolol[294] and propranolol[297]. The column showed remarkable stability despite the fact that very 'aggressive' mobile phases are used over a long period of time.

With all the drugs mentioned in this section, the degree of stereoselectivity is only moderate and can often vary depending on the ligand used to label the glycoprotein, the type of OMD used or the experimental conditions[250]. Thus the concept that a simple site represents the high affinity binding for drugs or certain other ligands can be neither supported nor disproved by these findings of stereoselectivty.

5.6 STRUCTURE AND LOCATION OF THE SINGLE BINDING SITE.

Although the primary structure of human OMD is known, little proven information is available on the possible structure and location of the single drug binding site. However, there is fairly good evidence that the carbohydrate

part is not critically involved as indicated by studies using disylated OMD and propranolol[321], progesterone[310] or dipyridamole[290]. In their work Kute and Westphal[310] ascribed the hydrophobic amino acid sequence 21-31 of OMD (-Ile-Thr-Gly-Lys-Trp-Phe-Ile-Ala-Ser-Ala-) as an important part of the binding of the binding site for progesterone. Halsall and co workers[80,322] have shown that two of the three tryptophans responsible for the inherent fluorescence of OMD are involved in drug binding. The two tryptophan residues are shielded indicating that the drug binding site can be viewed as a crevice in the protein structure with limited accessibility to water molecules.

While there is increasing evidence that sequence 21-31 is critically involved in the single drug binding site of OMD, there is also evidence to suggest other parts of the primary chain are also involved[232]. Therefore, it seems that the single high affinity site of OMD is finally formed by the tertiary structure of the protein.The site probably represents a large binding area with several sub sites which may be too close to allow co-binding but are distinct enough that their properties can be influenced differently by a variety of conditions like chemical modification, pH[30,286], ionic strength[31] and amino acid substitution. For these reasons when measuring binding and displacement of ligands to OMD as with all proteins as many of the parameters as possible must be kept constant. The binding forces involved at the binding site are thought to be dominated by hydrophobic interactions (Section 5.4) although H bonds and Van der Waals forces are also important. Unlike HSA, electrostatic forces seem to be poorly involved.

5.7 EXPERIMENTAL.

All procedures given in Chapter two and section 4.5 were followed.

All fluorescence measurements shown were taken in mes buffer pH 6.9 and recorded on the MPF44B for the preliminary work and the LS50 for all binding and displacement titrations.

All derivative spectra were obtained directly from the corresponding emission spectra using the derivatisation option on the FLDM package. Smoothing was carried out where necessary.

5.8 RESULTS AND DISCUSSION.

5.8.1 Preliminary Investigation of the Binding of the Dyes to OMD

As with the serum albumin studies in Chapter 4, all the dyes discussed previously were tested to see if any changes in their fluorescence properties resulted from their addition to α_1-acid glycoprotein. The results are summarized in Table 5.2 and it can be seen that the only dyes which showed any significant changes in fluorescence on addition of OMD and therefore possible binding were Rhodamine 800 and Nile Red.

Table 5.2: Changes in fluorescence of Dyes when added to OMD.

	% change in fluorescence intensity	Shift in emission wavelength (nm)
5μM DTTC:10μM OMD	0	0
3.5μM MC540:3μM OMD	0	+4
2μM ICG:10μM OMD	0	0
10μM R800:10μM OMD	+33	-6
5μM Nile Blue:10μM OMD	0	-2
2.5μM Nile Red:10μM OMD	+606	-25

Key: +/- fluorescence intensity means increase or decrease respectively.
+/- emission wavelength means shift to red ot shift to blue respectively.
0 for fluorescence indicates less than a 10% change.

The preliminary results achieved on the addition of 10 μM Rhodamine 800 to 10 μM and 30 μM OMD are shown in Fig. 5.2. On the initial addition of Rhodamine 800 to the protein, there was a fluorescence intensity enhancement and a shift to blue in emission wavelength compared to the fluorescence of the dye alone. These changes were similar to those found in increasing solvent polarity and suggest that

Rhodamine 800 is perhaps hydrophobically binding in a non polar environment of OMD. The trend continued at a higher protein concentration and it can also be seen that the fluorescence stability of the Rhodamine 800:OMD complex was also very good, not falling away at all over a sixty minute period. The concentrations used in Fig. 5.2 however, were much too high for any binding assay as the absorbance of the solution was much higher than 0.05 Abs. units allowed to minimize any "Inner Filter Effects".

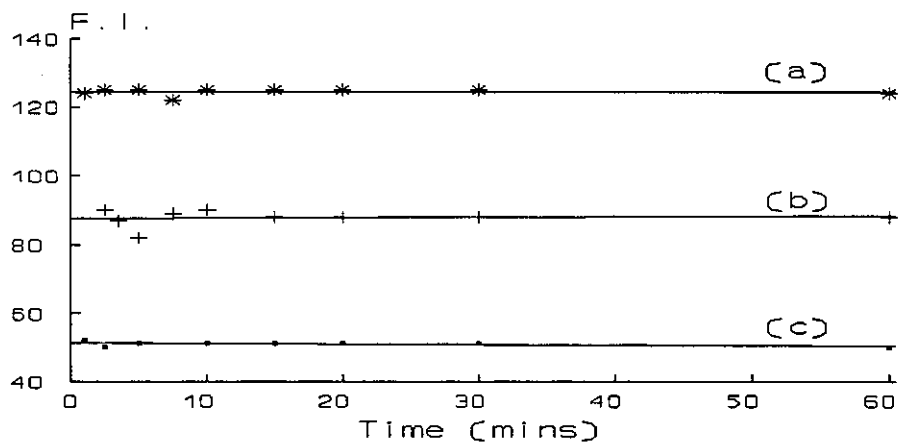


Fig. 5.2: Fluorescence changes of R800 in OMD.

Key: (a) 10μM R800:30μM OMD Em. W'length 701nm.
(b) 10μM R800:10μM OMD Em. W'length 704nm.
(c) 10μM R800 only.

Fig. 5.3 shows the results achieved with 1 μM OMD and increasing amounts of Rhodamine 800. The resultant changes in fluorescence would not be expected to be as large as those obtained with the higher concentrations in Fig 5.2. However, at these lower concentrations the addition of rhodamine 800 to OMD showed no enhancement of fluorescence intensity and although a slight shift in emission wavelength can be seen, it gradually decreases with increasing concentration of the dye. In the reverse experiment where the Rhodamine 800 was kept

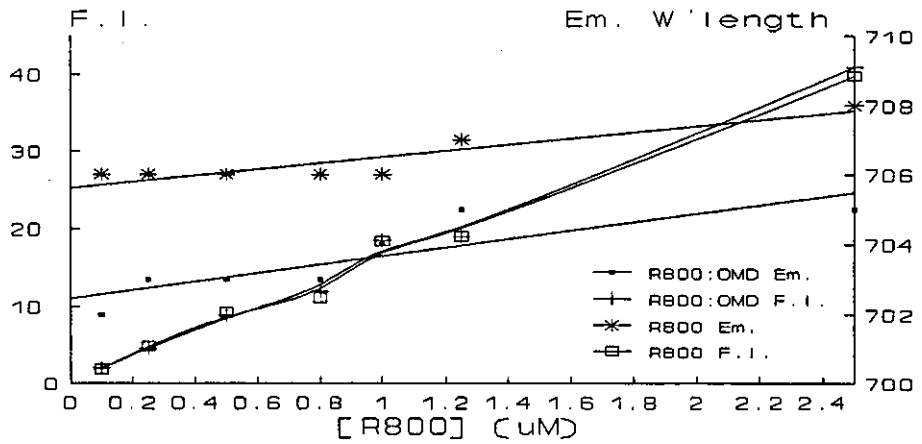


Fig. 5.3: Fluorescence changes of increasing R800 in OMD (1μM).

constant and the concentration of OMD increased (Fig. 5.4) fluorescence changes in both intensity and emission result simply because there are more protein molecules available to bind to. However, the fluorescence intensity enhancement is not large and even with a large excess of OMD compared to rhodamine 800 a two fold increase in intensity is not

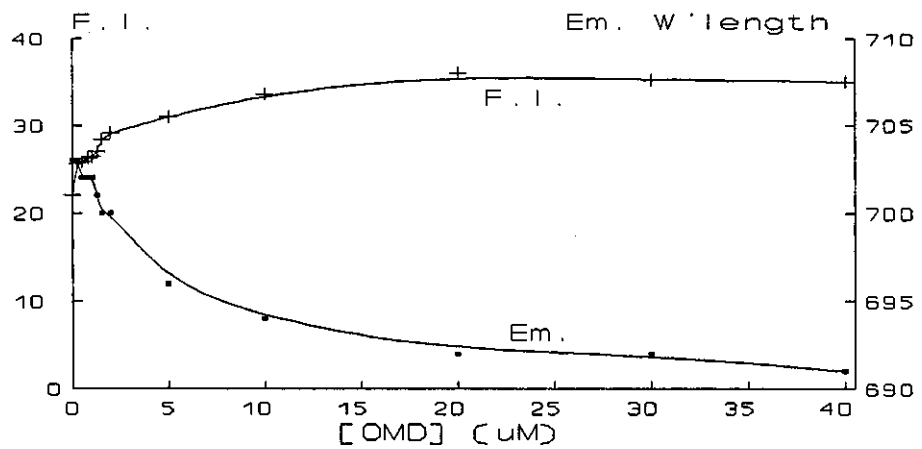


Fig. 5.4: Fluorescence changes of R800 (1μM) in increasing OMD.

achieved. The shift to blue in emission wavelength is much more significant and indicates that the binding of rhodamine 800 to OMD results in greater changes in the emission wavelength than fluorescence

intensity. However, for a good binding assay the opposite is necessary, thus Rhodamine 800 does not appear to be the ideal probe for OMD.

Fig. 5.5 shows the effect of the addition of 1 mg/ml (25 μM) OMD on the fluorescence of 10 μM Nile Red. In the presence of OMD, Nile Red undergoes an approximate six fold enhancement in fluorescence intensity and a ca. 25 nm shift to blue in emission wavelength suggesting it is binding hydrophobically to OMD. The stability of the complex is also fairly good and as with the serum albumins if twenty minutes incubation is allowed for the unbound Nile Red fluorescence to fall away the maximum fluorescence intensity enhancement will be observed.

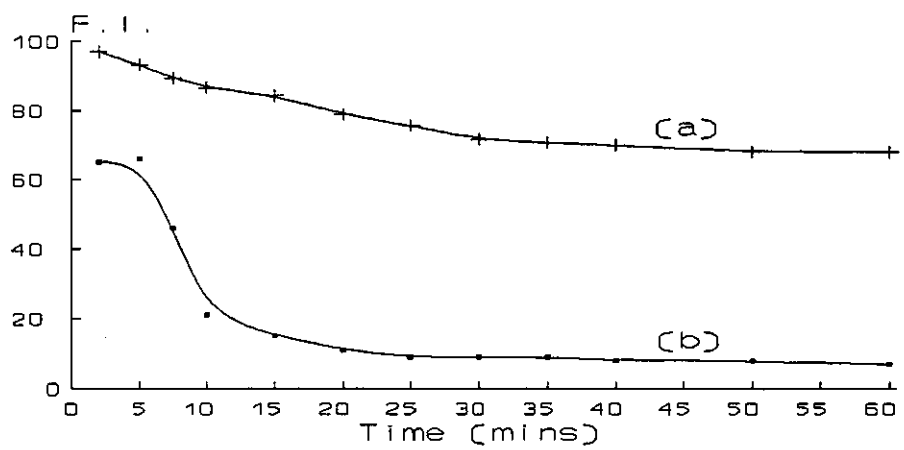


Fig 5.5: Fluorescence changes of Nile Red in OMD.

Key: (a) 2.5μM Nile Red:10μM OMD, Em. W'length 635nm.
(b) 2.5μM Nile Red only, Em. W'length 658nm.

In summary Nile Red was the best suited long wavelength dye to act as a fluorescence probe for α_1-acid glycoprotein, as it was for the serum albumins. Its shift in emission wavelength to blue and the fluorescence intensity enhancement when bound were much larger than anything achieved with the other dyes and indicate that it is binding to the protein hydrophobically. Further investigations at lower concentrations is necessary.

5.8.2 Nile Red Binding to OMD.

The binding of increasing concentrations of Nile Red to OMD are shown in Fig. 5.6 and compared to BSA and HSA. It can be seen that at these lower concentrations of Nile Red and OMD, which are necessary to avoid problems with the inner filter effect, the shift in emission to blue of 8nm is much smaller than the 25nm shown in Table 5.2. However the enhancement in intensity of Nile Red is still about four fold and adequate if not ideal for a binding assay.

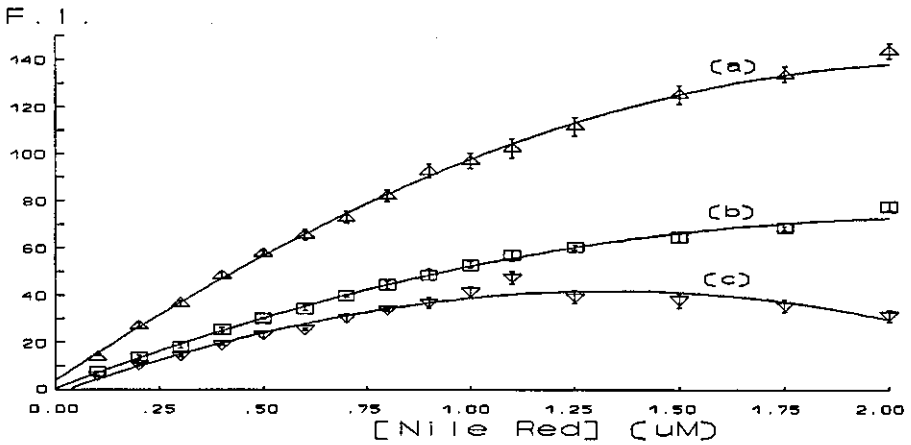


Fig 5.6: The binding of Nile Red to OMD, BSA and HSA.

Key: (a) 1μM HSA, Em. 640nm (b) 1μM BSA, Em. 645nm
(c) 1μM OMD, Em. 650nm.

The fluorescence changes of Nile Red bound to OMD are also much smaller than those associated with the serum albumins which indicates that the environment of Nile Red on OMD is not as hydrophobic as those on the serum albumins. The Scatchard plot of Nile Red binding to OMD is shown in Fig. 5.7 while the affinity constant and number of binding sites are given in Table 5.3.

