OBSERVATIONS ON THE NATURE OF LIGAND–ALBUMIN COMPLEXES

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ABSTRACT

In a comparative evaluation of several techniques, commercial modifications of equilibrium dialysis and ultrafiltration were found to give satisfactory values of ligand binding to albumin. However, continuous ultrafiltration yielded anomalously high estimates of binding. The fluorescent probe properties of warfarin and phenprocoumon were found to be useful for examination of the drugs' binding sites and particularly suitable for the monitoring of ligand competition for albumin binding.

Bovine Serum Albumin binding of medium chain fatty acids was investigated using ultrafiltration and computerised analysis of experimental data. The primary association constants increased smoothly, with chain length, up to heptanoate but the step to eight carbons was found to produce a particularly large increase in affinity. A model based upon two primary, chain length specific, fatty acid binding sites is proposed. Other observations suggest that a 'break point' at eight carbons may be a common feature of alkyl chain interactions with macromolecules. Thermodynamic data suggests that both electrostatic and hydrophobic bonding is involved in primary fatty acid complexes with albumin. Secondary binding increased linearly with chain length and was suggested to be purely hydrophobic in nature. The primary fatty acid binding sites on albumin have been shown to be dissimilar to those for coumarins and for carbamates whilst all of the ligands share some secondary sites.

Circular dichroism studies of a series of coumarin drugs has shown that they bind to albumins at the same sites, but in differing orientations. Lipophilicity was shown to be an important determinant of binding affinity, but
Ionic interactions seemed to be of little importance. Albumin binding of phenprocoumon and warfarin enantiomers was examined by dialysis and spectroscopic methods. The isomers were bound differently to albumin which was thought to be due to the spatial relationships of the coumarin rings and sidegroups to the binding sites. Bovine and Human albumins showed disparate stereospecificities for the coumarin drugs.
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This thesis is dedicated to my Parents and Frances
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CHAPTER ONE

AN INTRODUCTION TO DRUG-PROTEIN INTERACTIONS
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In living systems, biopolymer-small molecule interactions are ubiquitous and are of paramount importance to innumerable biological reactions. Such interactions cover the whole field of biology, ranging from the mechanisms of respiration through the action of receptors and repressors, the combination of enzyme and substrate to the subtle influence of electrolytes on proteins.

The discipline of Pharmacology is one which is inevitably, deeply concerned with the interaction of active small molecules with biopolymers. It was in the early years of the twentieth century that Ehrlich (1909) first proposed that drugs must combine with tissue constituents in order to exert any profound influence upon body function. The study of those macromolecules with which a drug interacts to produce its characteristic biological effects, the receptors, is therefore at the heart of Pharmacology.

Not only is a drug-receptor protein interaction necessary for the manifestation of pharmacological activity, but the combination of drugs with other tissue and plasma proteins profoundly influences the drug's fate in the body. It is to be hoped that studies of drug-protein interactions which have direct relevance in Pharmacology may also hold some relevant information for the wider field of small molecule-biopolymer interactions.
Blood is the medium by which drugs are transported between the
site of administration and their sites of action, metabolism and excretion. Drugs are carried either as free solution or suspension in the plasma water or bound to one of the components of blood. It is a fundamental, and often tacit, assumption, in all plasma binding studies, that it is only the free portion of drug which is able to exert a pharmacological effect or is available for metabolism and excretion. The inter-relation-
ship of free and bound drug with these processes may be illustrated as in figure 1:1.

Modern study of plasma binding was, perhaps, initiated by Benhold (1938) who suggested that plasma proteins had the function of transporting small molecules around the body. Whilst it is difficult to visualise the plasma proteins as a regimented or regulated transport system, they never-
theless do combine with a remarkable number of xenobiotic and natural substances. Goldstein's excellent review of 1949 served to emphasise the growing realisation of the importance of drug-plasma protein interactions. The increased interest in such interactions since that time is reflected in the amount of research published in the field, and a tabular presentation of all available data, such as Goldstein (1949) and later Meyer and Guttman (1968) provided, would now produce a most weighty volume. It is beyond the scope of a treatise such as this to consider more than a mere fraction of the information available to date, however several good reviews have been published, in addition to the two already mentioned, which cover most
Figure 1: The Fate of a Drug in the Body

- Site of Metabolism
- Storage or Tissue Binding Sites
- Receptor Sites
- Interstitial Fluid
- Elimination via Urine, Faeces, Respiration, Perspiration
- Free Drug
- Protein Bound Drug
- Blood
- Cells
- Metabolite(s)

- Protein Bound Drug → Interstitial Fluid
- Free Drug → Blood
- Blood → Cells
- Cells → Metabolite(s)
- Metabolite(s) → Site of Metabolism
- Site of Metabolism → Protein Bound Drug

It is important to realise that the plasma proteins are most important in the binding of endogenous substances as well as drugs. Often the term drug-protein could equally well be nutrient-protein or endogenous molecule-protein, however the phrase will be used throughout this work, rather than some more cumbersome term, with the realisation that the ligand involved need not necessarily be a pharmacologically active agent.
Blood is a complex mixture of proteins and cells, with a diversity in structure as manifold as their diversity in function. Excluding peptide hormones, erythrocytes and other cellular components, and tissue derived enzymes, plasma contains more than one hundred distinct proteins. With the exception of serum albumin only brief mention will be made of the constituents of blood where there is relevance to the binding of drugs. Further information on the structure and function of blood components can be obtained from the following articles: Putnam (1960), Neurath (1965), Schultze and Heremans (1966), Turner and Hulme (1971), Rothschild and Waldman (1970), Scanu and Wisdan (1972), Putnam (1975).
to the far smaller surface area presented by erythrocytes: \(3,200 \text{ m}^2\) in the blood of an adult man compared to \(130,000 - 725,000 \text{ m}^2\) for the plasma proteins (Bennhold, 1966). Compounds which have been reported to bind to the erythrocytes of various species include: promazine and chlorpromazine (Jähnchen et al., 1971; Girl and Peoples, 1975; Manian et al., 1974), pentazocine (Ehrnebo et al., 1974), steroids (Brinkman and van der Molen, 1972), sulphonamides (Maren, 1967), salicylate and phenobarbital (McArthur et al., 1971) and Phenprocoumon (Neider et al., 1975). Cruze and Meyer (1976) recently reported the binding of salicylate to bovine erythrocytes and the effects of haemolysis upon binding.

1:2:3 GLOBULINS, LIPOPROTEINS AND OTHER NON-ALBUMIN PLASMA PROTEINS

A list of some of the major non-albumin plasma proteins is presented in Table 1:1.

Thyroxin Binding Pre Albumin (TBPA) is the first plasma protein for which the complete amino acid sequence (Kanda et al., 1974) and three dimensional structure (Blake et al., 1974) were elucidated. One molecule of TBPA binds one molecule of Retinol Binding Protein (RBP), the vitamin A carrier of plasma, as well as combining with thyroxine. Thyroxine Binding Globulin (TBG) and albumin are the other carriers of thyroxine in plasma.

Laurell (1960) has reviewed the metal binding proteins, including Ceruloplasmin which binds more than 90% of plasma copper (Poulik and
Table 1: Some parameters of the major non-albumin plasma proteins from human plasma*

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular weight</th>
<th>Amount in plasma mg/100 ml</th>
<th>Substances transported (if any)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prealbumin (thyroxin binding)</td>
<td>54,980</td>
<td>10-40</td>
<td>Thyroxin, retinol binding protein</td>
</tr>
<tr>
<td>$\alpha_1$ Globulins:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retinol binding protein</td>
<td>21,000</td>
<td>3-6</td>
<td>Retinol (vitamin A)</td>
</tr>
<tr>
<td>$\alpha_1$ acid glycoprotein</td>
<td>40,000</td>
<td>55-140</td>
<td></td>
</tr>
<tr>
<td>Transcortin</td>
<td>55,700</td>
<td>7</td>
<td>Steroid hormones</td>
</tr>
<tr>
<td>$\alpha_1$ antitrypsin</td>
<td>54,000</td>
<td>200-400</td>
<td></td>
</tr>
<tr>
<td>Transcobalalbumin</td>
<td>?</td>
<td>?</td>
<td>Vitamin $B_{12}$</td>
</tr>
<tr>
<td>$\alpha_2$ Globulins:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>151,000</td>
<td>15-60</td>
<td>Copper</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>100,000-400,000</td>
<td>100-300</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>$\alpha_2$ Macroglobulin</td>
<td>725,000</td>
<td>150-420</td>
<td>Hormones</td>
</tr>
<tr>
<td>Thyroxine Binding globulin</td>
<td>58,000</td>
<td>1-2</td>
<td>Thyroxin</td>
</tr>
<tr>
<td>$\beta$ Globulins:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemopexin</td>
<td>57,000</td>
<td>50-100</td>
<td>Haem</td>
</tr>
<tr>
<td>Transferrin</td>
<td>76,500</td>
<td>200-320</td>
<td>Iron</td>
</tr>
<tr>
<td>Complement system</td>
<td>86,000-400,000</td>
<td>1-160</td>
<td></td>
</tr>
<tr>
<td>Immunoglobulins</td>
<td>160,000 and above</td>
<td>Up to 1800</td>
<td>Antigens (drugs?)</td>
</tr>
<tr>
<td>$\alpha_1 + \alpha_2$ lipoprotein</td>
<td>$1.5 \times 10^6$ to greater than $4 \times 10^8$</td>
<td>Variable</td>
<td>Phospholipid, fatty acid, cholesterol + ester</td>
</tr>
<tr>
<td>$\beta$ lipoprotein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coagulation proteins</td>
<td>various</td>
<td>Up to 450</td>
<td></td>
</tr>
</tbody>
</table>

*Data taken from Putnam 1975.*
Weiss, 1975), and Transferrin the specific iron binding protein of plasma (Putnam, 1975). A great deal of literature is available concerning the binding of corticosteroids to plasma proteins, much of which has been reviewed by Westphal (1971) including the roles of Transcortin and Corticosteroid Binding Globulin (CBG). Transcobalalbumin is a minor plasma protein with high affinity for vitamin B\textsubscript{12} (Hippe and Olsen, 1971), the binding of other vitamins to plasma proteins has been reviewed by Raoul (1966).

Haptoglobin and Hemopexin bind, in equimolar stoichiometry, hemo-globin and heme respectively (Jayle, 1962; Aisen, 1975) and together with Transferrin form a triumvirate of proteins involved in the conservation of hemoglobin and its metabolites.

The plasma lipoproteins form an extremely heterogeneous group of proteins, complexed in variable proportions with lipid and carbohydrate. The majority of plasma lipid, in the form of mono, di and triglycerides, phospholipids, cholesterol and cholesterol esters is transported in complex with lipoproteins (Scanu et al., 1975). Under conditions of high plasma free fatty acid concentrations these also may be carried in association with lipoprotein (Mora et al., 1955; Polonovski, 1966). Bilirubin and prostaglandins, normally associated with albumin in plasma, can combine with lipoproteins at high ligand concentrations (Cooke and Roberts, 1969; Raz, 1972). The drugs tetrahydrocannabinol (Wahlquist et al., 1970; Widman et al., 1973) and recently quinidine (Nilsen, 1976) have been reported to bind to a small degree to plasma lipoprotein.

The proteins of the complement and coagulation systems have not been
reported to interact with drugs, and whilst the same can be said of the
immunoglobulins it is important to realise that drugs may act as haptens
or even antigens resulting in the production of specific antibodies capable
of binding avidly to the drug. This is a possibility which is often ignored
in drug binding studies.

1:2:4 ALBUMIN - GENERAL ASPECTS

Albumin is the most plentiful of the plasma proteins, accounting for
more than 50% of total protein in human plasma. It is the principle agent
responsible for the osmotic pressure of blood and for the sequestration and
transport of many endogenous compounds: fatty acids, bilirubin, amino
acids, various hormones etc. etc. The concentration of albumin in plasma
is generally a good measure of health, or as Peters (1975) has it: "Happiness
is a normal albumin level".

The ability of albumin to enter into reversible combination with an
enormous range of compounds with differing chemical structure and physical
characteristics is unrivalled throughout all biological systems. The relative
ease of isolation and purification of albumin, coupled with the fact that it
makes the predominant contribution to plasma binding for the majority of
drugs has made it particularly suitable and popular for use in studies of
drug-plasma binding and as a model for general ligand-protein interactions.

The literature on albumin is so extensive as to have promoted several
reviews, those of Foster (1960), Putnam (1965) and Peters (1975) concern
mainly structural aspects whilst the biosynthesis, metabolism and physio-
logical role of albumin have been covered by Anker (1960); Burke (1969); Rothschild and Waldman (1970) and Peters (1970). Most of the next three sections will be concerned with Human Serum Albumin (HSA, syn. Human Plasma Albumin, HPA) with reference to other albumins, particularly that of the major commercial source, the cow; Bovine Serum Albumin (BSA), where pertinent.

The figures $35 \, \text{g} \cdot \text{l}^{-1}$ to $45 \, \text{g} \cdot \text{l}^{-1}$ (Marks, 1972) are now generally accepted as the limits of albumin concentration in the plasma of normal, healthy male adults, giving a total intravascular mass of about 140 g for a 75 kg man. Intravascular albumin accounts for only 30% to 40% of total body albumin and is in continuous, free exchange with other body fluids. The half life of albumin in the body is about 19 days, with a turnover of about 14 g.day$^{-1}$ (Schulze and Heremans, 1966; Peters, 1970). The liver is the major site of synthesis, with the thyroid gland producing minor quantities (Rothschild et al., 1972). Catabolism seems to be a general phenomenon, being faster in organs of high metabolic rate such as liver, kidney, spleen and lymph nodes. Muscle, however, with its large mass, contributes much to the overall rate (Hawkins, 1961; MacFarlane, 1963). Changes in albumin levels with age and disease, and the influence upon plasma protein binding will be discussed in a later section. The plasma concentration of albumin is subject to some diurnal variations, mainly under the influence of the nutritional status, exercise and posture.

It has already been mentioned that albumin may be a useful model to mirror general ligand-protein or enzyme-substrate interactions, but it
is not generally appreciated that albumin may actually have some inherent enzymatic activity. Most enzymatic activity associated with albumin preparations can be removed by rigorous purification, such activities include acid protease (Wilson and Foster, 1971); nuclease (Arai et al., 1972) and phospholipase (Elsbach and Pettis, 1973). However purified albumin has been reported to catalyse the breakdown of ferrihaems (Brown et al., 1974); the Messenheimer complex (Taylor and Chau, 1975); S-lauroyl-mercaptoethanol (Kurooka and Yoshimura, 1973); p-nitrophenyl acetate (Tildon and Ogilvie, 1972) and several other esters (see previous citation for references). Additionally, albumin is thought to possess some thyroid hormone synthesising ability (Thomas-Morvan, 1976) and has been reported to catalyse the oxidative dimerisation of a synthetic derivative of trinitrobenzene (Chavez and Benjamin, 1975). The significance of such catalytic activities is at present unknown, but the phenomena must merit further study.

1 : 2 : 5 ALBUMIN - STRUCTURE

Serum albumin is a simple protein, being a polypeptide chain of about 580 residues, with small interspecies variations. It is alone among the plasma proteins in having no associated carbohydrate, a criterion which is used to demonstrate its purity (Cohn, 1947). The molecular weights of albumins have been variously reported to be between 66,000 and 69,000. The molecular weight of HSA, calculated from the amino acid composition is 66,500 (Meloun et al., 1975), the best estimate from physical measurements is 66,700 obtained using the ultracentrifuge by Squire et al., 1968.
Albumin is characterised by smaller size, but greater solubility, total charge, net negative charge, stability and flexibility than other proteins. According to hydrodynamic data, albumin is a prolate ellipsoid with major and minor axes of 14 nm and 4 nm.

Albumin has more than 200 positive and negative charges, distributed throughout the molecule, giving a hydrophilic character and leading to a high solubility in aqueous media. The isotonic point of albumin is about pH 5.2 which is also the point of maximum total charge with 101 positive and negative charges (Hughes, 1954). At pH 7.4, albumin has a net negative charge of 18 (White et al., 1968).