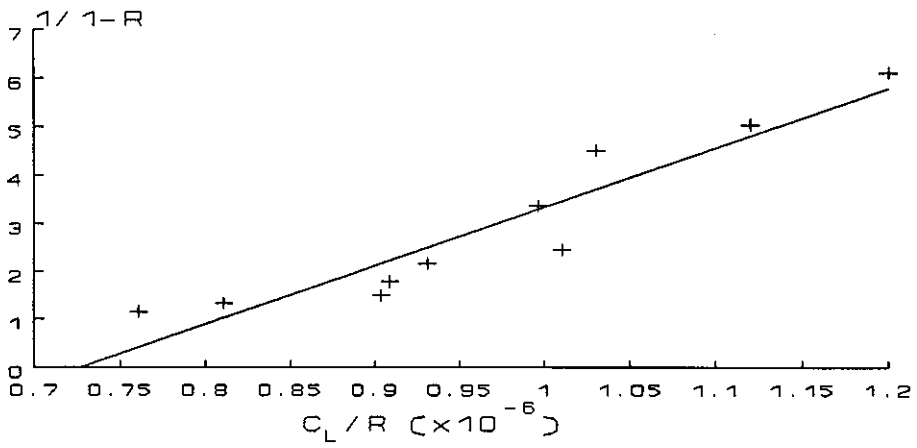


Fig. 5.7: Scatchard plot of Nile Red binding to OMD.

Table 5.3: Binding parameters of Nile Red in OMD.

	K_a M^{-1}	n	Correlation Coefficient
OMD	12261000	0.73	0.949

Nile Red unsurprisingly appears to have one high affinity site on OMD, the K_a of which has a similar order of magnitude to that of the high affinity site on HSA.

Fig. 5.8 show the effects of increasing concentration of OMD on the fluorescence of 1 μM Nile Red. With an increase in protein concentration there is a fluorescence intensity enhancement, the explanation for this effect being the same as that proposed for this phenomenon with serum albumin. However, OMD differs from the serum albumins in that the increases in fluorescence intensity obeys a linear relationship with increasing concentration, the straight line having a correlation coefficient of 0.995. This effect is not exceptional and was found to occur at different concentrations of Nile Red with increasing OMD. With this linear relationship it could be possible to quantitatively assess the amount of OMD in solution by adding Nile Red and measuring the fluorescence.

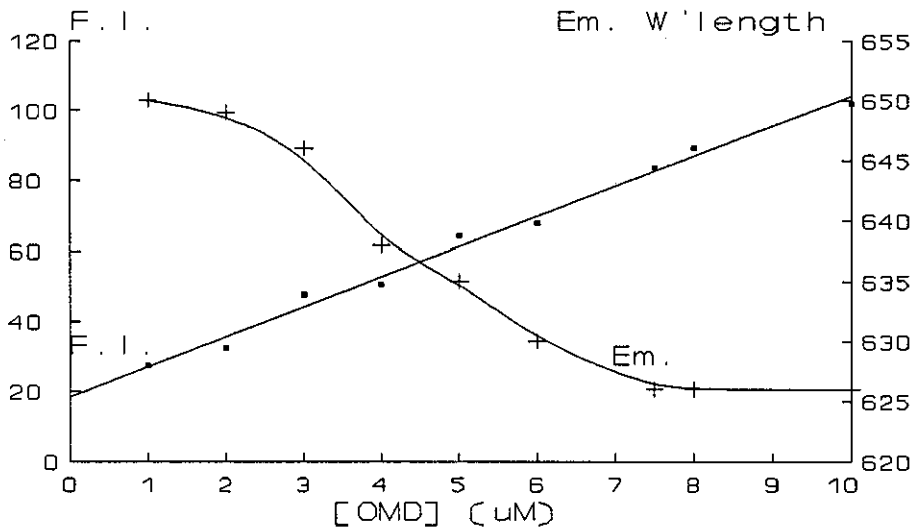


Fig. 5.8: Fluorescence changes of Nile Red (1μM) in increasing concentrations of OMD.

The shift in emission wavelength associated with increasing OMD concentration shown in Fig. 5.8 is not as simple as those associated with the serum albumins. It is not just a simple shift to blue in wavelength, because at certain OMD concentrations the occurrence of shoulders as well as peaks can be observed. As the concentration of the OMD increases the original shoulders become peaks and the former peaks become shoulders. This effect is shown in Fig. 5.9.

The wavelength shifts shown in Fig. 5.8 are the shifts associated with the peaks as is the fluorescence intensity. This effect will be discussed in the following section.

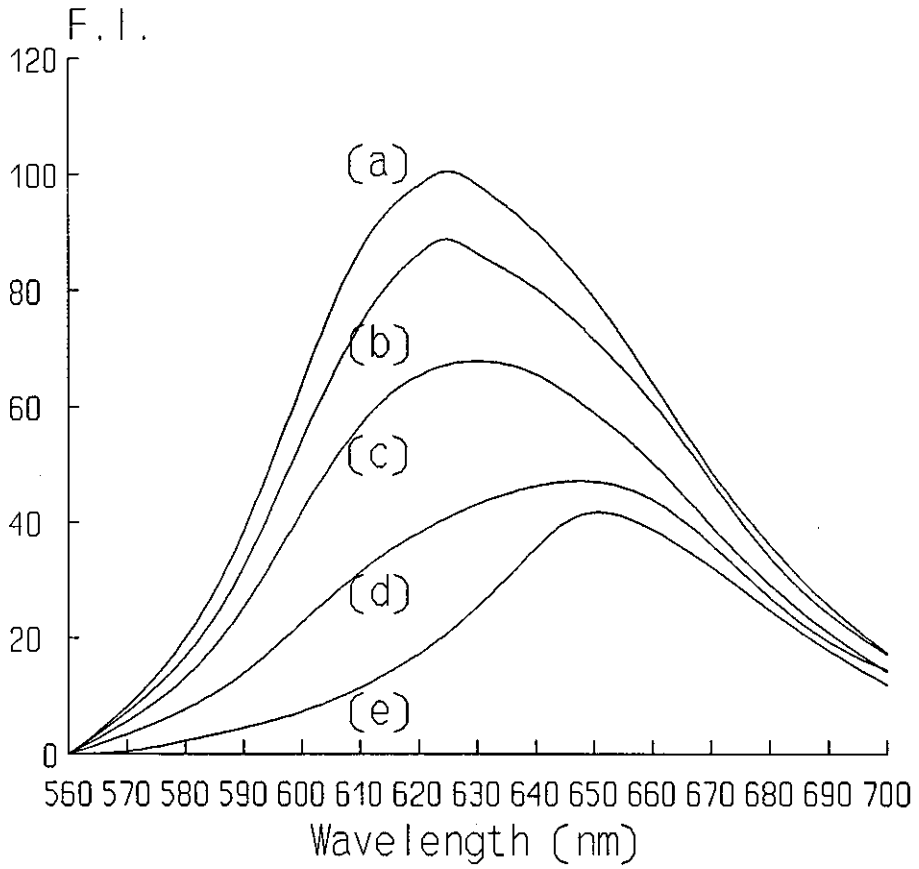


Fig. 5.9: Emission spectra of OMD:Nile Red complexes.

Key: (a) 10μM OMD: 1μM Nile Red. (b) 8μM OMD: 1μM Nile Red.
(c) 6μM OMD: 1μM Nile Red. (d) 3μM OMD: 1μM Nile Red.
(e) 1μM OMD: 1μM Nile Red.

The fluorescence stability of the Nile Red OMD complexes are shown in Fig 5.10. As with the serum albumins, the stability of the complexes increase as the amount of OMD increases compared to Nile Red, supporting the theory that at higher protein concentrations the dye favours binding rather than aggregation.

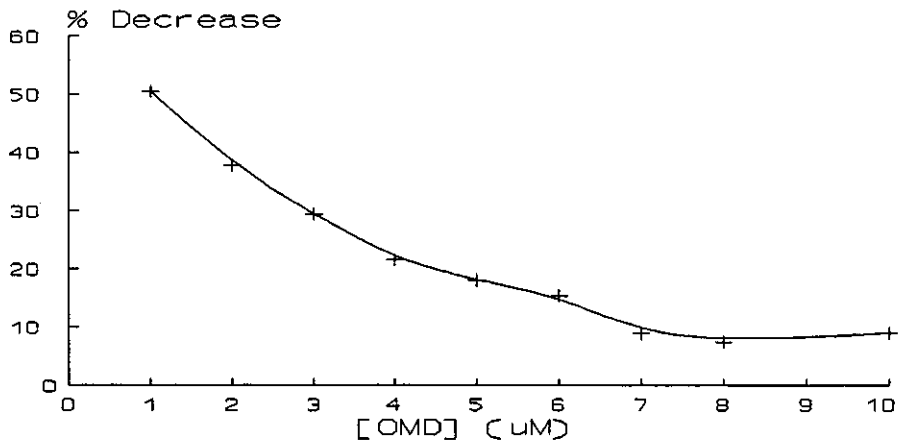


Fig. 5.10: Percentage decrease in the fluorescence of Nile Red (1μM) and OMD complexes after twenty minutes.

5.8.3 Derivatisation Spectroscopy.

Derivative spectroscopy is essentially a re-arrangement of spectroscopic data. It thus enhances narrow structure, bringing about an effective enhancement of resolution and a discrimination against broad background features, but at the expense of a decrease in the signal-to-noise (S/N) ratio.

The characteristic features of a derivative spectrum in the first and second order are shown in Fig. 5.11 with a theoretical symmetrical fluorescence emission peak.

The points of inflection in the original spectrum result in a maximum or minimum in the first derivative, with the maximum becoming a so-called "zero crossing". In the second derivative the points of inflection of the original spectrum appear as maxima while the emission maximum becomes a minimum. Due to a differentiation process, the positions of the zero crossing in the first derivative and the minimum in the second derivative are very slightly shifted in the direction of recording when compared to the position of the emission maximum in the original

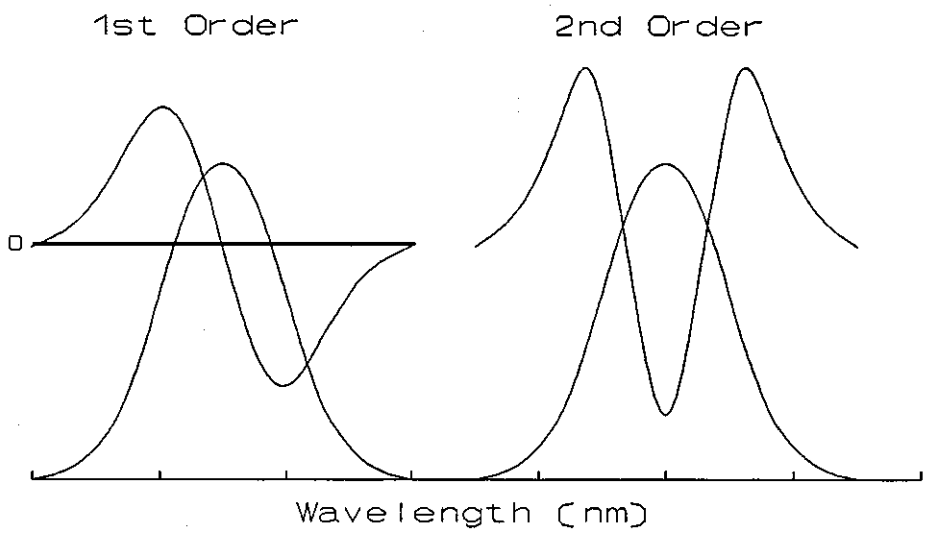


Fig 5.11: Symmetrical emission peaks and first and second order derivatives calculated theoretically.

spectrum. However, this is insignificant for the quantitative and qualitative evaluation of the derivative spectra.

In the first and second derivatives, the ordinate deflections are not proportional to the fluorescence value, but rather to the slope in the original spectrum. Since the slope in the normal spectrum may be positive as well as negative the derivative spectrum will show either positive or negative ordinate deflections, or maxima or minima, depending on the type of slope. The distance between these maxima and minima being a characteristic of the particular compound under examination and can be often used in its evaluation.

Absorption or emission spectra are generally comprised of several bands which overlap so strongly that their individual features are not prominent. Very often the derivative technique will produce a distinct feature for each of the overlapping bands in a spectrum. The resulting spectra are more useful than the initial spectra both as a fingerprint and as a way for assessing small spectral differences quantitatively.

Derivative spectra have the further effect of favouring sharp features in the spectrum, with the discrimination against broad features increasing exponentially with the order of the derivative. This effect is useful for observing sharp bands buried in a broad background, for measuring concentrations in the presence of a broad background interference and most importantly, as far as this work is concerned, for precisely assigning wavelengths to shoulders. In general, these advantages are best realised at high orders, however they are limited by low S/N ratio and a weak but sharp structure in the background.

Many reviews exist on the use of derivatisation spectroscopy and its applications[324-328], not only in the UV/visible region but also in the infra red, in flame emission and absorption spectral photometry and in many forms of luminescence. For fluorescence second order derivatisation is probably the most commonly used derivatisation technique. It is frequently used in the evaluation of the emission spectra of proteins[329,330] allowing the detection of the tyrosine residue where strong tryptophan emission and energy transfer would normally make this difficult.

As stated previously, with a set concentration of 1 μM Nile Red and an increasing concentration of OMD there is a fluorescence enhancement and a large shift in emission wavelength to blue (Fig. 5.8). The trend is easily explained by the fact that with more protein present more Nile Red is binding (Section 5.8.2) and resulting in a greater change in fluorescence. However, as the emission maxima shifts to blue shoulders begin to appear on the spectra. Fig. 5.9 shows some of the resultant spectra.

It was very difficult to determine the exact wavelengths of the shoulders and peaks from the original spectra. Therefore, second order derivatisation was carried out on the original spectra to distinguish between and resolve the two areas on the spectra. Fig. 5.12 shows

examples of some second order derivatisation spectra obtained from the original spectra from Fig. 5.9.