The complete amino acid sequences of Bovine and Human Serum Albumins have recently been elucidated, largely by the efforts of Brown and his co-workers (Brown, 1974, 1975; Behrens et al., 1975). This group have also partially sequenced Ovine and Porcine Albumins. Many other workers, from various laboratories have contributed significantly to the deciphering of these sequences. Peters (1975) has recently reviewed the relevant literature. The amino acid sequences of Bovine and Human Albumins are shown in figures 1:2 and 1:3 as determined by Brown and colleagues, and in the configuration proposed by them (Brown, 1975; Behrens et al., 1975). The group of Meloun has also published the complete sequence of HSA (Meloun et al., 1975) and considerable differences exist between these two reports. Perhaps the most important discrepancy is the deletion of Phe 157 from the structure of HSA determined by Behrens et al. (1975) resulting in one too few residues in their sequence. It is likely that the sequence of Meloun and co-workers is the more accurate,
Figure 1:2  The amino acid sequence of Bovine Serum Albumin

The sequence is displayed in a model showing the proposed linking of cystines to form multiple double loops. From Brown (1975).
Figure 1:3  The amino acid sequence of Human Serum Albumin.

Residues assigned by analogy with BSA are shown in lower case letters. From Behrens et al. (1975).
since their data agrees well with that of other groups and also much of 
the sequence of HSA proposed by Behrens et al., was from analogy 
with that of BSA.

Both albumin molecules are composed of 9 major loops, each being 
made up of two minor loops, which are bonded by disulphide linkages. 
There are six long major loops and three short in each case, occurring in 
the repeating sequence long short long - long short long - long short long. 
This arrangement was proposed by Brown (1975) from the primary sequence 
for reasons which may be summarised as follows:- There are seven 
Cys-Cys pairs in each molecule (loops 2,3,4,6,7,8) each of which is 
situated 7 to 10 residues from a lone Cys and a greater distance from a 
second lone Cys. Take for instance, major loop 9 of BSA (residues 504-
581, isolated independently by Peters and Hawn, 1967, and designated 
'Phe'). The peptide contains two lone Cys residues, 513 and 566 and a 
pair 557-558. The conformational rigidity of the backbone prevents a 
disulphide link being formed between Cys 557 and Cys 558. Of the two 
other possible permutations of disulphide bonds the pairing 513-558 and 
566-557 was chosen to increase the length of the short minor loop, thus 
decreasing its conformational restrictions.

There are two departures from the typical disulphide pairing structure, 
short loop 5 has its Cys pair separated by three residues rather than 
adjacent and loop 1 has no lone Cys in the region 8-11 and only a single 
Cys at 53-54 rather than the expected pair. The residues 8-52 are never-
theless represented as the long minor loop of major loop 1, even though 
they are not held by a disulphide link.
The existence of independent double loops, as proposed here, is supported by the various peptide fragments which have been formed by digestion of the albumin molecule. The relationship of many of the fragments, reported by various workers, to the model of Brown (1975) is shown in figure 1:4. Further reference to these peptides and to the distribution of amino acids with regard to function will be made in the next section.

The secondary structure of albumin has been reported to contain 50-55% α helix, about 15-20% β pleated sheet and the remainder random coil. (Sjoholm and Ljungstedt, 1973; Sogami and Foster, 1968; Reed et al., 1975), but recently, Chen (1976) could find no evidence of β sheet in BSA using the technique of laser-Raman spectroscopy. The effects of the many S-S bridges upon helical content is difficult to assess but the two sets of data are not incompatible. Little is known of the tertiary structure of albumin, but some predictions can be made from what is known of the primary and secondary structure. These predictions will remain unproven until X-ray crystalographic studies, now in their infancy (McClure and Craven, 1974) reach a more mature state.

The observed compactness of the molecule at neutral pH can be assumed to be a consequence of associations between the loops. Brown (1975) has suggested that BSA is in the form of a pentahedral cylinder formed by five helices. It is likely that association of loops form globular portions of the molecule, or domains. The existence of a series of domains, linearly arranged with transverse clefts was proposed by Anderson and Weber (1969) from fluorescence polarisation data of the interaction of ANS with BSA.
Figure 1: The peptides of albumin which have been used in small molecule binding studies.

**HUMAN**

(a) BCA CNBr

| 1 | B | 124 | C | 297 | A | 584 |

**BOVINE**

(b) Peps

| 1 | 24 | 49 | 185 | 306 | 405 | 453 | 503 | 581 |

(c) Peps

| 1 | 405 |

(d) Peps

| 1 | B | 306 | A | 581 |

All fragments were isolated without breaking S-S bonds.

CNBr and Peps signify cleavage by cyanogen bromide and pepsin respectively. The fragments are named according to the workers who isolated them.

References are: (a) McMenamy *et al.* (1971); (b) Peters and Hawn (1967), and Feldhoff and Peters (1975); (c) Feldhoff and Peters (1975); (d) King (1973).
Peters (1975) has proposed a system of domains for albumin which divide the molecule into four portions; loops 123, loops 4 and 5, loop 6 and loops 789, based upon the net charge of each loop and the susceptibility of linkages to peptic or tryptic attack. The division between loops 4, 5 and loop 6 was the least clearly defined.

The theory of domains has been invoked to explain the transition which albumin undergoes below pH 4. A lowering of pH from 4 to 2 is followed by the transition of albumin from a normal (N) form to a faster (F) migrating form. Further lowering of the pH causes continued acid unfolding (Foster, 1960). This acid expansion has been explained by a separation of the domains such as those proposed by Peters (1975). The separation has variously been suggested to produce 2, 3 or 4 sections (Foster, 1960; Bloomfield, 1966; Weber and Young, 1964).

A commercial preparation of albumin is generally a heterogeneous mix, containing simple contaminants such as α and β Globulins or endonucleases but also many more subtle additions which manifest themselves by producing a range of apparently different albumins. One class of impurities are those which are complexed with the single free sulphydryl group of albumin (Cysteine 34). About 20% of crystalline albumin is complexed with half cysteine and about 5% with half glutathione (King, 1961; Andersson, 1966). Albumin which contains one free sulphydryl group per molecule is termed mercaptalbumin.

Foster et al. (1965) reported a form of microheterogeneity of albumin associated with differences in the pH at which the species undergo the
N→F transition. These differences are not due to sulphydryl interactions, but may be explained by the presence of tightly bound fatty acids (McMenamy, 1967; Wong and Foster, 1969). Other possible explanations include the presence of dimers or polymers, differences in amide content or location (Spencer and King, 1973) or so called molecular ageing. The ageing of circulating Human Albumin is thought to include such processes as the acetylation of ε amino groups by aspirin (see next section).

The optical properties of albumin including ultraviolet and visible light absorption, fluorescence and circular dichroism characteristics will be covered in subsequent chapters.

1:2:6 ALBUMIN - NATURE OF BINDING SITES

Albumin has some 200 charged residues, equally divided between negative and positive groups with a net negative charge at pH 7.4. In the absence of any other evidence it would seem likely that these charged residues would provide a multitude of sites for interaction with charged small molecules. One would also expect a disposition toward the binding of cations at neutral pH, due to the net negative charge. In reality this is far from the truth, for most small molecules the number of primary binding sites on albumin total less than ten, often only one or two. Also, albumin generally shows a greater affinity for anionic compounds rather than those with a positive charge. These surprising findings have led to much interest in the nature and location of binding sites in albumin.