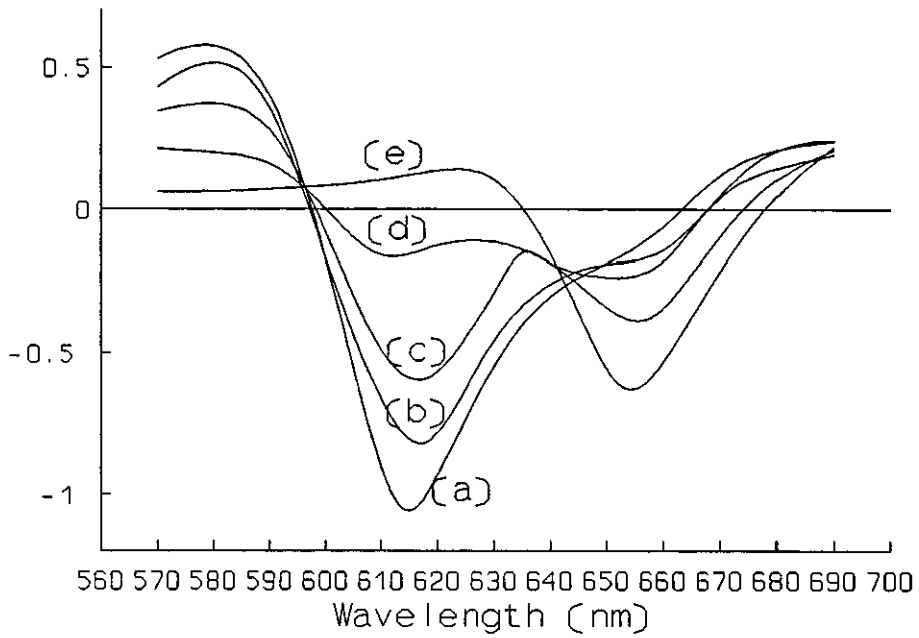


Fig. 5.12: Second order derivatisation of emission spectra of OMD:Nile Red complexes.

Key: (a) 10μM OMD: 1μM Nile Red. (b) 8μM OMD: 1μM Nile Red.
(c) 6μM OMD: 1μM Nile Red. (d) 3μM OMD: 1μM Nile Red.
(e) 1μM OMD: 1μM Nile Red.

This technique helped to resolve what appears to be two binding areas of Nile Red on OMD. There is a high wavelength binding site at ca. 655nm, its high wavelength suggests it is the least hydrophobic of the two binding sites probably because it is closer to the polar water molecules and it is protected from them. The lower wavelength binding site at ca. 615 nm is obviously a much more hydrophobic binding site and probably more in the interior of the protein rather than on the outside like the higher wavelength binding site. Table 5.4 shows the change in the two binding sites of Nile Red with increasing OMD concentration. The wavelengths of the shoulders and peaks were found from second order derivatisation spectra and the intensity directly from the original spectra.

Table 5.4: Fluorescence Intensity of Nile Red in varying concentration of α_1-acid glycoprotein.

[OMD] μM	Fluorescence intensity	
	Low Wavelength Binding site	High Wavelength Binding site
1	3.6	11.4
2	19.6	32.3
3	35.8	47.7
4	47.6	50.2
5	61.3	56.5
6	67.8	59.4
8	89.3	71.2
10	97.8	73.4

5.8.4 Drug Displacement of Nile Red from OMD.

Preliminary investigation of drug binding to α_1-acid glycoprotein using Nile Red involved the study of the effects on the addition of a basic drug like propranolol and an acidic drug like flufenamic acid. Figs. 5.13 and 5.14 show that both drugs cause a drop in fluorescence intensity and a shift to red in the emission wavelength of Nile Red indicating it has been displaced from its binding site.

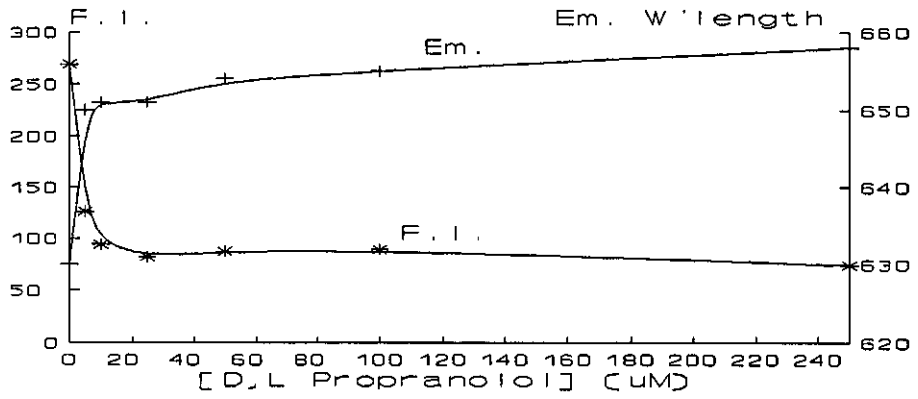


Fig 5.13: Displacement of 1μM Nile Red from 6μM OMD by DL-Propranolol.

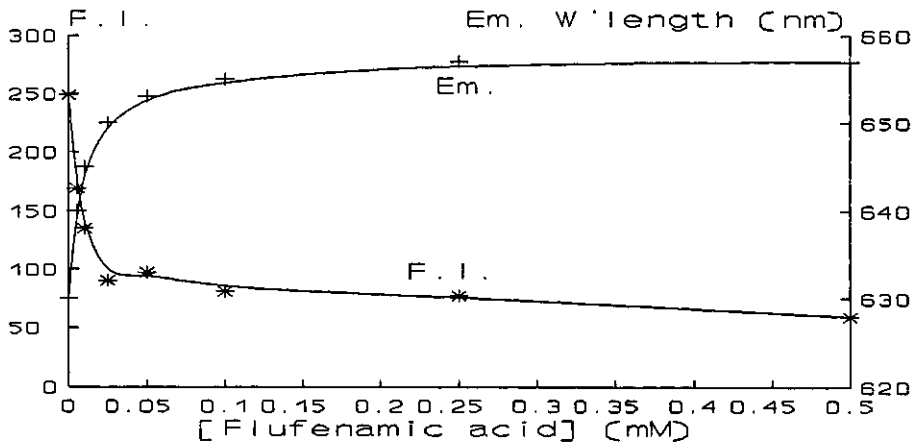


Fig 5.14: Displacement of 1μM Nile Red from 6μM OMD by Flufenamic acid.

The concentration of OMD and Nile Red (6 μM:1 μM) used in the experiments ensured that Nile Red was binding to the low wavelength binding site. The results confirm that basic and acidic drugs bind to similar sites on OMD and as they are known to bind to the high affinity single site, this must be the low wavelength binding site that Nile Red is attached to. For any future binding or displacement titrations fluorescence measurments were taken at the wavelength of this binding site.

As discussed in section 5.5, α_1-acid glycoprotein is reported to show a certain degree of enantiomeric selectivity. Fig. 5.15 shows the displacement of Nile Red with D,L, propranolol and its racemic forms R-(-)-propranolol and S-(+)-propranolol.

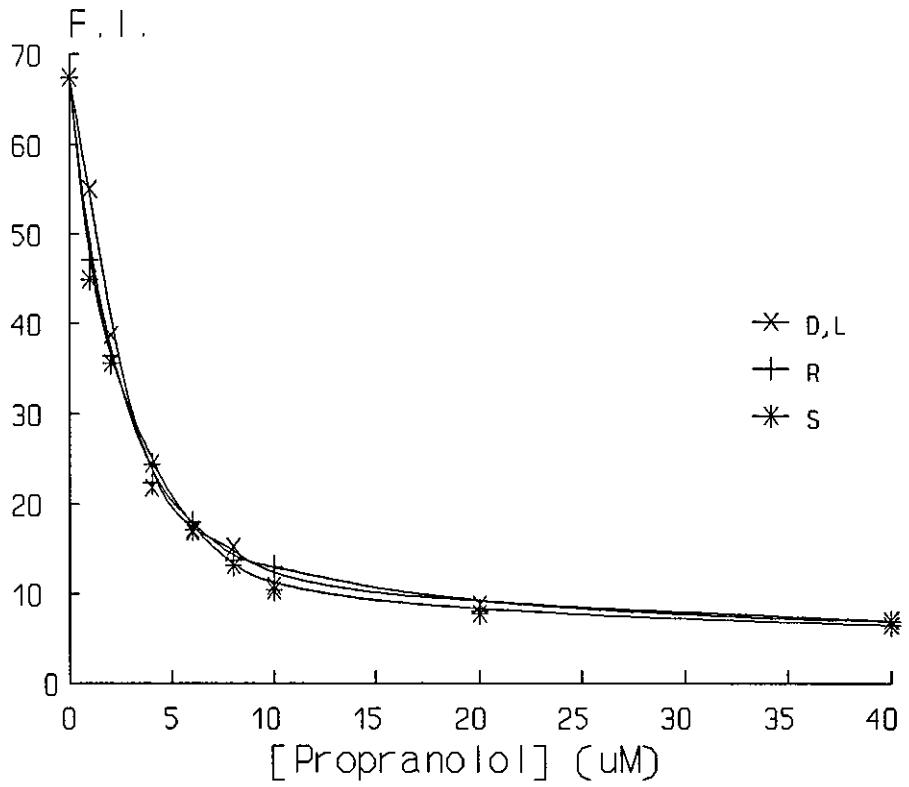


Fig. 5.15: Displacement of 2μM Nile Red from 4μM OMD by various enantiomeric forms of propranolol.

The results show that there appears to be no preferential displacement of Nile Red bound to bovine serum OMD by DL-propranolol or either of its racemers. This is further supported by the affinity constants for the different forms of propranolol given in Table 5.5 which all appear to be of the same order of magnitude and not significantly different.

Table 5.5: Affintiy constants for different enantiomeric forms of propranolol bound to OMD.

Propranolol	K_a M^{-1}	Correlation coefficient
D,L	3556000	0.935
R	3862000	0.936
S	3996000	0.949

The K_a values are similar to those found in the literature for human[87,299] and rat[302] for enanatiomeric mixtures of propranolol and suggest that although no stereoselectivity of propranolol binding to bovine OMD could be found the affinity of the drug for this type of OMD is similar to that of the others.

A similar experiment at the same concentrations was caried out with another basic drug ephedrine and racemic forms. The calculated affinity constants are shown in Table 5.6.

Table 5.6: Affintiy constants for different enantiomeric forms of ephedrine bound to OMD.

Ephedrine	K_a M^{-1}	Correlation coefficient
+/-	78850	0.971
+	22680	0.970
-	89510	0.951

The results show that (+)-ephedrine has a smaller affinity than (±)-ephedrine and (-)-ephedrine for the Nile Red binding site on OMD. It could therefore be possible that OMD is showing enantiomeric selectivity for ephedrine.

5.8.5 Double Probe Binding to OMD.

α_1-acid glycoprotein is believed to have a wide and flexible area for its high affinity binding of drugs. Acidic and basic drugs are known to bind in this area at different sub sites (Section 5.6). As with the serum albumins the effect of ANS on the binding of Nile Red to OMD was studied to see what further information could be obtained about this binding area. The binding of ANS is shown in Figs. 5.16 and 5.17.

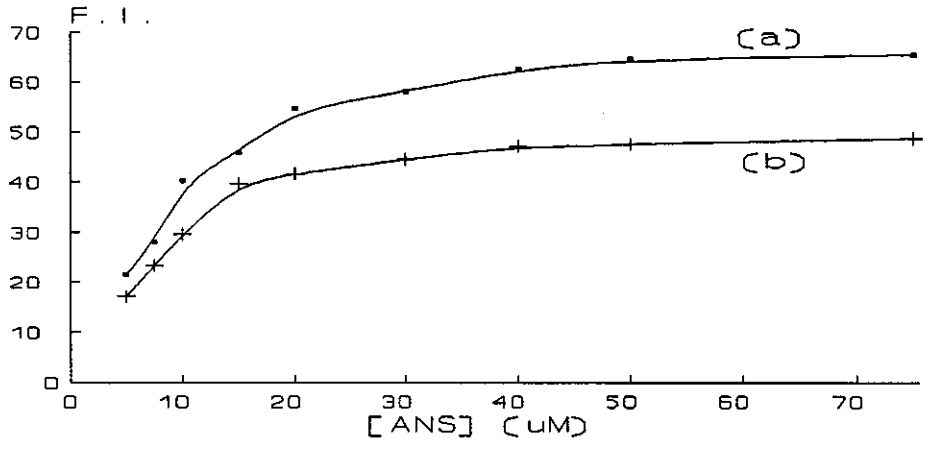


Fig. 5.16: ANS binding to OMD (1μM) (a) without or (b) with the presence of Nile Red (Fluorescence intensity).

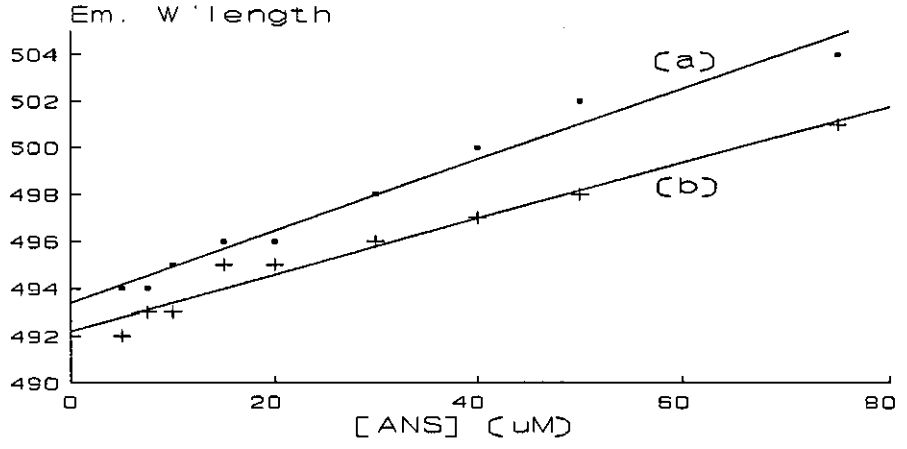


Fig 5.17: ANS binding to OMD (1μM) (a) with or (b) without the presence of Nile Red (Emission wavelength).

The first conclusion to be drawn is the difference in fluorescence ANS shows when binding to OMD in comparison to the serum albumins. At a 10 μM:1 μM (OMD:ANS) level ANS shows a fluorescence intensity of ca. 50*arb* fluorescence units and an emission maxima of 495nm, with ANS bound to BSA, however, at an equivalent concentration a fluorescence intensity of ca. 800 fluorescence units and an emission wavelength of ca. 470nm. The binding site of ANS on OMD would appear to be a lot less hydrophobic than that of BSA, with the three tryptophan residues on OMD compared to the two on BSA having little effect. It is obvious and expected that acidic ANS does not bind as well

to OMD as it does to the serum albumins, for this reason it was difficult to obtain any binding parameters.

As with the serum albumins the difference in fluorescence of ANS with and without the presence of Nile Red can be explained by an absorption re-emission mechanism from the ANS to the Nile Red as the difference is removed upon dilution. This indicates that the two probes do not bind within 80Å of each other and thus are probably not in the same binding area. The difference in emission wavelengths, however, which was not observed with the serum albumins may be explained by a certain amount of displacement. ANS had no effect on the binding of Nile Red to OMD at the concentrations used.

The fluorescence stabilities of both probes with and without the presence of the other are displayed in Table 5.7. The only significant difference of the stabilities in any system is that ANS bound to OMD is not as stable in the presence of Nile Red as it is on its own.

Table 5.7: Fluorescence stability of probes bound to OMD (1μM Nile Red:20μM ANS:1μM OMD).

	% decrease in fluorescence after 60mins
ANS	
ANS only	17.4 ±0.91
Nile Red added first	36.9 ±1.21
Nile Red added last	33.4 ±1.71
Nile Red	
Nile Red only	50.2 ±2.14
ANS added first	52.6 ±2.29
ANS added last	49.3 ±2.31

This may indicate that Nile Red may be displacing a certain amount of ANS over a period of time. However, the effect is not substantial as there is no change in emission wavelength associated with it.

The results of the competition between Nile Red and ANS and their binding to α_1-acid glycoprotein would indicate that both probes can bind simultaneously although a slight displacement of ANS may be observed.Thus both probes can be used in ligand displacement experiments. It has been previously reported[87] that ANS has more than one binding site on OMD and it is possible that any displacement is due to the competition at one of these sites by Nile Red. This site is likely to be the high affinity site previously discussed in section 5.6.

5.8.6 Drug Displacement of Double Probe Complex.

The results of the displacement of Nile Red and ANS by both acidic and basic drugs are shown in Table 5.8.

The results show that at the concentrations used most drugs that displace Nile Red also affect ANS. The most noticeable exception being (±)- ephedrine which causes substantial displacement of Nile Red while not significantly affecting ANS. Sulphadiazine and Sulphamethaoxazole, also appear to displace Nile Red a little but have no affect on ANS. Salicylic acid displaces neither Nile Red or ANS.

The fact that both acidic and basic drugs displace Nile Red supports the theory that both types of drugs share the same binding site. However, some of the acidic and basic drugs also displace ANS which indicates that they may also have more than one point of attachment to OMD.

Table 5.8: Displacement of 2 μM Nile Red and 8 μM ANS from 5 μM α_1-acid glycoprotein by 250 μM concentration of various drugs.

DRUGS	Nile Red		ANS	
	% Displacement	Change in λ (nm)	% Displacement	Change in λ (nm)
Basic				
Amitriptyline	85.1	9	64.8	13
Ephedrine	62.6	7	24.5	0
Propranolol	86.8	11	66.6	17
Acidic				
Ethacrynic acid	64.5	6	61.9	13
Flufenamic acid	84.6	10	74.5	17
Phenylbutazone	70.2	7	54.6	11
Salicylic acid	10.6	0	6.6	0
Sulphadiazine	19.9	2	15.2	0
Sulphamethaoxazole	23.2	2	11.5	0
Warfarin	55.3	6	53.4	11

NB Changes in emission wavelength correspond to shifts to red.

5.9 CONCLUSIONS.

As in the study of serum albumins, Nile red was the best suited of all the compounds tested to act as a fluorescence probe for OMD.

Binding titrations involving Nile Red produced a Scatchard plot showing evidence of one high affinity site. However, examination of the fluorescence spectra indicated the presence of two possible areas of attachment for Nile Red on OMD. Using second order derivatisation the two areas were assigned emission maxima at ca. 615nm and ca. 655nm. The lower wavelength binding area obviously being more hydrophobic and thus more in the interior of the

OMD than the higher wavelength site. The results showed how useful the technique of second order derivatisation of emission spectra was for identifying two overlapping peaks.

The displacement of Nile Red was achieved by many types of drugs both acidic and basic indicating that unlike the serum albumins, ligand binding to OMD would appear to be largely hydrophobic in nature. Stereoselectivity was possibly shown for the basic drug ephedrine although none could be found for the various racemic forms of propranolol despite the fact it has been reported for several species types of OMD.

Again the double probe technique provided additional information on the binding sites of the Nile Red in relation to those of ANS.

CHAPTER SIX

ß-LACTOGLOBULIN

6.1 INTRODUCTION.

Milk is a significant source of animal protein for humans. It is generally considered to consist of approximately 87% water, 5% lactose, 4% fat, 1% ash and just over 3% protein[331]. Table 6.1 lists the proteins in bovine milk[332].

Table 6.1: Proteins of Bovine Milk.

Protein	% of the milk
Caseins#	2.5
ß-lactoglobulin	0.3
α-lactalbumin	0.07
Blood serum albumins	0.03
Euglobulin	0.03
Pseudoglobulin	0.03
Other albumins and globulins	0.13
Fat globule protein	0.02
	3.11

components of casein
α-casein
α_s Ca sensitive stabilised by κ
κ Ca insensitive acted upon by renin
ß-casein
γ-casein

Generally when discussing the proteins of milk it is necessary to distinguish between the proteins in milk and those obtained from milk by various physical and chemical procedures. Due to the ease of which casein can be isolated from milk, the earliest sub division of milk proteins was casein or curds and whey proteins. However, this implies that casein exists in milk in the same form as it does in the isolated state, which is not true. In milk, casein exists in the form of a complex or micelle, consisting of calcium caseinate with phosphate, additional calcium, magnesium and citrate. Casein may in fact be defined as the protein precipitated by acidifying milk to a pH value near 4.6[333] and is properly called hydrogen caseinate.

The proteins remaining in solution after the curds have been removed from milk are known as the whey proteins or milk serum proteins and are shown in Table 6.1. Initially the whey proteins were divided up into the lactalbumin fraction and the lactoglobulin fraction and were considered to be single chemical entities. However, in 1934 Palmer[334] discovered that a crystalline protein with a low water solubility could be separated out from the classical lactalbumin fraction. In 1942, Cannan *et al.,*[335] proposed the name ß-lactoglobulin by which the protein is known today, while at a similar time a protein known as α-lactalbumin was also fractionated from the classic lactalbumin fraction[336].

ß-lactoglobulin was shown to be the most abundant of whey proteins and because of the fine crystals of the protein that could be isolated from cow milk, ß-lactoglobulin was considered to be a convenient pure protein to use in physical and chemical studies. In fact it was used in the first complete analysis of a fairly large protein. Reviews of this early work have been written by McKenzie[337], McMeekin[338] and Tilley[339]. An important discovery however was made by Aschaffenberg and Drewry[340] who discovered that bovine milk could contain either a mixture or one of two types of ß-lactoglobulin. It was possible to isolate each type in a state of high purity and although at first they were initially called $ß_1$- and $ß_2$- lactoglobulin later they were known as 'A' and 'B'. It was shown that the occurrence of these variants was genetically determined, the genes controlling the synthesis of the protein being autosomal alleles without dominance. This was the first demonstration of genetic variation in milk proteins.

6.2 STRUCTURE AND FUNCTION OF ß-LACTOGLOBULIN.

ß-lactoglobulin is the predominant whey protein in the milk of many species including ruminants and non-ruminants such as horse, pig, dolphin, kangaroo and possibly man, although the bovine form is the most studied[341].

The general amino acid sequence of bovine ß-lactoglobulin is shown in Fig. 6.1[342]. The protein has a molecular weight of 18, 400 corresponding to a polypeptide chain of 162 residues.

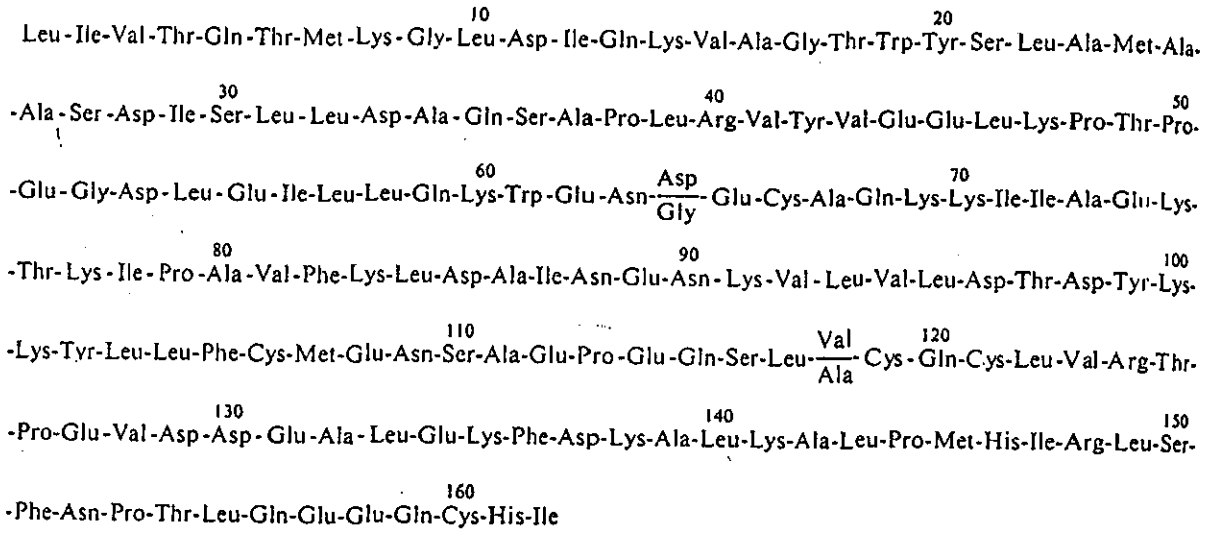


Fig. 6.1: Amino acid sequence of ß-lactoglobulin (A/B).

There are two disulphide bridges and a free cysteine in the sequence Cys-Gln-Cys (119-121) the exact residue being uncertain although it may be an equal mixture of the two[337,343]. Bovine ß-lactoglobulin as with the other species is believed to exist as a dimer and has genetic variants thought to be in the forms ß-lactoglobulin 'A' and 'B' which only differ by a few amino acid residues (Fig. 6.1) and are thought to be very closely physically and chemically related.

The structure of the protein molecule consists of anti-parallel ß sheets, formed by 9 strands wrapped round to form a flattened cone or calyx[344]. The core of the molecule, an eight stranded, antiparallel ß-barrel (Fig. 6.2) is unusual and has only been observed before in retinol binding protein.

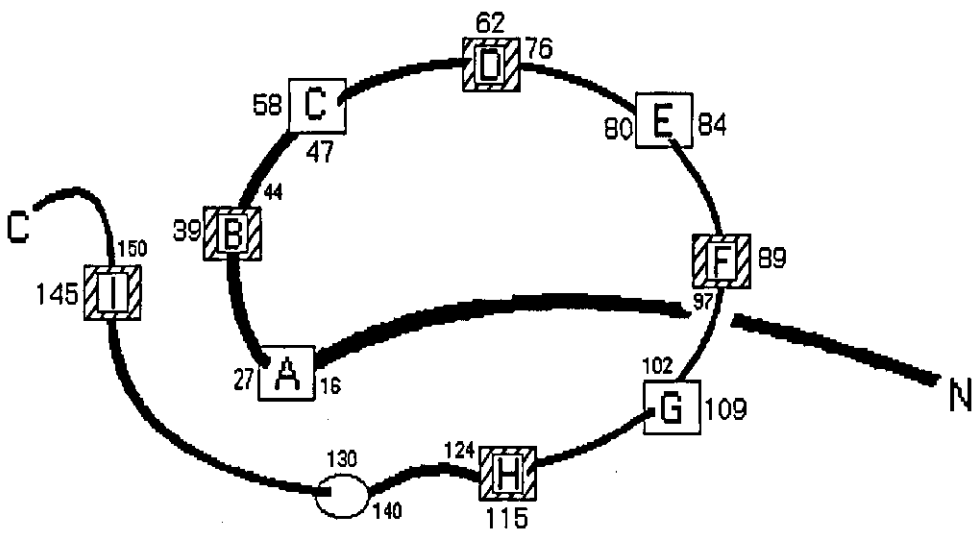


Fig. 6.2: The topology of ß-lactoglobulin with the end termini hatched to emphasize their anti-parallel nature.

The strands A-I comprise of residues 16-27, 39-44, 47-58, 62-76, 80-84, 89-97, 102-109, 115-124 and 145-150. The upper sheet strands E, F, G, H and I are flanked by a three turn helix (130-140). The lower sheet comprises of strands A,B,C and D with strand A bent through a right angle to participate in both upper and lower sheets. Strand E makes several contacts with strand D, thus helping the formation of the calyx. Reverse turns occur at residues 44-47, 59-62, 78-81 and 84-88 but the turns at 98-102 and 111-115 are not true ß-bends. Strand I is involved in the formation of the dimer by making antiparallel interactions with the dyad related strand. The interface between monomers also involves hydrophobic interactions between Ile 29 and Ile 147 and stacking of the imidazoles of symmetry related His 146 residues. The free sulphydryl is buried in the sheet helix interface and the two disulphide bonds exist between residues 106-119 and 66-160. The former linking strand H to G and the latter links strand D to the C terminal region. In summary 50% of the

peptide residues in ß-lactoglobulin are anti parallel ß sheets, 15% are α helix, 20% turns and the remaining 15% represents amino acids in a random non repetitive arrangement.

The exact function of ß-lactoglobulin is still really unknown and many theories have been postulated. It was previously though as with all milk proteins it may act as a source of amino acids for nutrition in young ruminants or that it has an enzymatic role in milk biosynthesis[338]. None of these theories have been proved although ß-lactoglobulin has been found to act as a substrate for a certain enzyme[345], suggesting it may have an enzymatic function.

More recent work has involved the theory that ß-lactoglobulin may be a transport protein. Studies of small globular proteins termed lipocalins, which interact with certain hydrophobic ligands suggests that these proteins can be classified in the "superfamily" of hydrophobic molecule transporters[346]. ß-Lactoglobulin, retinol binding protein, bilin binding protein and insecticyanin are the best known proteins of this class. All these proteins are known to have similar structures[347] especially ß-lactoglobulin and retinol binding protein[344]. In fact it has been found in-vitro that retinol has a greater affinity for bovine ß-lactoglobulin than it does for bovine retinol binding protein. This prompted speculation that ß-lactoglobulin is involved with vitamin A transport and in fact Papis *et al.*,[344] reported the discovery of ß-lactoglobulin receptors in the small intestine of the neonate calf which may be important in vitamin uptake. However, in milk, retinol is associated with fat globules[348] and retinol bound to ß-lactoglobulin has not been detected. Thus the binding of retinol in-vitro could only reflect a general affinity for small hydrophobic molecules.

ß-lactoglobulin has also been shown to bind certain fatty acids in-vitro[349]. Other proteins which specialize in fatty acid transport, like α-fetoprotein[350] are also able to bind retinol in-vitro, in a binding site that overlaps one of the fatty acid binding sites[351]. This indicates that ß-lactoglobulin may have a role in fatty acid transport.

It has also been determined that ß-lactoglobulin shows certain homology with some intracellular fatty acid binding proteins like Z protein and P_2 protein[352]. These proteins with a tissue-specific distribution have different ligand affinities suggesting they may have evolved to serve distinct functions all related to lipid metabolism, which is another possible function for ß-lactoglobulin.