The binding of some metallic cations is well documented, but generally
little is known of the binding sites for positively charged molecules. The amino terminal nitrogen along with the first two peptide nitrogens and the nitrogen of the imidazole ring His 3 form a square planar chelate ring for both \( \text{Cu}^{2+} \) and \( \text{Ni}^{2+} \) (Bradshaw, 1968). The sulphydryl group of Cys 34 forms covalent bonds with \( \text{Ag}^{+} \), \( \text{Hg}^{2+} \) and \( \text{RHg}^{+} \), and, as was mentioned previously, can form an S - S bond with other sulphur containing ligands. The metal ions already listed, plus manganese which is bound at a single site (Mildvan and Cohn, 1963), are examples of elemental ions which have specific binding sites on albumin. Many other such ions have a large number of weaker interactions with the protein e.g. \( \text{Co}^{2+} \), \( \text{Zn}^{2+} \), \( \text{Cu}^{2+} \), \( \text{Ca}^{2+} \), \( \text{F}^- \), \( \text{Br}^- \), and \( \text{I}^- \). Thorpe (1964) considered that the N-terminal region acts as a binding site for pyridoxal phosphate, fatty acids, thyroxine and various other ligands, but recent evidence largely contradicts this theory. Affinity labelling has suggested that pyridoxal phosphate is attached at Lys 223 of BSA, at the tip of loop 4 (Andersson et al., 1971). Acetyl salicylic acid (Aspirin) is known to acetylate a specific lysine amino group in albumin, although it may bind to other sites with equal or greater avidity but without any chemical modification resulting. Hawkins et al. (1969) isolated an acetylated peptide from HSA, after aspirin pretreatment, with the sequence leu-lys*-ser (glu,ala,leu) lys. Gambhir et al. (1975) have recently sequenced a 107 residue fragment of HSA, including the acetylated Lys and the lone tryptophan. From the sequence of HSA by Meloun et al. (1975) the acetylated lysine can be placed at position 199 in the sequence leu-lys*-cys-ala-ser-leu-gln-lys, which lies in the cleft between loops 3 and 4 in the model of Behrens et al. (1975).
The binding sites already mentioned have been single amino acids, it is very likely however that most interactions cannot be considered to involve just one residue, but larger regions of the protein.

Further work on the nature of drug binding sites has been largely concerned with the binding of organic anions to albumin. It has been suggested many times that such binding sites would contain a cationic head group(s) and a pocket of non polar amino acids. The structure of albumin seems to provide many such sites, with the clefts between the loops providing areas of hydrophobicity and several charged amino acids at the tips of the loops, particularly on the 'right side' of the molecule e.g. Arg 81, 143, 144, 334, 335, 410 etc. etc. Lys 223, 272, 293 etc. (all residues refer to BSA). Brown (1975) has suggested that the binding site for anionic compounds is a right side hole in the cylinder structure which he also postulated for BSA. Swaney and Klotz (1970) sequenced a short peptide containing the lone tryptophan of HSA and suggested that certain cationic groups surrounding a cluster of non polar residues forms an ideal binding site. Their sequence of lys-ala-trp-ala-val-ala-arg fits into the HSA sequence of Meloun et al. (1975) as residues 212-218, and, in the scheme of Behrens et al. (1975), between loops 3 and 4 (see fig. 1:3). A similar region is present in the BSA molecule. As evidence for the presence of tryptophan at a binding site of HSA Swaney and Klotz cited the findings that tryptophan is perturbed by the binding of sodium dodecyl sulphate (SDS) (Herskovits and Laskowski, 1962; Williams et al., 1965) and the steroids testosterone, progesterone and cortisol (Ryan, 1968; Ryan and Gibbs, 1970). Further reference will be made to the perturbation of tryptophan and its possible involvement in binding.
The area around the 'reactive' tyrosine of albumin (Sanger, 1960, cited by Steinhardt et al., 1971) also meets the requirement for an organic anion binding site. The sequences of arg-tyr-thr-argin in BSA and arg-trp-thr-lys in HSA can be found around residues 407-413 between the loops 6 and 7.

Anderson and Weber (1969) postulated the existence of clefts perpendicular to the major axis of albumin which would bind ANS, in order to explain the fluorescence polarisation of ANS when bound to BSA. The areas between loops 3 and 4 (including the tryptophan containing site of Swaney and Klotz) and loops 6 and 7 (including the reactive tyrosine of Sanger) are two possible clefts. The facts that ANS strongly quenches the fluorescence of HSA (Santos and Spector, 1974) and BSA (Jun et al., 1975) and that modification of the Arg residues of BSA causes a 100 fold diminishing of ANS binding (Jonas and Weber, 1971) give further weight to this argument. Reed et al. (1975) found ANS to bind mostly to large fragments of BSA and to loop 3. The same workers reported the bilirubin binding site to be located in residues 186-238 of BSA, this area is much of loop 4 and again contains the tryptophan site of Swaney and Klotz. They also suggested that salicylate was bound in a similar region. These observations are consistent with the pronounced quenching of the fluorescence of BSA and HSA by bilirubin (Chen, 1971, quoted by Chignell, 1972), and the competition of salicylate and bilirubin for binding sites (Odell, 1973).

The site of binding of acetyl salicylic acid (residue 199 of HSA) and the findings of Jacobsen (1972, 1975) that histidyl, tyrosyl, arginyl and lysyl residues are involved in the binding of bilirubin to HSA.
are also quite compatible with these views. Studies involving Tryptophan, the only amino acid bound with plasma albumin to any extent, have yielded some interesting results. Using three cyanogen bromide fragments of HSA A (residues 299-585, loops 6,7,8,9) B (residues 1-124, loops 1 and 2) and C (residues 125-298, loops 3,4,5) two groups have studied the tryptophan binding site. Sjöholm and Lungstedt (1973) reported that fragment C contained the main tryptophan binding site, whilst A did have some affinity for tryptophan. They also found the drugs phenylbutazone, Librium and Propiomazine (a phenothiazine) bound to fragments A and C but not B. The group of McMenamy (McMenamy et al., 1971; Gambhir and McMenamy, 1973; Gambhir et al., 1975) localised the primary tryptophan binding site near His 146 by affinity labeling with bromoacetyl-L-tryptophan. They also reported Lys 190 (189 in Behren's sequence) to be involved, and that a tyrosyl hydroxyl of fragment A could also be implicated in tryptophan binding. These observations place the primary tryptophan binding site between loops 3 and 4. This group considered that within fragments C (loops 3,4,5) and A-phe (a section of A probably containing loops 6 + 7) exist the major binding sites of HSA, a conclusion which fits well with the previous discussion.

Albumin binding sites for fatty acids are of particular interest in this thesis, and have received much attention from other workers. The influence of chain length and conformational changes upon binding sites will be discussed in Chapter 4. King (1973) studied the binding of octanoate to two fragments of BSA equivalent to loops 1,2,3,4,5 (named B) and 6,7,8,9 (A). He reported loop 6,7,8,9 to contain one high affinity site with two
lower affinity sites on the remainder of the molecule. King (1973) also reported D and L tryptophan to compete for the high affinity site, this is possibly the same site reported for secondary binding of L-tryptophan. The binding of fatty acids to fragments of BSA produced by limited proteolysis has recently been reported by Reed et al. (1975). The three strongest binding sites for palmitate were found to be in the carboxy terminal two thirds of the molecule as follows; site 1 within residues 377 to 503 (loops 7 and 8) site 2 239-306 (loops 4 and 5) and site 3 within 307-377 (loop 6).

In studies using trinitrobenzene sulphonylic acid, a peptide was isolated labelled with TNP which had the sequence leu-al-a-glu-lys-tyr (Andersson et al., 1971). It was found that this site in BSA was not included in the primary binding site for caprylate or palmitate but was involved in secondary binding. This sequence does not occur in the sequence of BSA proposed by Brown (1975) but may well be equivalent to residues 346 to 351: leu-al-a-lys-glu-tyr in loop 6 which would be consistent with site 3 proposed by Reed et al. (1975). Lysine has also been reported to be involved in fatty acid binding by Green (1963).

Noel and Hunter (1972) considered that at least some of the binding of fatty acid to BSA was to a cleft which contained the single sulphhydryl group (residue 34) since the binding of unsaturated fatty acid potentiated the oxidation of the sulphhydryl group. They also suggested that a tryptophanyl residue was near this binding site, this is probably Trp 134 of loop 3 (not that of Swaney and Klotz) which is brought into close proximity to Cys 34 by a folding together of loops 1 and 3. It is interesting that L-thyroxine is thought to bind near Cys_{34} (Ohkubo, 1969) and that
L-thyroxine competes for a secondary fatty acid binding site (Tabachnick, 1964).