6.3 SPECIES DIFFERENCES AND GENETIC VARIATIONS.

As stated previously the first discovery of genetic variants of ß-lactoglobulin was made by Aschaffenburg and Drewry in cows. In the present day ß-lactoglobulin is known to vary between species and there may even be several different forms per species. Sheep, horse, pig, goat, kangaroo and many other ruminant ß-lactoglobulins have all been studied[353] although bovine ß-lactoglobulin and its generic forms is the most commonly discussed in the literature.

Since the discovery of the two variants of bovine ß-lactoglobulin by their differing mobility on filter paper electrophoresis at pH 8.6, 'A' having a higher mobility than 'B', other forms have also been discovered in various breeds of cattle. In 1962, Bell[354] showed the existence of a third variant in Australian Jersey cows. This variant had a mobility less than the 'A' or 'B' variants and was shown to be genetically determined. It was called variant 'C' and its presence was later confirmed in Jersey cattle in North America, British and other European herds. The fact that it could not be found in any Guernsey herd inferred that the two breeds differed in origin and must have arrived in the Channel Islands by different routes.

In 1966 two other variants were announced, variant 'D' was found in Montbeliarde cattle in France[355] with its presence later confirmed in many other European herds. The second new variant was isolated from Australian Drought Master beef cattle[356] and was given the name ß-lactoglobulin$_{Dr}$. This nomenclature was chosen since the variant has the same amino acid

composition as the bovine 'A' variant but a carbohydrate moiety attached to it.

The differences in the bovine variants are small and all of them are believed to have a monomeric molecular weight of around 18,400 apart from the droughtmaster form which has a weight of ca. 20,000 because of its carbohydrate content. Table 6.2 shows the differences in amino acids residues between the variants. Variants 'A' and 'B' are believed to be much more common than the other forms and the most commercially available ß-lactoglobulin is a combination of these.

Table 6.2: Difference in Number of Amino Acid Residues per Monomer Compared with Monomer B[357].

Residue	Bovine			
	A	C	D	Dr
Gly	-1			-1
Ala	-1			-1
Ser				
Val	+1			+1
Ile				
Leu				
Tyr				
Asp	+1			+1
Gln		-1	a	
His		+1		
Lys				
Carbohydrate				b

Key: (a) D has 1 Gln substituted for 1 Glu
(b) Droughtmaster has 1.0 N-acetylneuraminic acid, 4.3 hexosamine and 2.7 hexose residues.

6.4 EFFECT OF PH ON ß-LACTOGLOBULIN.

ß-lactoglobulin shows a remarkable stability to low pH resisting denaturation even at pH 2, however, it denatures at alkaline pH. At intermediate values it aggregates and undergoes conformational changes some of which have been detected by spectroscopic techniques[358-364] and are discussed below.

pH Range 1.8-3.5.

In this range all the variants appear to undergo a rapid monomer↔dimer equilibrium with increasing dissociation as the pH is lowered.

pH Range 3.5-5.2.

Initially, ß-lactoglobulin was considered to exist as a molecular weight unit of 36,000 near pH 5.2 and this was considered to be the monomer unit. Eventually, however, it became clear that this was in fact the dimeric unit of two identical chains held together by non-covalent forces. It is now apparent that even at pH 5.2 the dimer is very weakly dissociated to the monomer. As the pH is lowered there is an increasing tendency for dissociation. This type of behaviour has been observed for all the bovine variants, but the bovine 'A' variant also undergoes an additional type of reaction. At around pH 4.6, the 'A' variant is largely associated above the dimer in fact an octamer formation is preferred. The dimer-octamer equilibrium is rapid and unstable and is favoured at a temperature of 4 °C. At pH values above and below pH 4.6 the amount of octamer falls away. The 'B' variant can form mixed octamers with the 'A' variant but by itself it only octamerizes weakly. It is thought that the formation of the octamer involves carboxyl groups and hydrogen bonding[357,365].

pH Range 5.2-9.2.

Between pH 5.2 and 6.0 the protein changes from the acidic Q form to the native N form[358]. The species larger than the dimer decrease in relative amounts and the dimeric form is predominant at pH 6.0. At pH 7.5, there is a reversible transition (known as the Tanford transition[366]), which is characterised by the release of a buried carboxyl group, an increase in the

reactivity of a free sulphydryl group and a change in environment of a tyrosine residue. The released carboxyl group is generally considered to be the ionization linkage of the transition and the increase in reactivity of the sulphydryl parallels the degree of dissociation of dimer to monomer (variant 'A' > 'B' > 'C').

Above pH 7.5 then the protein exists as a monomer while at pH 8.0 and above several time-dependent changes involving conformational and new aggregation processes occur and are related to the irreversible denaturation of the protein.

6.5 BINDING PROPERTIES OF ß-LACTOGLOBULIN.

As mentioned previously, an interesting property of ß-lactoglobulin is its ability in-vitro to bind a variety of hydrophobic substances which could be related to its putatative physiological role. It has been shown that the protein can bind long chain fatty acids[349] and triglycerides[367]. The binding site for the fatty acids was proposed by Spector and Fletcher[368] as a hydrophobic pocket with a positively charged amino acid near the entrance. The changes in hydrophobicity induced by this binding have been measured using hydrophobic low wavelength fluorescence probes like cis-paranaric acid and ANS[369]. These binding properties have been exploited by using ß-lactoglobulin as an emulsifying agent in food technology[370] and as a fatty acid carrier in cell culture[371].

It has also been reported that ß-lactoglobulin from several species can bind retinol and some of its derivatives especially retinoic acid which is one of the key morphagen molecules in developing embryos[347]. This property has been related to a possible vitamin A transport function of ß-lactoglobulin. Retinol itself is believed to bind to the protein in a 1:1 molar ratio with a dissociation constant in the range of 10^{-8} M. Initially it was believed that retinol was binding in the ß-barrel of ß-lactoglobulin with a tryptophan residue interacting with the ß-ionone moiety of retinol[372]. However X-ray diffraction studies have

shown that the binding site is a superficial hydrophobic pocket rather than a ß-barrel[373] and that this pocket may be the same as that associated with the fatty acids[374].

The interaction of ellipticine with ß-lactoglobulin has also been studied[375]. Ellipticine is an anti-tumour, DNA intercalating agent that is strongly hydrophobic in its neutral form. It has been reported to bind to neutral micelles, liposomes[376] and certain cyclodextrins[377]. It was thus thought to be a good candidate for interaction with ß-lactoglobulin. It was found that ß-lactoglobulins A and B both bind neutral ellipticine with an affinity constant of $7\pm3 \times 10^5$ M^{-1}, which is comparable to that of DNA. The attachment site was not the ß-barrel or the hydrophobic site identified as the retinol site but a domain located at the interface of two monomeric units of the protein, the ligand lying close to the Trp 61 residues of both polypeptide chains. The binding site was highly hydrophobic as the other charged forms of the drugs did not bind.

Current work involves the study of drug binding to ß-lactoglobulin because of the problems associated with drugs taken orally, such as total breakdown of the compound within the stomach or major irritation to the stomach lining. A molecule which could carry such a drug compound throughout the stomach and release it further down the gut would be of great advantage.
ß-lactoglobulin is known to be strongly resistant to the low pH values associated with the gut and is also reported to be resistant to the acid proteases found in the stomach[378] and so is believed to be a possible future drug delivery system. The binding of long wavelength fluorescence probes to ß-lactoglobulin could be a useful tool in studying such interactions.

6.6 EXPERIMENTAL.

All procedures given in chapter two were followed.

The MPF 44B was used for all preliminary work involving the different dyes. The LS50 was used for all work involving Nile Red. All excitation wavelengths used have been discussed previously.

MES buffer was used at pH 6.9 unless otherwise stated, in which case pH 8.75 was used. All ß-lactoglobulin used was the commercially available ß-lactoglobulin containing a mixture of variants 'A' and 'B', unless otherwise stated.

6.7 RESULTS AND DISCUSSION.

6.7.1 Preliminary Investigation of the Binding of the Dyes to ß-Lactoglobulin.

As with the serum albumins and α_1-acid glycoprotein all the dyes were tested to see if any under went a change in fluorescence emission or intensity when they were added to ß-lactoglobulin. Table 6.3 gives a summary of the results. It is obvious that again Nile Red seems the most suitable fluorescent probe for the investigation of ß-lactoglobulin.

Table 6.3: Changes in Fluorescence Properties of Dyes when added to ß-lactoglobulin.

DYE	Concentration Dye:Protein μM	Change in emission wavelength nm	% Change in Fluorescence Intensity
DTTC	2.5:6	0	0
IR125	2:10	0	0
MC 540	3.5:3	0	+30.4
R800	10:30	0	+14.3
Nile Blue	5:10	0	0
Nile Red	2.5:5.4	-45	+406.3

NB Any changes in F.I. less than 10% are listed as 0. Negative change in emission wavelength means a shift to blue, positive fluorescence intensity indicates a fluorescence enhancement.

Fig. 6.3 details one of the preliminary experiments performed with ß-lactoglobulin and Nile Red. As the concentration of the protein increases there is a shift to blue and increase in fluorescence intensity which is common to the effect seen with Nile Red fluorescence in decreasing solvent polarity. Nile Red is acting as a hydrophobic fluorescence probe for ß-lactoglobulin. However, the concentrations used in Fig. 6.3 produce an absorbance over 0.05 and so further investigation was carried out at much lower concentrations.

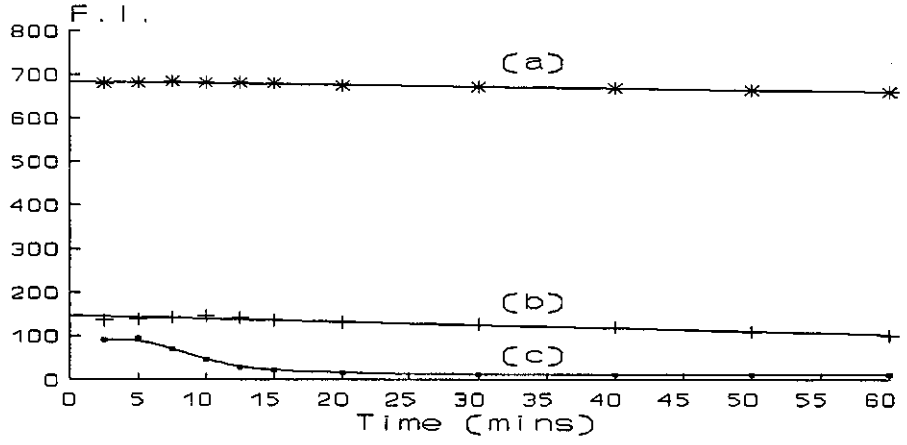


Fig. 6.3: Fluorescence changes of Nile Red in ß-lactoglobulin.

Key: (a) 2.5µM Nile Red:27µM ß-lactoglobulin Em. W'length 610nm.
(b) 2.5µM Nile Red:5.4µM ß-lactoglobulin Em. W'length 615nm.
(c) 2.5µM Nile Red only Em. W'length 658nm.

6.7.2 Nile Red Binding to ß-Lactoglobulin.

The titration of Nile Red with ß-lactoglobulin (1 µM) is shown in Fig. 6.4 with the binding parameters in Table 6.4 which show the binding of Nile Red to one binding site on ß-lactoglobulin.

Table 6.4: Binding parameters of Nile Red in ß-lactoglobulin.

K_a M^{-1}	n	Correlation Coefficient
8976000	0.523	0.932

However examination of the resultant spectra one of which is shown in Fig. 6.5 showed evidence of two distinct regions of Nile Red binding, at ca. 610nm and 650nm which were so intelligible that second order derivatisation of the spectra was unnecessary.

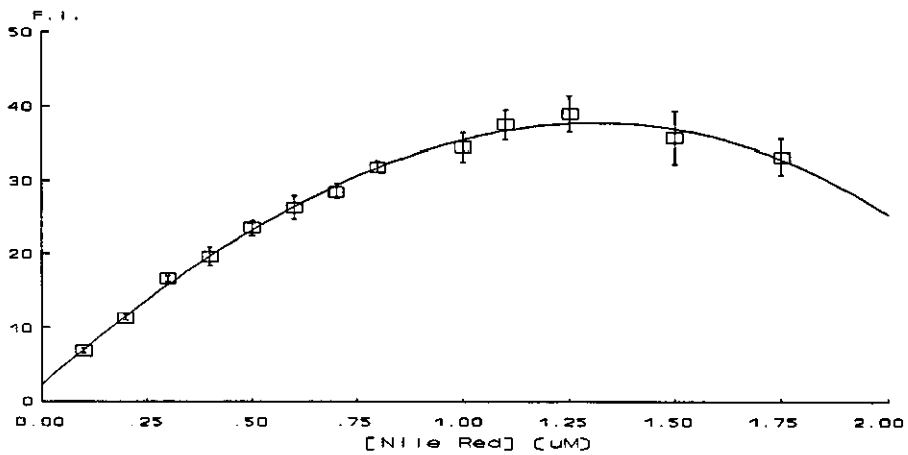


Fig. 6.4: Nile Red binding to 1μM ß-lactoglobulin.

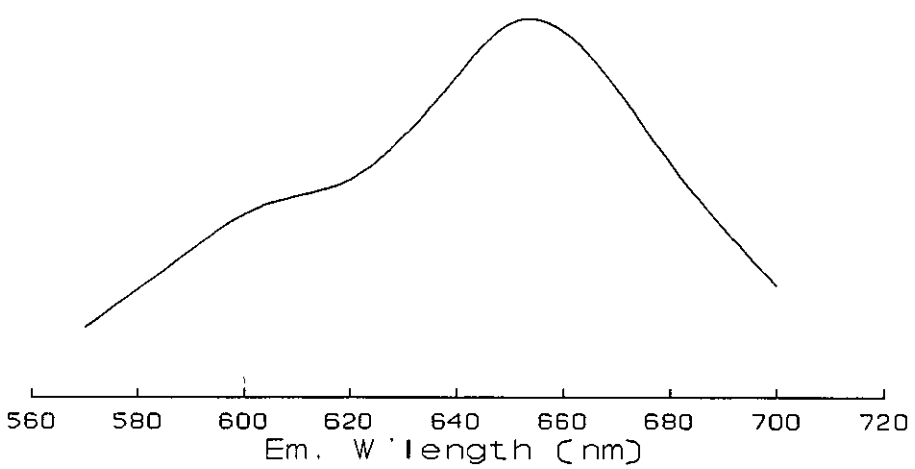


Fig. 6.5: Emission spectrum of 1μM Nile Red: 1μM ß-lactoglobulin.

Further study of the two possible binding sites was carried out by using reverse titration of 1μM Nile Red and increasing concentration of ß-lactoglobulin. Examples of the spectra are shown in Fig. 6.6, as with OMD, as the concentration of ß-lactoglobulin increases the lower wavelength binding area becomes more prominent.

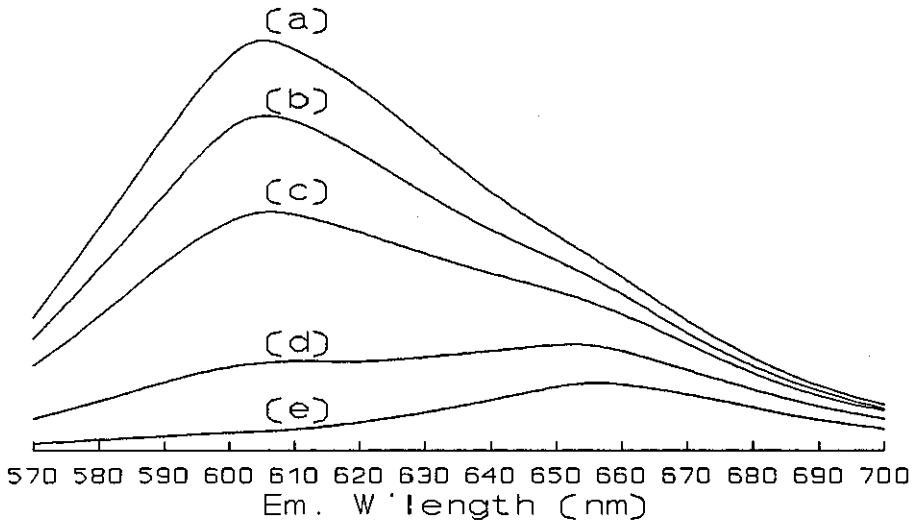


Fig 6.6: Emission spectra of Nile Red:ß-lactoglobulin complexes.

Key: (a) 10µM ß-lactoglobulin:1µM Nile Red.
(b) 8µM ß-lactoglobulin:1µM Nile Red.
(c) 5µM ß-lactoglobulin:1µM Nile Red.
(d) 2µM ß-lactoglobulin:1µM Nile Red.
(e) 0.5µM ß-lactoglobulin:1µM Nile Red.

The fluorescence intensity increase at both the low and high wavelength binding areas (Fig. 6.7) obey a straight line relationship with increasing ß-lactoglobulin concentration and have the correlation coefficients of 0.994 and 0.986 respectively. These results indicate that Nile Red could be used in a fluorescence assay for ß-lactoglobulin in milk whey (Section 6.7.5).

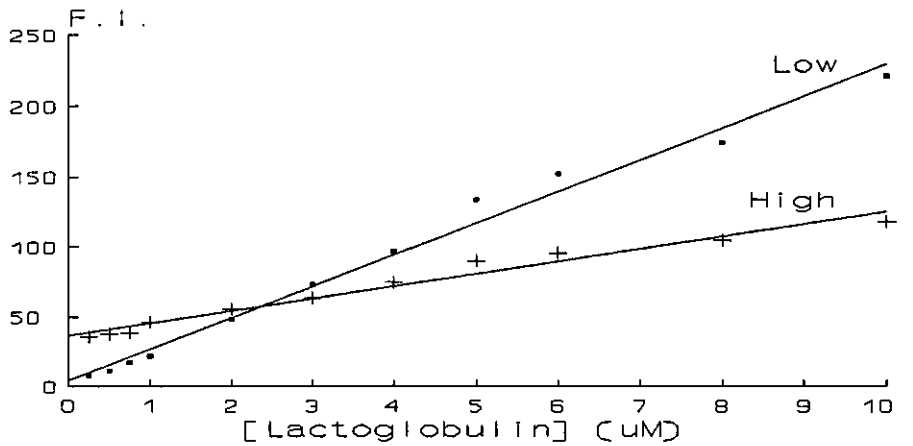


Fig. 6.7: Fluorescence intensity increase of Nile Red (1µM) bound to increasing concentrations of ß-lactoglobulin at low and high wavelength binding site.

From Fig. 6.7 it can be seen that above a 2:1μM (protein:Nile Red) ratio the intensity at the low wavelength binding area is the largest indicating that the attachment of Nile Red here is favoured at higher protein concentrations.

The fluorescence stabilities of the two binding sites are shown in Fig. 6.8. Initially at low protein concentrations the low wavelength binding site is not as stable as the high wavelength binding site probably because the Nile Red does not preferably bind here. However at higher protein concentrations the fluorescence stability of the low wavelength binding site is comparable to that of the high, the stabilities of both binding sites improving for reasons explained previously.

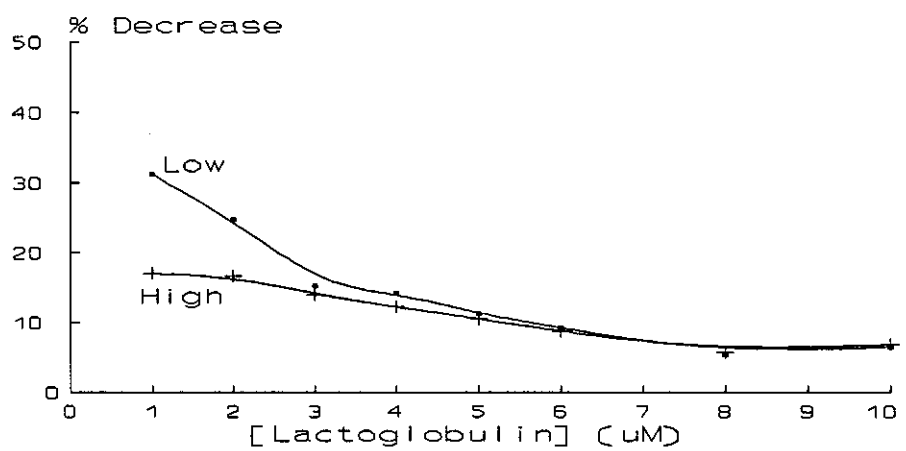


Fig 6.8: Percentage decrease in fluorescence of Nile Red (1μM) and ß-lactoglobulin complexes after twenty minutes.

The results obtained in this reverse titration support the findings found in the direct titration that there are two binding areas or sites for Nile Red on ß-lactoglobulin. The lower wavelength binding site is probably more hydrophobic in nature and so is likely to be in the interior of the protein perhaps in some hydrophobic crevice. The binding site at 655 nm is obviously not as hydrophobic and is probably more on the exterior of the protein closer to the polar water molecules. However, binding of the two sites does not remain the same, at low ß-lactoglobulin

concentrations Nile Red binding is favoured at the high wavelength binding site while at higher concentrations the reverse is true. ß-lactoglobulin is known to exist as a dimer at pH 6.9, as the protein concentration increases more dimers or aggregates will be apparent. As binding of the 608 nm site increases in these concentrations by a much larger proportion than at the 655 nm binding site it could be that Nile Red is binding in between the two monomeric units of the dimer much the same as ellipticine is believed to do[375]. This would explain the greater hydrophobicity of the site and why binding increases at this site by a much greater proportion than at the higher wavelength site.

This theory is supported by the fluorescence stability findings where the stability of the low wavelength binding site increases by a much larger amount than that of the high wavelength binding site with increasing ß-lactoglobulin concentration.

6.7.3 Nile Red Binding to Different Generic Forms of ß-Lactoglobulin.

Reverse titrations, as used previously, were carried out with Nile Red and ß-lactoglobulin 'A', ß-lactoglobulin 'B' and an equimolar mixture of the two and compared with the results of the commercially bought mixture of ß-lactoglobulin 'A' and 'B' (Section 6.7.2). Table 6.5 shows a comparison of the results at a standard (1:3 μM) concentration.

With either form of ß-lactoglobulin or mixtures of the two, the high wavelength site does not appear to differ in emission wavelength and it remains at ca. 650nm. There is a little difference in terms of the fluorescence intensity at this site with the 'A' form having the smallest, the 'B' form the highest and the mixture being in between. However these differences are not large and it would appear that this binding site is very similar on ß-lactoglobulin 'A' and ß-lactoglobulin 'B'.

Table 6.5: Nile Red (1 μM) binding to different forms of ß-lactoglobulin (3 μM).

Lactoglobulin	Low wavelength binding site		High wavelength binding site	
	Emission wavelength	Fluorescence intensity	Emission Wavelength	Fluorescence Intensity
ß-lactoglobulin A	604	51.5	650	55.3
ß-lactoglobulin B	610	95.1	650	69
Commercial ß-lactoglobulin A and B	608	73.2	650	63.8
Prepared ß-lactoglobulin A and B	608	70.0	650	60.9

With the low wavelength binding site the fluorescence of Nile Red differs depending on what form of ß-lactoglobulin it is attached to. ß-lactoglobulin 'A' has the lowest emission wavelength and the smallest fluorescence intensity while the opposite is true for ß-lactoglobulin 'B'. Again the emission maximum and fluorescence intensity for the mixtures lie between these limits.

The results for both of the binding sites are confirmed by Figs. 6.9 and 6.10, which show the effects of increasing protein concentration on the fluorescence of Nile Red at the different binding sites for the different forms of ß-lactoglobulin.

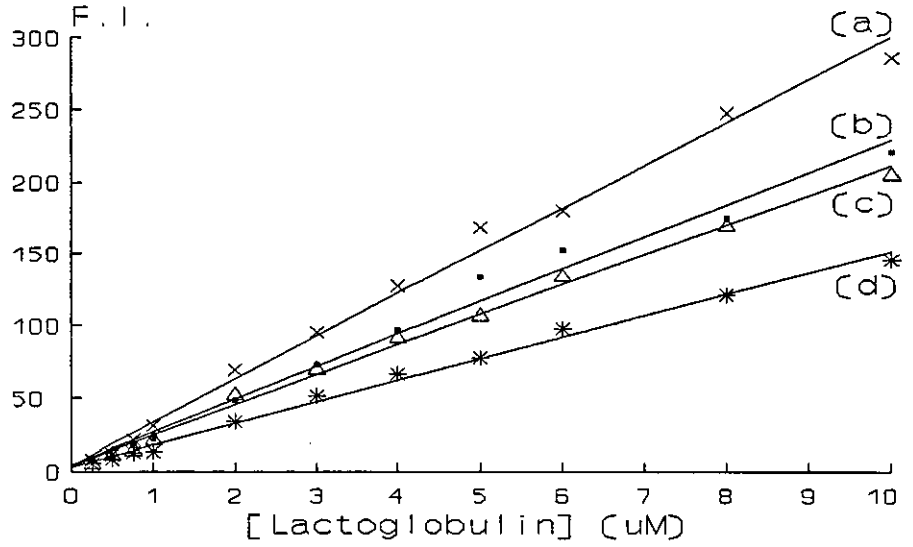


Fig. 6.9: Fluorescence intensity increase of Nile Red (1μM) bound to increasing concentrations of different forms of ß-lactoglobulin at the low wavelength binding site.

Key:
(a) 1µM Nile Red: ß-lactoglobulin B — Em. W'length 610nm.
(b) 1µM Nile Red: ß-lactoglobulin A + B (commercial) — Em. W'length 608nm.
(c) 1µM Nile Red: ß-lactoglobulin A + B (prepared) — Em. W'length 608nm.
(d) 1µM Nile Red: ß-lactoglobulin A — Em. W'length 604nm.

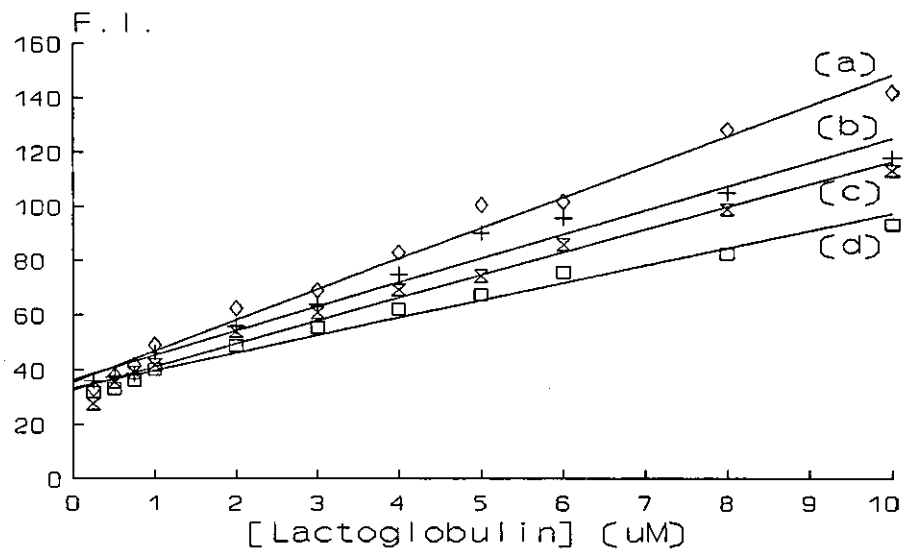


Fig. 6.10: Fluorescence intensity increase of Nile Red (1µM) bound to increasing concentrations of different forms of ß-lactoglobulin at the high wavelength binding site.

Key:
(a) 1µM Nile Red: ß-lactoglobulin B.
(b) 1µM Nile Red: ß-lactoglobulin A + B (commercial).
(c) 1µM Nile Red: ß-lactoglobulin A + B (prepared).
(d) 1µM Nile Red: ß-lactoglobulin A.
(All Em. W'lengths at 650nm).

As found with the commercially available ß-lactoglobulin mixture the fluorescence of Nile Red at each binding site undergoes a linear relationship with increasing protein concentration. The correlation coefficients of which are given in Table 6.6. The figures show that the low wavelength binding site is more reactive to change of ß-lactoglobulin concentration than the high wavelength binding area and that the difference in Nile Red fluorescence bound to the different forms is much bigger at the low wavelength binding site than at the high.

Table 6.6: Correlation coefficients of straight line fits of the fluorescence of Nile Red with increasing concentration of ß-lactoglobulin.

Lactoglobulin	Correlation Coefficients	
	Low wavelength binding site	High wavelength binding site
ß-lactoglobulin A	0.997	0.993
ß-lactoglobulin B	0.996	0.990
Commercial ß-lactoglobulin A and B	0.994	0.986
Prepared ß-lactoglobulin A and B	0.994	0.993

The fluorescence stabilities for the 'A' and 'B' forms (Figs. 6.11 and 6.12) were the same as those found for the commercially available mixture in that initially at low concentrations the low wavelength binding site was the most unstable. However, at higher protein concentrations when binding was favoured here the stability at this site increased to the level of the high wavelength binding site.

At low protein concentrations the 'B' form had a slightly poorer fluorescence stability than the 'A' form at both sites. This was not

suprising as the fluorescence intensity of Nile Red bound to the 'B' form was greater than that of the 'A' and so had the most amount to lose.