It is likely that the area around the exposed Trp of HSA (214) and BSA (212) does not provide a primary binding site for fatty acid, since the addition of 2 or 3 moles of long chain fatty acid does not cause any change in the fluorescence or absorption of HSA tryptophan. (Spector and John, 1968). Palmitate can reduce the quenching of HSA trp caused by iodide, but this is probably due to a conformational change (Spector et al., 1973). This site probably does constitute a secondary site since a high concentration of palmitate will displace ANS (Santos and Spector 1972, 1974) and L-tryptophan (McMenamy and Oncley, 1958) both thought to bind to this region and will alter the transitions of Trp at high concentrations (Steinhardt et al., 1972). Conclusions are much the same with BSA, however, fluorescence data is confused by the presence of a second tryptophan residue. The free tryptophan, which is quenched by iodine and is not near the free -SH group (Noel and Hunter, 1972) is not a primary binding site. The second tryptophan, which is not quenched by iodine but is affected by oxidation of the lone -SH group seems to be involved (Noel and Hunter, 1972) and probably accounts for the 45% quenching of BSA fluorescence upon the addition of fatty acid (Spector and John, 1968). The involvement of tyrosine residues in the binding of fatty acid, as reported by several groups is confirmed by a perturbation of tyrosine absorption with fatty acid binding (Zakrzewski and Goch, 1968; Steinhardt et al., 1972).

Figure 1:6 is an attempt to summarise the postulated locations of binding sites on the albumin molecule. Some other locations may be
Figure 1: The location of some binding sites on albumin

a) Specific amino acids

1. N terminal (1,2,3): Cu$^{2+}$, Ni$^{2+}$.

b) Larger binding areas

1. Around the lone Cys and buried Trp: secondary fatty acid, Thyroxin.
2. Around the exposed Trp: ANS, Bilirubin, Salicylate, L-Tryptophan.
3. Fatty acid binding sites of Reed et al. (1975)
   - Site 2, 239-306
   - Site 3, 307-377
   - Site 1, 307-377
4. Around the reactive Tyr: ANS, D and L Tryptophan secondary.

Residues refer to BSA, unless otherwise stated. All these binding sites are fully discussed in the text. ■ = Cys 34  ▲ = Trp 134 (BSA only)  ● = Trp 212 (214 in HSA).
inferred from competition studies. Those involving fatty acids and warfarin will be discussed later, in the relevant chapters (4 and 5).

Having considered the information available pertaining to binding sites it must be said that some workers believe that it may be a misconception to think of binding sites in terms of specific areas of the molecule. Karush (1950) first proposed the idea of 'conformational adaptability' which includes the concept that binding sites may be very flexible, moulding to suit the ligand involved, and that the binding of a ligand can induce conformational changes which create or destroy binding sites. There is considerable evidence to support the idea that albumin undergoes some conformational changes with the binding of ligands, reflected by changes in physical properties such as viscosity, light absorption, stability etc. Conformational changes may also explain the interference of one ligand's binding by a second ligand in a 'non competitive' fashion (see section and chapter 3). We must be careful, then, not to apply a too rigid approach to the allocation of drug binding sites to portions of the albumin model.
1:3 BONDS INVOLVED IN DRUG-PROTEIN INTERACTIONS

The original concept of the molecular nature of drug-albumin interactions was one of electrostatic attraction and bonding by ionic bonds. When the ionic nature of many drugs, and the number of charged groups on the protein are considered it is not surprising that this view was taken. It has however become increasingly evident that ionic interactions play only a minor role in most associations with albumin. It is pertinent, then, to consider the various types of bond which may be formed between protein and ligand.

1:3:1 COVALENT BONDS

Covalent bonds are formed when two molecules share a pair of electrons. This type of interaction is a chemical combination rather than an association and is generally not important in drug-protein interactions. Covalent bonds are very stable, with a bond energy of \( \sim 100 \text{ kcal.m}^{-1} \) and are thus essentially irreversible.

There are only a few examples of covalent bonds being formed between albumin and drugs, probably the best known examples are the interaction of acetylsalicylic acid and TNBS with the \( \varepsilon \) amino groups of lysine in albumin (see section 1:2:6). There may be many more covalent interactions between drugs and albumin than are generally recognised, since few binding experiments are designed to display the presence of this type of binding. There are certain groups of drugs which, either in their native form, or after metabolic conversion, are known to bind covalently to macromolecules. The alkylating agents are one such group which have
the carbonium ion as the reactive species and which bind to nucleic acids and various cellular and tissue proteins. Compounds which form epoxides as metabolic intermediates are also known to bind covalently to macromolecules. The study of reactive species binding to macromolecules is a separate and fast expanding field which is beyond the scope of this introduction. It should be pointed out, however, that the covalent binding of a drug to albumin may produce a molecule which acts as an antigen, eliciting an immunological response.

Albumin contains a single free cysteine residue which may be available for oxidation and mercaptide formation. This residue is normally present in the reduced state or complexed with either half cysteine or half glutathione. However, the formation of a covalent link with exogenous compounds is a distinct possibility. Heavy metals and compounds which contain sulphhydryl groups are just two types of molecule which can react with free cysteine.

The coordinate bond is a particular form of covalent bond in which both electrons are donated from one atom. The elements nitrogen, oxygen and sulphur, when bonded normally, have an unshared pair of \( \frac{1}{2} \) orbital electrons which are available for coordinate bond formation. Often bonds are formed with metal ions such as \( \text{Na}^+, \text{Mg}^{2+}, \text{K}^+, \text{Ca}^{2+}, \text{Cu}^+, \text{or} \text{Zn}^{2+} \) and also the transition metals e.g. Co, Mn, Fe have a peculiar ability to accept electrons in unfilled inner orbitals.

1:3:2 THE IONIC BOND

The ionic bond is the result of coulombic forces (electrostatic attraction) between oppositely charged ions. The bond has an average energy of
5 k cal.mole\(^{-1}\) and the attraction diminishes with the square root of the separating distance. The ionisable amino acids are listed in table 1:2 with the numbers present in Bovine and Human Albumins. The mere presence of an ionisable amino acid does not mean that it is necessarily available for ionic interactions with a ligand. The surrounding structure of the protein may make it inaccessible; the ion may be involved in intra-molecular bonding or the amino acid may be predominantly in the unionised form. Many drugs contain charged groups which are capable of forming ionic bonds with the protein. The charged group need not necessarily be totally ionisable, the carbonyl group for example \(R - C=O\) has a small negative charge on the oxygen and the opposite charge on the carbon atom.

1:3:3 THE HYDROGEN BOND

The hydrogen bond may be considered as a particular type of ionic bond, despite the fact that both contributors to the bond may be uncharged when apart. The bond is formed between a hydrogen atom, which is covalently bound, and an electronegative receptor atom. The average hydrogen bond bridges approximately 3\(\AA\) and has a strength of 2 - 5 k cal mole\(^{-1}\). Despite the rather weak nature of the bond, many such interactions can be formed between two ligands giving considerable stability to the complex. The most common forms of hydrogen bond in ligand-protein interactions occur between the hydroxyl groups of the protein and terminal sulphur, nitrogen or oxygen atoms of the ligand. Hydrogen bonds are highly directional, the electronegative centre must lie in line with the hydrogen covalent link before a bond can be formed.
Table 1: The ionizable groups present on albumin

<table>
<thead>
<tr>
<th>Amino acid residues</th>
<th>Ionic group</th>
<th>pK a (25°C)</th>
<th>Number of residues present on albumin</th>
<th>b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>HSA</td>
<td>BSA</td>
</tr>
<tr>
<td>C-terminal</td>
<td>α-carboxyl</td>
<td>2.09</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>N-terminal</td>
<td>α-amino</td>
<td>9.65</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>β-carboxyl</td>
<td>3.86</td>
<td>39</td>
<td>41</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>γ-carboxyl</td>
<td>4.25</td>
<td>60</td>
<td>59</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>phenolic</td>
<td>10.35</td>
<td>18</td>
<td>19</td>
</tr>
<tr>
<td>Lysine</td>
<td>ε-amino</td>
<td>10.53</td>
<td>58</td>
<td>59</td>
</tr>
<tr>
<td>Arginine</td>
<td>guanidino</td>
<td>12.48</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>Histidine</td>
<td>imidazole</td>
<td>6.00</td>
<td>16</td>
<td>17</td>
</tr>
</tbody>
</table>

a. From Lehninger (1970)