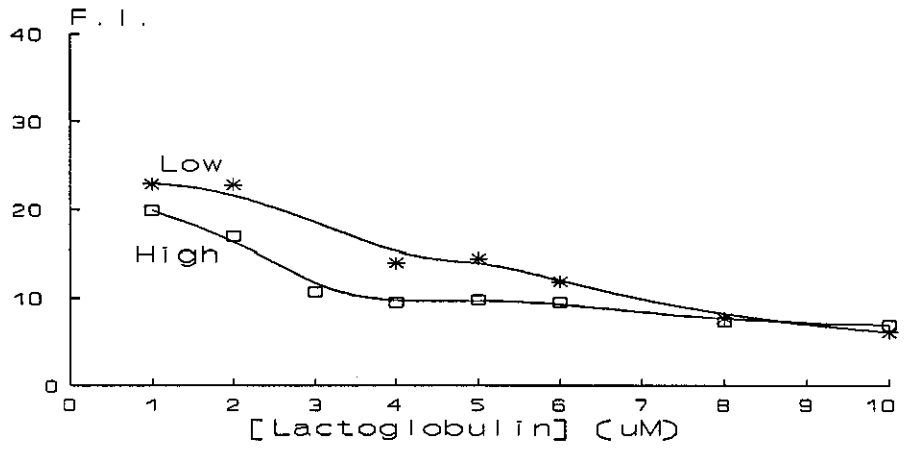


Fig. 6.11: Percentage decrease in fluorescence of Nile Red (1μM) and ß-lactoglobulin A complexes after twenty minutes.

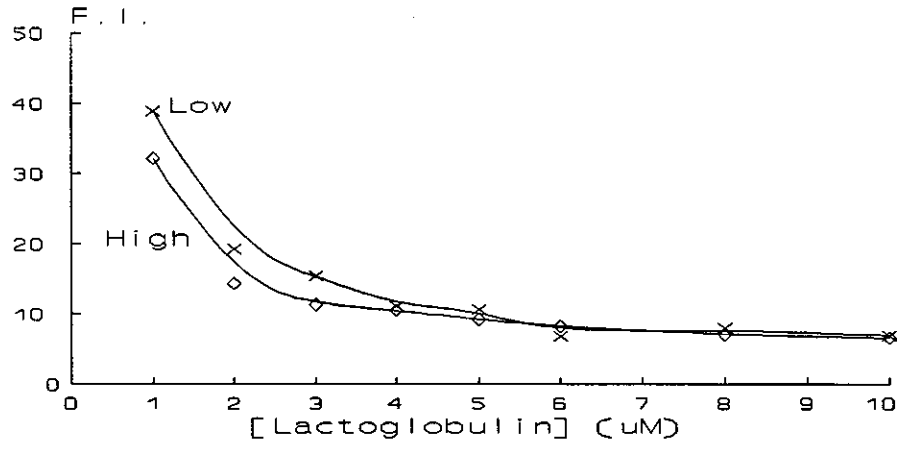


Fig. 6.12: Percentage decrease in fluorescence of Nile Red (1μM) and ß-lactoglobulin B complexes after twenty minutes.

Generally, the fluorescence stability of Nile Red bound to ß-lactoglobulin increases with increasing protein concnetration, for the reasons explained for serum albumin and OMD. The fact that the stability at the low wavelength was improved the most at these higher protein concentrations supports the theory that binding is favoured here in these conditions.

The resultant data discussed in this section indicates that the high wavelength binding site of Nile Red is fairly independent of the form of ß-lactoglobulin. This suggests that the binding site is probably in an area on the outside of the protein where ß-lactoglobulin 'A' and ß-lactoglobulin 'B' do not differ in amino acid residues. Otherwise any difference in residues in this binding area would cause a difference in Nile Red fluorescence at this site. However, at the low wavelength site the fluorescence of Nile Red varies depending on whether it is attached to the 'A' or 'B' form. This implies that the binding site is different on the two forms and irrelevant of whether it is a hydrophobic crevice in a monomeric or dimeric unit different amino acids are present in the binding area. These different residues could cause a change in the hydrophobicity of the environment of Nile Red or have an energy transfer effect, although the latter is not likely because of the high wavelength of the dye.

The difference in fluorescence of Nile Red when bound to the two forms of ß-lactoglobulin as with the serum albumins, showed that Nile Red can distinguish between different forms of a single protein just by the changes in its environment. This effect could well be used in future assays for these different generic forms.

6.7.4 Effect of Varying pH on the Binding of Nile Red to ß-Lactoglobulin.

Reverse titrations were carried out on the commercially available ß-lactoglobulin mixture at pH 8.75 and compared to the results at pH 6.9. The results are shown in Figs. 6.13-6.16.

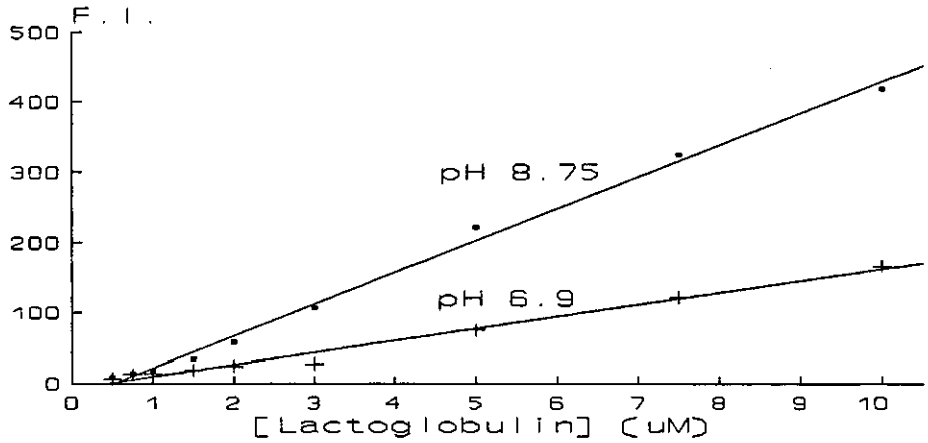


Fig. 6.13: The effect of pH on 1μM Nile Red binding to increasing ß-lactoglobulin at the low wavelength binding site (F.I.).

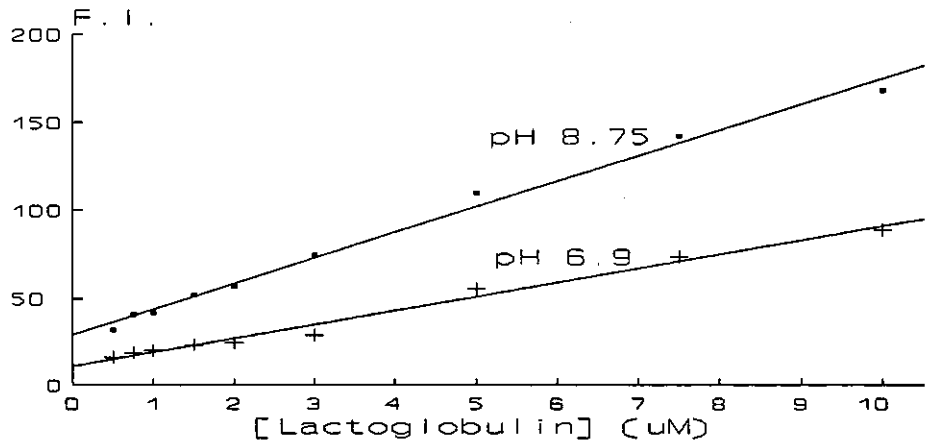


Fig. 6.14: The effect of pH on 1μM Nile Red binding to increasing ß-lactoglobulin at the high wavelength binding site (F.I.).

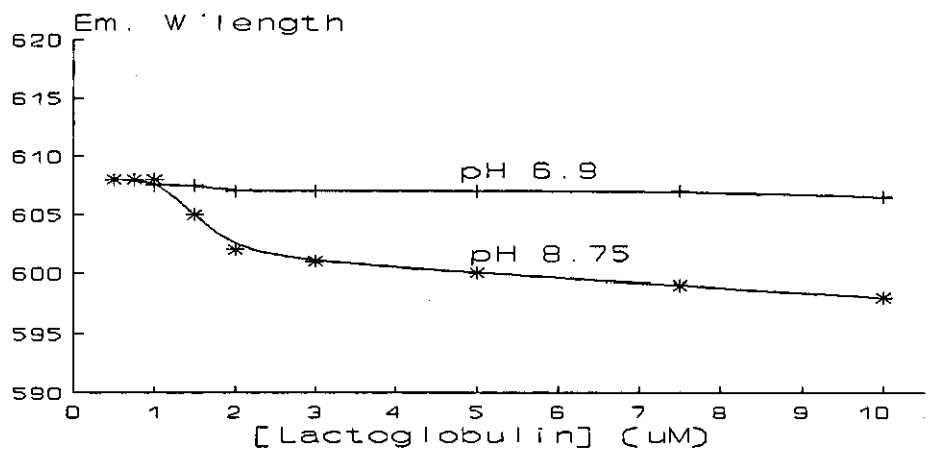


Fig. 6.15: The effect of pH on 1μM Nile Red binding to increasing ß-lactoglobulin at the low wavelength binding site (Em. W'length).

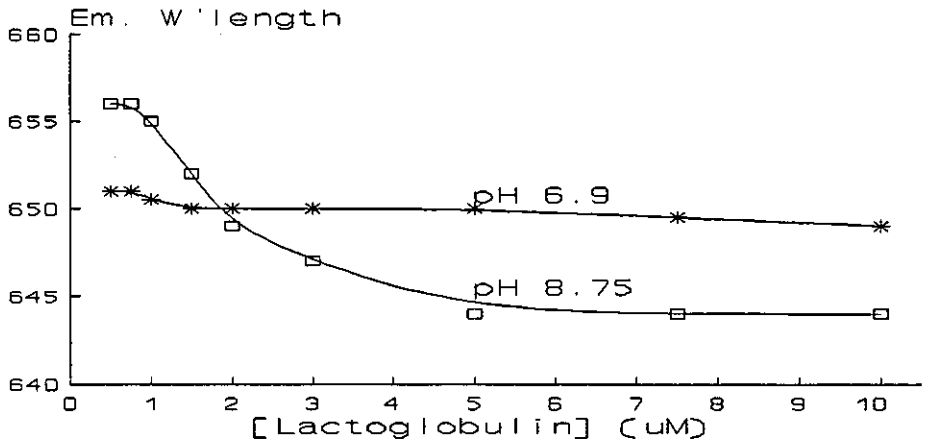


Fig. 6.16: The effect of pH on 1μM Nile Red binding to increasing ß-lactoglobulin at the high wavelength binding site (Em. W'length).

The results show that at high protein concentrations the higher pH causes fluorescence intensity enhancement at both sites much greater than occurs at the lower pH. In addition, the higher pH also causes a shift in emission wavelength to blue at both sites with increasing protein concentration which does not occur at the lower pH. The increases in fluorescence intensity at the higher pH is slightly more at the low wavelength binding site than the high, while the shift in emission wavelength is approximately the same at either site.

At pH 6.9 the favoured conformation of ß-lactoglobulin is a dimer while at pH 8.75 alkaline denaturation is believed to occur. In this process initially aggregation is believed to occur, followed by the unfolding of the α-helices and "exposed" ß-strands[362]. Finally, the rest of the ß-strands unfold. It is hard to put an exact conformation of ß-lactoglobulin at pH 8.75 but it could exist as an expanded monomer[364].

The results indicate that the binding of Nile Red is much greater at either binding site at the higher pH rather than the low. This indicates that the binding sites at pH 8.75 are more hydrophobic in nature than

those at pH 6.9. This is probably due to the unfolding of the protein which could result in a change in conformation of the binding sites or exposure of hydrophobic environments as was found with Nile Red binding to calmodulin[62].

The fact that the binding of Nile Red increases at the higher pH would also indicate that perhaps the low wavelength binding site is not between monomeric units of a dimer but just in a hydrophobic crevice of the monomer. As discussed above dimers are more likely to exist at pH 6.9 than pH 8.75 and so if dimer binding was occurring Nile Red fluorescence would be expected to be greater at pH 6.9 and not the opposite which occurred. However, alkaline denaturation can result in aggregation and so the possibility cannot be ruled out as in this case greater binding at pH 8.75 would be expected. Nevertheless because of the fluorescence stability of Nile Red over this pH range[52] it is obvious that the probe is monitoring a pH induced conformational change in the structure of ß-lactoglobulin and that this could be very useful in other studies.

6.7.5 Nile Red Binding to α-Lactalbumin and Immunoglobulin G.

As shown in Table 6.1 there are other proteins in milk whey as well as ß-lactoglobulin. For an assay for ß-lactoglobulin in milk whey to be devised, the binding of Nile Red to the other proteins in whey should be studied, to see if any separation steps will be necessary before the ß-lactoglobulin is determined.

The binding of Nile Red to serum albumins has already been discussed in Chapter four, and the next most abundant proteins are probably α-lactalbumin and the many types of globulins. For the globulins, the antibody immunoglobulin was studied because of the reported homology of the immunoglobulins with OMD (Section 5.2) and the fact that any

Nile Red binding may be useful for any future work involving immunoassay.

Reverse titrations involving 1μM Nile Red and increasing concentrations of the proteins are shown in Fig. 6.17 and compared to the low wavelength binding site of ß-lactoglobulin. Both proteins show a very small amount of fluorescence enhancement and shift in emission wavelength and thus binding. These changes however were too small to perform any binding titrations. The changes are very small compared with those of ß-lactoglobulin and would be negligible if they were monitored at the ca. 610nm emission of the low wavelength binding site of ß-lactoglobulin. It would therefore appear that the serum albumins are the only proteins that show any significant binding of Nile Red, and their removal could be the only possible separation step necessary for any future ß-lactoglobulin assay.

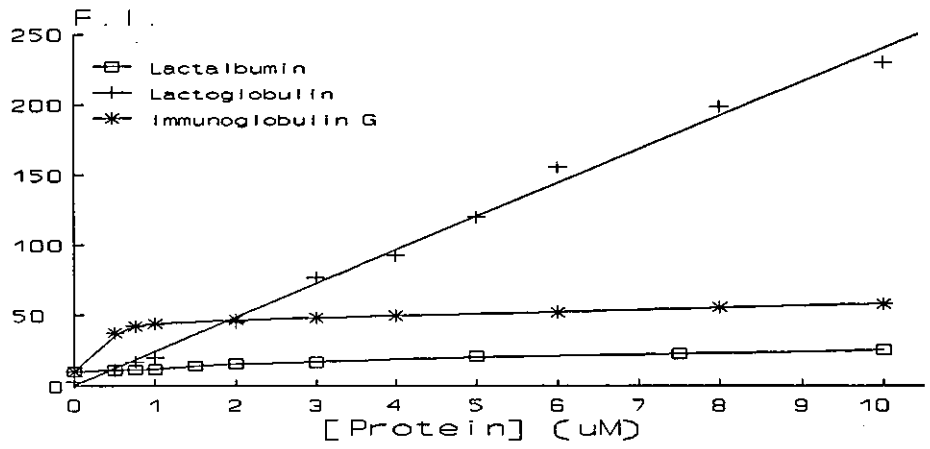


Fig. 6.17: Fluorescence change of Nile Red (1μM) in increasing concentrations of α-lactalbumin, ß-lactoglobulin and immunoglobulin G.