b. Based upon the sequences shown in figures 1:2 and 1:3
Van der Waals forces are a group of weak electrostatic interactions including attractions between dipoles and induced dipoles and the repulsion between similar atoms. The repulsion is known as the Born force and is due to the repulsion of the electron clouds which surround atoms and molecules. The Born force is only important when atoms are in close proximity and the electron clouds overlap. Keesom and Debye attractive forces require a permanent dipole and are thus not often important in drug-protein interactions. Permanent dipoles occur in electrically neutral molecules due to the uneven distribution of charge around covalently linked atoms of different electronegativity. Keesom forces are due to the attraction of two permanent dipoles whilst Debye forces occur between permanent and induced dipoles. London forces occur between neutral atoms with no permanent dipole and are the most important of the Van der Waals forces involved in ligand-protein interactions. The electrons surrounding a neutral atom are not fixed and can move from orbital to orbital producing transitory asymmetries in the charge distribution, i.e. a dipole. Although such a dipole is very short lived, it can induce a second dipole in a close, second electric field which results in an attraction. Such attractions are continuously formed and are responsible for the permanent attraction between two atoms, in the absence of any other electrical processes. Van der Waal's forces are very short ranged, diminishing with the sixth power of the distance between participating atoms and have a very small bond energy. Complexes of considerable stability can be formed, however, since Van der Waal's forces are additive and molecules can participate in many such interactions, a good example of 'strength in numbers'.
The term 'hydrophobic bond' is much used in the jargon of protein-binding researchers, but it is a somewhat misleading title and requires clarification. Hydrophobic bonding has been defined as the tendency for non-polar groups to aggregate together in aqueous solution. This process does of course occur, but is not due to some special type of bond, rather due to a maximisation of Van der Waal's and ionic interactions. Thus, if a hydrocarbon is mixed with water, the water molecules will associate to form the maximum number of hydrogen bonds and the hydrocarbon molecules will associate to give the maximum number of London-Van der Waal's interactions. Even in a system as simple as hydrocarbon in water the preceding explanation is, however, only an approximation of the processes involved. The precise nature of hydrocarbon-water interactions is an area of contention at the present time (Hermann, 1971; 1972). The term hydrophobic bond is then a convenient short hand phrase to describe a complex mixture of interactions.

As a postscript to this section it is informative to consider the order of reactions as a ligand is introduced into a protein solution. Providing the ligand is soluble, it will be taken up into solution, in other words will be surrounded by water molecules. (The surface of the protein is already coated with water and consequently binding must involve the squeezing out of some of these water molecules). Once diffusion processes have brought the ligand into the vicinity of the protein, thermal agitations will ensure that the ligand and protein will collide. If the ligand is ionised the first attachment to the protein will be via ionic bonds, since other attractive
forces are effective only over a short range. Once in very close proximity to the binding site a combination of hydrogen, Van der Waal's and hydrophobic bonds will determine the strength of the interaction. In most cases the binding will not be so tight as to preclude dissociation and an equilibrium will be set up which will obey the laws of mass action.
One of the main goals of protein binding research is to reach a stage at which it will be possible to predict the affinity of a new drug for albumin solely from its structure. Whilst this stage is still many years in the future, which is perhaps not surprising considering the number of bond types and the multiplicity of sites involved in binding, certain general conclusions have been made concerning the influence of drug structure on binding. These conclusions concern three broad types of modification in drug structure which may affect protein binding, the three types are illustrated in an early piece of work by Klotz et al. (1952). This report concerned the binding to albumin of the dye methyl orange and its derivatives.

The affinity of albumin for the compound was found to change if:

a) the dimethyl amino group was lengthened to diethyl, dipropyl, or dibutyl, there was in fact a reduction in affinity with increasing chain length.

b) the anionic residue sulphate was modified to carboxylate, phosphate or arsonate and 

c) if the position of the anionic group on the phenyl ring was changed. The three types of modification are, then: changes in the hydrophobicity of the molecule, changes in the ionisable group and steric or isomeric changes. Of these three, the first category is by far the most studied.
There have been many reports of a correlation between lipophilic character of a ligand and its degree of binding, virtually all of which have reported increased binding with increasing lipophilicity, in contrast to the findings of Klotz et al. (1952). Bridges and Wilson (1976) have recently reviewed the literature in this field, with reference to the albumin binding of steroid hormones, cardenolides, penicillins, other antibiotics and particularly phenothiazines and sulphonamides. In such studies, lipophilicity is often measured as the partition coefficient \( P \), usually quoted as \( \log P \) of the compound between octanol and water. The correlation between \( \log P \) and affinity of binding to albumin was first noted by Hanch and co-workers (see Leo et al., 1971) for a series of neutral molecules. Wilson (1974) showed a similar correlation between \( \log P \) and the 'total binding constant' \( nK \), for a series of aliphatic carbamates. In a detailed account of alkane binding to BSA, Wishina (1964; Wishina and Finder, 1964) concluded that the binding was akin to gas in liquid solubility and that alkane binding was a good test for the presence of hydrophobic areas on the surface of proteins.

Similar relationships between binding and lipophilicity have also been reported for amphiphilic compounds, i.e. those with ionic and hydrophobic portions. The acidic sulphates and sulphonates (Karush and Sonenburg, 1949; Ray et al., 1966; Reynolds et al., 1967) are two examples and the fatty acids are also well studied, but will be discussed in chapter 4. For the basic phenothiazines (Kriegstein et al., 1972a, b; Nambu and Nagai, 1972) a correlation has been found whilst there was no relationship for tricyclic antidepressant binding (Jorgensen et al., 1973).
The overwhelming volume of data leads to the conclusion that lipophilic character is a prime determinant of the strength of binding for a wide range of compounds. There are however deviations from the norm. The binding of methyl orange derivatives (Klotz et al., 1952) has already been reported as one such exception. Dunn (1973) reported a parabolic relationship between log.P and binding for carboxylic acids, p-amino benzene sulphonamides, anti-inflammatory and uricouric agents. The binding of uncharged ligands, such as alkenes, alcohols and carbamates are typified by a larger number of binding sites (often greater than 5) and a lower affinity (often $K_a < 1 \times 10^4 M^{-1}$) than the equivalent charged molecule, which suggests some involvement of ionic interactions in the binding reaction of charged compounds.

The nature of ionisable groups in a ligand can profoundly affect the binding to albumin. Klotz et al. (1952) found the carboxyl derivative of methyl orange had a similar binding whilst the phosphate and arsonate derivatives had much reduced binding compared to the parent molecule. For a straight, hydrocarbon chain the affinity of binding is increased as the polar head is modified in the order $OH < COO^- < SO_3^- < SO_4^-$ (Steinhard and Reynolds, 1969). Generally, anionic compounds bind more avidly to albumin than cationic molecules, the addition of amino or N-heterocyclic groups to a ligand frequently diminishes binding. Any modification of an ionic ligand which affects the degree of dissociation may affect the ligand's binding characteristics.

The arrangement of functional groups in a ligand is often as important as the nature of the groups in determining binding characteristics. Generalisations are hard to make in this area because of the diverse structural nature of drugs, but for aromatic compounds Klotz et al. (1948) has
suggested ortho substituents may affect binding more than the meta or para equivalents because the latter two can interact with solvent molecules to a greater extent. Ortho aminobenzoate and ortho hydroxyphenylacetate are both more highly bound than their para counterparts (Klotz, 1946; Luck and Schmidt, 1948). The binding of various hydroxylated warfarin derivatives is also consistent with the theory of Klotz et al. (1948).

Warfarin

Hydroxylation in positions 4', 6, 7 or 8, where interaction with water molecules is likely, produces a diminution of binding whilst 5-hydroxylation increases binding. O'Reilly (1973) suggests that a hydrogen bonded ring, involving the 5 and 4 hydroxyl's may account for this increased binding.

Optical isomers, as well as structural isomers, may also have dissimilar binding characteristics. Müller and Wollert (1975) reported a stereo specific binding of D and L oxazepam hemisuccinate. L tryptophan is known to bind to HSA with a higher affinity than the D isomer (McMenamy and Oncley, 1958) Koga et al. (1975) studied the binding of dansyl alanine and dansyl glycine and found the D isomer to have a higher affinity for BSA in each case. Stereo specific binding in general, and more specifically the binding of the enantiomers of warfarin and phenprocoumon will be discussed in chapter 6.
Despite the wealth of information available concerning drug-albumin interactions, the significance of binding in drug mediated therapy is still poorly understood. Several authors have reviewed the field of plasma protein binding with reference to pharmacodynamics and pharmacokinetics: Goldstein, 1949; Brodie, 1966; Meyer and Guttman, 1968; Keen, 1971; Anton and Solomon, 1973; Gillette, 1975; Shand et al., 1975; Wilson and Bridges, 1976.

Little is known of the influence of protein binding on the absorption of drugs administered enterally or parenterally. Indeed, interaction with plasma or interstitial proteins is seldom considered in drug absorption studies. Brodie (1966) has suggested that aqueous insoluble drugs will precipitate in the gastrointestinal tract giving a situation where the limiting factor of absorption is the rate of solution of the drug crystals. If the drug is highly bound to plasma proteins, freshly dissolved drug is quickly removed and the rate of solutions is enhanced. Brodie asserts, furthermore, that without a high affinity for plasma proteins, highly lipophilic drugs such as dicoumerol would not be absorbed. There have however, been no definitive experiments to establish the role of plasma binding in absorption from the G.I. tract. The rate of absorption of a series of barbituric acids has been correlated with their plasma protein binding, but the authors (Kakemi et al., 1969) concluded that binding to the mucosal membrane was far more important.
It is possible that the parenteral administration of aqueous insoluble drugs would be impractical if they were not bound to plasma proteins since the drug would precipitate in small blood vessels.

1:5:2 DISTRIBUTION AND ACTIVITY

After administration to the body, a drug will distribute, down a concentration gradient, throughout the body, just as if it were a volume of water providing that the drug can pass freely across cellular membranes and that it is not associated with any body components. This situation rarely occurs in practice since most drugs associate to some degree with the macromolecules of plasma and tissue limiting their availability to other compartments of the body which may include the receptor sites. An equilibrium between compartments is set up which may be illustrated simply as below or more comprehensively as in figure 1:1.

![Diagram](image)

It is a fundamental, and often tacit, assumption of pharmacology and pharmacokinetics that the activity of a drug is related to the free concentration in plasma. The bound drug does not contribute directly to activity since only the free form is in equilibrium with the receptor (Goldstein, 1949; Brodie, 1966; Goldstein et al., 1968).

Whilst this premise is certainly correct for the majority of drugs and is supported by the many observations of reduced potency with high plasma
protein binding (for example sulphonamides, Kruger-Thiemer et al., 1965; or penicillins, Rolinson and Sutherland, 1965) the possibility that bound drugs may still retain some activity should not be ignored. Many small molecules and drugs, when covalently linked to albumin, can elicit an antigenic response and thus the bound molecule must be available to receptors of the immunological system. There is no firm evidence to prove that other receptors cannot interact with bound drugs in a similar manner.

Benhold (1938) hypothesised that drugs would have to be bound to plasma proteins in order to pass into cells. This view is now largely discounted, but it may actually have some foundation. Fatty acids must be bound to albumin for efficient uptake and removal from adipose tissue in vivo and adipose cells in culture (Spector, 1975). Drugs which are otherwise unable to cross the cell membrane may be taken up bound to albumin by pinocytosis. The binding of the drugs phalloidin (Barbanti-Brodano et al., 1974) and amanitin (Derenzini et al., 1973) causes a much enhanced toxicity of the compounds due to this phenomenon. It must be remembered in this context that albumin is available to cells as a source of amino acids via pinocytosis. As much as 10% of protein metabolism may be carried on in this manner (Peters, 1975).

Drug bound to plasma or tissue proteins should not be considered as lost, but in storage. Metabolism and excretion will reduce the free plasma concentration which promotes the dissociation of the drug-protein complex thus replenishing the free drug. In this way protein binding prolongs the action of many drugs at a reasonably constant free plasma concentration. Without this effect, drugs which are rapidly metabolised or excreted would have to be given with great frequency in order to maintain therapeutic levels.
In addition, protein binding can act as a buffer to prevent large fluctuations in free drug concentrations with the administration of a dose which might otherwise lead to toxic levels.

It is interesting to observe at this point that the clinical estimation of plasma drug concentrations is almost always one of total drug (unless bioassay is used in which case only free drug may be measured). It should be obvious from preceding comments that total plasma levels of drug may bear little relation to the availability of drug at the receptor site. Even bioassay or other free drug estimation may give misleading results if diluted plasma is used in the assay since drug binding is not necessarily independent of protein concentration.

Several workers have produced mathematical models to mimic the effects of plasma protein binding upon drug distribution: Martin, 1965a, b; Keen, 1971; Shoenemann et al., 1973; Wagner, 1973; Gillette, 1975; Shand et al., 1975; Julkunen et al., 1976. Most of these reports include complex pharmacokinetic models beyond the scope of this thesis, but the conclusions of Martin provide some simple generalisations: plasma protein binding will only have an appreciable effect upon distribution if the association constant of the drug-protein complex is greater than $10^4 \text{ M}^{-1}$. Drugs with a high affinity will be localised mainly in the plasma compartment at low concentrations, but the fraction in plasma will be markedly reduced at higher concentrations. Such drugs will also have a narrow dosage range above which a small addition of drug will result in a large increase in free concentration. The treatment of Martin (1965a, b) is oversimplified, ignoring such important factors as metabolism and excretion, but the final statement that plasma protein binding is an important modifier of drug distribution only for drugs
with high affinity at high concentrations is a good rule-of-thumb.

Little mention has been made of the influence of tissue binding upon drug distribution. Despite the fact that the mass of extravascular protein available to drugs easily outweighs the plasma proteins, the balance of research into the two areas is tipped firmly away from tissue binding. Because tissue protein is so abundant, it is quite likely to be a more important binding component than plasma, at least for some drugs. Experimental data is, however, scarce; Burns et al. (1953) found drug levels in a patient who had been receiving phenylbutazone for some time to be high in several extravascular sites. The concentrations of drug in heart, lung and muscle were 32, 24 and 19 times higher than the total plasma concentration. Brodie (1952) reported the concentration of thiopentone to be 10 times higher in adipose tissue than in plasma, three hours after intravenous injection.

A number of body compartments are separated from the circulation by membranes dissimilar to the normal plasma membrane. Areas such as the brain, the eye, the mammary gland and the foetus have very slow equilibration with plasma free drug because of the need to pass across several membranes. The final concentration of drug on the extravascular side of the membrane will depend largely upon the local protein concentration and its affinity for the drug in question. The brain, for example, generally attains only low concentrations of highly plasma protein bound drugs.

The transfer of small molecules across the placenta is being closely studied, due to concern over the chemical teratogenicity of environmental agents. Drugs can normally pass through the placenta but equilibration
is slow, since only the free drug of maternal plasma is available for transfer (Goldstein et al., 1968). The binding of drugs in fetal plasma will be covered in section 1:6:1.

1:5:3 METABOLISM

Just as it is assumed that only the free fraction of a drug is biologically active, it is also generally thought that it is the same fraction which is available for metabolism. However, from the few studies which have been made of the rate of dissociation of a drug-albumin complex it seems likely that this process would not be rate limiting in metabolism (Thorp, 1964; Meyer and Gutman, 1968). A theoretical approach to the influence of plasma protein binding on the rate of drug metabolism in the liver has been made by Gillette (1971, 1973, 1975) and also Shand and colleagues (1975). They conclude that binding can have diverse effects depending upon the $K_A$ of the interaction, the $K_M$ and $V_{max}$ of the metabolising system, the degree of tissue binding and the dose of the drug. To illustrate this diversity in effect consider two drugs, one which has a 'first pass' type metabolism, the other being only slowly transformed. Plasma binding will enhance the rate of metabolism for the first drug, since it is effectively transporting drug to the liver, whilst for the second, slowly metabolised compound, binding will act as a storage mechanism, increasing the half life of the drug (Gillette, 1975). The situation is further complicated by tissue binding, where plasma binding may act to transfer drug from the tissues to the site of metabolism.