Key: Shift in emission wavelength to blue of α-lactalbumin is 3nm from 1→10μM.
Shift in emission wavelength to blue of immunoglobulin G is 3nm from 1→10μM.

6.8 CONCLUSIONS.

Nile Red when added to ß-lactoglobulin, showed the largest changes in fluorescence of all the dyes considered enabling its use as a probe to study the protein.

Nile Red was found from adapted Scatchard plots to have one binding site on ß-lactoglobulin however the fluorescence spectra consisted of two overlapping peaks at ca. 610nm and 650nm the lower wavelength site being more in the interior of the protein, possibly even between the two protein units of the dimer. The fluorescence intensity of each of these wavelengths was found to increase linearly with increasing ß-lactoglobulin, the lower wavelength site being more susceptible to change in the protein concentration than that of the high.

The fluorescence of Nile Red was also found to significantly differ between the two generic forms of 'A' and 'B' ß-lactoglobulin, even though the two forms are only slightly different.

The pH stability of Nile Red fluorescence also enabled its use to monitor pH induced conformational changes in the ß-lactoglobulin and determined that the low wavelength binding site was probably in the monomeric rather than the dimeric unit.

The linear fluorescence intensity increase of Nile Red in ß-lactoglobulin suggests the use of Nile Red in an assay for ß-lactoglobulin in milk whey. Investigation of two other common proteins in milk whey showed that neither α-lactalbumin or immunoglobulin G cause significant Nile Red fluorescence. Thus it would appear for any future assay the only possible interference would come from the serum albumins which significantly bind Nile Red. However, the actual concentration of serum albumin in milk whey, in terms of molarity, is low and so any interference in an assay would be negligible. It would therefore appear that the concentration of ß-lactoglobulin in milk whey could

be determined directly with no separation step necessary i.e., the assay would be homogeneous.

CHAPTER SEVEN

CONCLUSIONS

7.1 CONCLUSIONS.

Drug-protein binding is of obvious importance both therapeutically and analytically. The presence and nature of this binding affects the absorption, activity, metabolism and excretion of the drug which can lead to a change in its duration of action and its analytical response in many measurement methods. Different drugs or ligands may also compete for the same binding site and any displacement or inhibition that may occur could affect the therapeutic action and toxicity of the drugs.

Spectrofluorimetry in the long wavelength region (600-1000nm) is a recent development in photoluminescence spectroscopy and has many advantages compared with more conventional measurements made in the ultraviolet and visible spectral regions. These include: decreased background fluorescence when dealing with biological samples; decreased scattering; decreased photodecomposition and the availability of inexpensive, solid state optical components which operate in this spectral region.

Numerous compounds were studied to investigate the possibility of their use as a long wavelength fluorescent molecule for non-covalent attachment to a protein binding site, i.e., to act as fluorescent probe. Despite its poor solubility and sticking properties in aqueous media Nile Red was found to the most suitable of all the dyes tested for use in this area. It is a strongly hydrophobic molecule and polarity studies showed that its fluorescence was strongly dependent on the polarity of its environment. An increase in fluorescence intensity and a shift to blue in emission wavelength of Nile Red was found with increasing polarity.

Similar changes resulted from the addition of Nile Red to various protein solutions indicating a hydrophobic attachment, the different fluorescence values associated with varying hydrophobic environments of Nile Red in the binding site. As the fluorescence of unbound Nile Red was found to decrease in aqueous media due to its aggregation, a twenty minute incubation period

was allowed to ensure that the fluorescence of unbound Nile Red was at a minimum and was considered to be negligible.

The fluorescence of Nile Red was found to be very sensitive to its environment. In fact the fluorescence changes that occurred with bound Nile Red enabled the identification of different binding sites on a number of serum albumins and also on different generic forms of ß-lactoglobulin. The two forms of ß-lactoglobulin only differing by a few amino acids and are thought to be closely physically and chemically related.

The binding titrations of Nile Red and protein were precise and extremely repeatable as can be seen from the small error bars in the graphs. The resultant Scatchard plots indicated two types of binding sites on serum albumin while one was found for OMD and ß-lactoglobulin. However, using reverse titration where the protein concentration rather than the Nile Red concentration was increased, two overlapping fluorescence peaks were found with OMD and ß-lactoglobulin. With the aid of second order derivatisation when necessary these peaks were easily separated. It is believed that the two peaks indicate two different areas of attachment of Nile Red on the proteins. The lower wavelength binding site is probably more in the interior of the protein and more hydrophobic in nature while the higher wavelength site is on the outside of the protein where it is more exposed to the polar, water molecules. It is possible that the low wavelength site on ß-lactoglobulin is in fact located between two monomeric units of the protein which combine to form a dimer at neutral pH.

The fluorescence of Nile Red bound to increasing concentrations of ß-lactoglobulin was found to undergo a linear relationship at both sites indicating that the probe could be used in a future assay for ß-lactoglobulin in milk whey. Nile Red because of its stability to pH was also used to monitor pH induced conformational changes in ß-lactoglobulin.

Drug and other ligand displacement of Nile Red from its binding site was achieved with both the serum albumins and OMD enabling the calculation of association constants. It was found that on the addition of both acidic and basic drugs to the Nile Red:OMD complex the fluorescence of Nile Red was shifted back to its unbound form, indicating displacement from its binding site on the protein. The results suggest that this is the high affinity binding site on OMD, previously discussed in the literature, where both acidic and basic drugs bind. The fact that displacement occurs with both acidic and basic drugs also shows that the site is more hydrophobic and less electrostatic in nature.

Stereoselective binding of the basic drugs propranolol and ephedrine was also studied using their displacement of Nile Red from OMD. Although no difference could be found with the racemic forms of propranolol the (+) form of ephedrine was found to have a smaller K_a than the (-) form and racemic mixture. The results show that a long wavelength probe like Nile Red could be used in the study of such interactions.

With the serum albumins the only ligands that achieved displacement of Nile Red in both the human and bovine forms were flufenamic acid and stearic acid. With the former, the mechanism for displacement was obviously not the same between the two types of albumin indicating a difference in attachment between the two. Digitoxin also achieved displacement of Nile Red from HSA, while the addition of diazepam, ethacrynic acid, warfarin and phenylbutazone caused an enhancement in Nile Red fluorescence. The enhancement showing that the binding of these drugs was probably causing an allosteric effect resulting in a change in conformation of the Nile Red binding site. The poor displacement of Nile Red from the serum albumins suggesting that binding to the protein is less hydrophobic and more electrostatic in nature.

The use of two fluorescent probes was a fairly novel technique designed to produce additional information on the binding sites for Nile Red on the

proteins. For two probes such as ANS and Nile Red with overlapping excitation and emission spectra the resultant fluorescence data can indicate where the probes bind in relation to each other. For both the serum albumins and OMD an energy transfer effect was observed between the two probes, which was lost upon dilution. This means that the mechanism was not that of a singlet-singlet resonance energy transfer but a simple absorption re-emission. The two probes were therefore not within 80Å of each other and probably on different protein molecules indicating that the probes were not attached to the same binding area on any of the proteins.

The poor solubility and lack of fluorescence of Nile Red after it has been mixed in aqueous media made the development of a gradient titration FIA method for the determination of drug-protein interactions difficult. However, a system was designed which produced suitable displacement troughs that could possibly be used in the calculation of the binding parameters. The technique being more rapid and less inexpensive than the standard static measurement. Problems arose trying to calibrate the system to enable the use of the suitable equations to determine the concentrations at any particular time produced by the dispersion coil. This was not possible in the time available and future work is necessary on this system.

This project has shown that the study of ligand-protein interactions is possible in the long wavelength region. The technique has the benefits of the sensitivity and specificity of fluorescence as well as all the advantages of measurement in the long wavelength region. Work has involved the use of second order derivatisation and a novel double probe technique both of which provided valuable information and could be very useful in future fluorescence assays. Further investigation for compounds with improved fluorescent probe properties and the study of other proteins to which they can become attached could result in the widespread use of the techniques discussed in this thesis.

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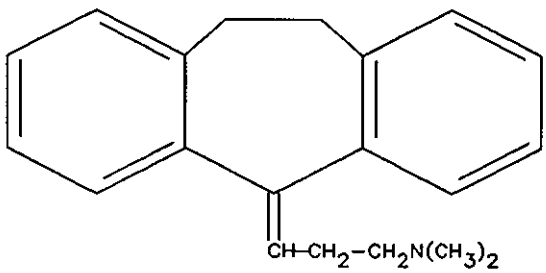
APPENDIX I

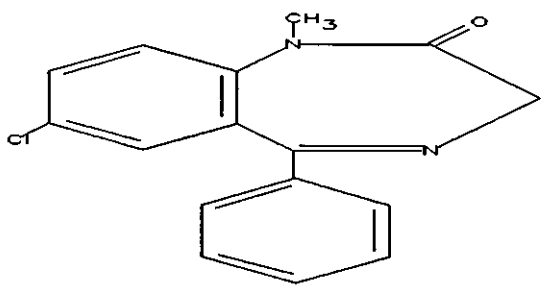
Amitriptyline

Tricyclic antidepressant



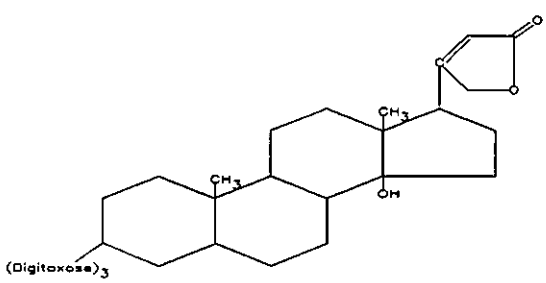
Diazepam

Minor tranquillizer



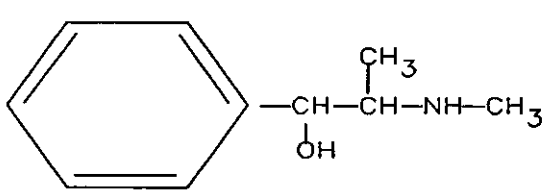
Digitoxin

Cardiotonic



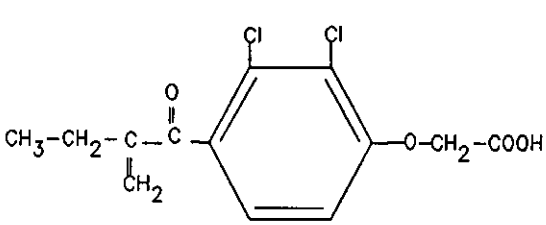
Ephedrine

Indirectly acting sympathomimetic



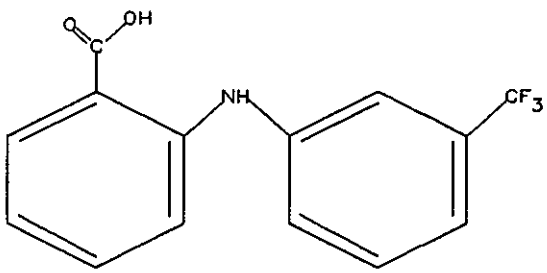
Ethacrynic Acid

Diuretic



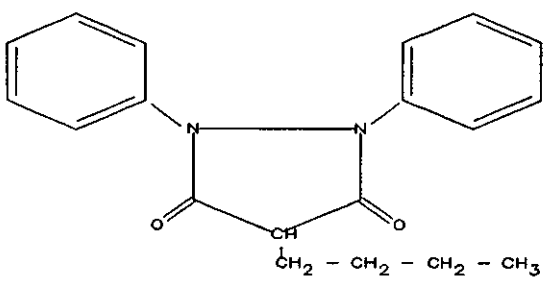
Flufenamic Acid

Anti-inflammatory



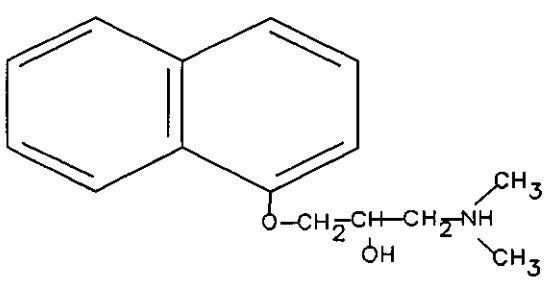
Phenylbutazone

Anti-inflammatory



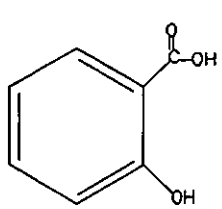
Propranolol

Non-selective ß-receptor antagonist



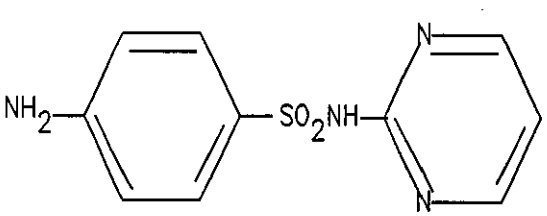
Salicylic Acid

Analgesic



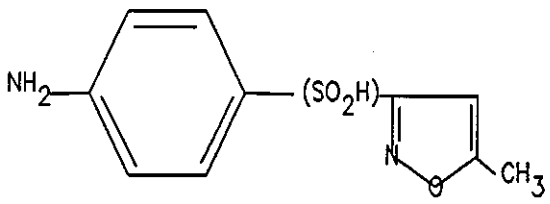
Sulphadiazine

Anti-bacterial



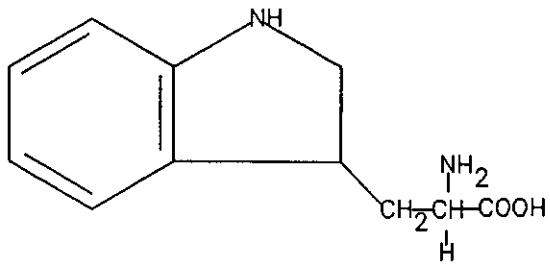
Sulphamethoxazole

Anti-bacterial



Tryptophan (essential amino acid)

Anti-depressant



Warfarin

Anti-coagulant