The rate of diffusion of a drug into hepatocytes may be limiting for highly active liver enzymes systems. It has been suggested (Gillette, 1973) that
the hepatic cytoplasmic proteins Y (ligandin) and Z (FABP or amino azo dye BPA) may be involved in the binding of drugs, thus maintaining a concentration gradient and possibly increasing the availability of drugs to enzymes in the centre of cells. Y and Z proteins have been shown to bind bilirubin, bromosulphophthalein, indocyanine green (Levi et al., 1969), corticosteroids (Litwack et al., 1972), haem (Meuwissen et al., 1972), fatty acids, steroids, and various carcinogens (Tipping et al., 1976). These proteins may also be involved in the flux of molecules from liver cells into the bile (Litwack et al., 1971).

There have been relatively few experimental studies of the effects of plasma protein binding upon metabolism, Newbold and Kilpatrick (1960), showed a decreased rate of acetylation of two sulphonamides in rabbit perfused liver with the addition of plasma to the perfusate. Later work also showed correlations between the protein binding and metabolism of sulphonamides both in vitro (Anton and Boyle, 1964) and in vivo (Wiseman and Nelson, 1964). Levy (1973) has suggested that the rate of metabolism of dicoumerol is regulated by protein binding determining the distribution of the drug to the liver.

One interesting aspect of the metabolism-binding relationship is that metabolites may compete with the native compound for binding sites. This could result in a displacement of the drug and possibly an increase in metabolism leading to more metabolite being formed.
EXCRETION

The removal of drugs from the body is usually accomplished either by renal elimination and disposal in the urine, or biliary elimination and excretion in the faeces. Three processes contribute to renal excretion: glomerular filtration, tubular secretion and tubular resorption. The pores of the glomerulus allow free passage of small molecules but not proteins, therefore if no secretion or resorption of a drug takes place then renal elimination will be a function of the free drug concentration. The elimination of tetracyclines is an example of such a situation, with an inverse relationship between protein binding and rate of renal excretion (Kunin et al., 1959; Fabre et al., 1971). Drugs which are subject to tubular secretion are rapidly and continuously removed from the tubules and thus protein binding is probably not important unless it is considered that high protein binding acts to transport the drug to the site of elimination (Keen, 1971). The opposing view has been put forward by Kruger-Theimer (1968) who suggested that tubular secretion is a function of free drug. The findings that penicillins are rapidly secreted via tubular secretion, independent of their high protein binding (Rolinson, 1967; Notari, 1973) lends support to the former theory. Tubular resorption takes place passively through the intact tubular membrane and is a function only of the hydrophobicity and ionisation of the drug. The three processes can interact to produce a complex pattern of elimination which is well illustrated by the excretion of sulphonamides via the kidneys which some workers have found unconnected with protein binding (Newbold and Kilpatrick, 1960; Rieder, 1963) whilst others have shown a positive correlation (Arita et al., 1971).
Since bile is secreted from liver cells, those factors, including protein binding, which influence drug uptake into liver cells may also affect biliary elimination. The biliary excretion of several compounds has been correlated with protein binding, including steroids (Sandberg and Slaunwhite, 1956), organic, carboxylic acids (Brauer, 1959) and azo dyes (Preistly and O'Reilly, 1966). However, all these compounds are known to bind to ligandin (or aminoaizodye binding protein A) and it may actually be a correlation between ligandin binding and biliary excretion which was observed by these authors.
1:6 MODIFIERS OF DRUG-PLASMA PROTEIN BINDING

1:6:1 AGE

The plasma protein binding of many drugs is reduced both in the very young and in senior citizens. This trend is at least partially a reflection of the plasma albumin levels at various stages of maturity. The human, foetal plasma albumin level at 26 weeks is 18 g.L\(^{-1}\) rising to 35.7 g.L\(^{-1}\) immediately before birth (Gitlin and Boesman, 1966). The normal adult level of about 35 g.L\(^{-1}\) falls off after the age of 50 to as little as 22 g.L\(^{-1}\) in septuagenarians (Weber and Cohen, 1975).

Many workers have reported a reduced binding of drugs in foetal, neonatal or cord human plasma compared to adult plasma. These drugs include sulphonamides and salicylate (Ganshorn and Kutz, 1968), diphenylhydantoin, phenobarbitone, ampicillin and benzylpenicillin (Ehnebo et al., 1971), sulphaphenazole (Chignell et al., 1971). These workers suggested that the binding differences were due to reduced albumin concentration in immature plasma and a higher concentration of competing ligands such as bilirubin. However Krasner and co-workers (Krasner et al., 1973) found purified foetal albumin bound salicylate less strongly, but bilirubin more avidly, than adult albumin. Similar findings were reported by Windorfer et al. (1974) for neonatal and adult plasmas which had been diluted to the same albumin concentration and adjusted to the same bilirubin level. Salicylate was bound less strongly in neonatal plasma and was more easily displaced by bilirubin. It seems, then, that there is a genuine discrepancy between the binding abilities of immature albumin and adult albumin, but
the origin of the difference is unknown.

The preceding results suggest that drug concentrations on the foetal side of the placenta will be lower than the maternal side due to the reduced binding. This is based upon an adult: neonatal or foetal comparison, recent findings have suggested that foetal binding should be compared with the corresponding maternal parameter. Garrettson et al. (1975) found a maternal: foetal salicylate ratio of 1:1.6 in the case of a mother who had taken large daily doses of salicylate throughout pregnancy. Later work (Levy et al., 1975) has shown salicylate to be more extensively bound in foetal plasma than the corresponding maternal plasma, and it was postulated that the difference was because of reduced maternal albumin levels, and higher maternal plasma free fatty acid concentration.

1:6:2 DISEASE

The most obvious way in which disease can affect protein binding is by altering the plasma concentration of the binding macromolecule. Hypoalbuminaemia is a rare genetic disorder but a similar condition can arise in a variety of clinical conditions including hepatitis and cirrhosis of the liver and impaired renal function. Diseases of the liver and kidneys have been well studied in recent years with relevance to the effect on protein binding. Reidenberg (1974) has reviewed the field whilst Black and Arias (1975) have covered the implications of chronic renal failure.

It has been reported that most organic, anionic, drugs normally associated with plasma proteins, show reduced binding in uremic plasma, whereas basic
or neutral compounds are less affected (Reidenburg, 1974; Reidenburg and Affrime, 1973). This is probably explained by the increase in nitrogenous and acidic waste products in uremia as well as the reduced albumin levels. Whether the binding properties of albumin are actually altered in uremia is a contentious matter, some groups have concluded that the albumin of uremic plasma is intrinsically different to that of normal plasma (Anton and Corey, 1971; Gohneim and Pandya, 1975). Phenytoin (syn diphenylhydantoin, DPH) is bound less in uremic plasma than normal (Reidenburg et al., 1971, Odar-Cederlof and Borga, 1976) and Shoeman et al. (1973) have suggested that this may be due to qualitative differences in the respective albumins. It is interesting to note that uremia is often treated by haemodialysis which entails the administration of large amounts of heparin to the patient. Storstein and Jannsen (1976) have shown that heparin given to both normal and uremic subjects causes a significant reduction in the binding of digoxin and digitoxin, due to the release of free fatty acids.

Many drugs have been reported to be less bound in the plasma of patients with hepatic failure. The binding of DPH in acute viral hepatitis (Blaschke et al., 1975) and thiopental in hepatic cirrhosis (Gohneim and Pandya, 1975) are both reduced, probably due to lower albumin levels and a higher bilirubin concentration. Hepatic failure not only affects albumin synthesis but may influence drug metabolism which may in turn affect protein binding by alteration of metabolite levels.

1:6:3 COMPETITION

It has been shown, from a consideration of the nature of albumin
(see 1;2;6) that there are only a limited number of binding sites available on the molecule. It is inevitable, then, that drugs or endogenous substances will compete for binding sites under some circumstances. Competition can also occur for binding sites on other proteins or on tissues but these are less well documented. Co-administered drugs may compete with each other or a drug may compete with an endogenous compound, or more unusually two endogenous compounds may have the same binding sites. Several review articles have appeared dealing with competition and displacement, penned by the following authors: Brodie, 1966; Meyer and Guttman, 1968; Hartshorn, 1970; Sellers and Koch-Weser, 1970a,b; Swidler, 1971; Wardell, 1974; Koch-Weser and Sellers, 1976b. Solomon (1971) has considered competition between drugs and endogenous compounds for tissue and plasma binding sites. Specific drug interactions with warfarin will be considered in chapter 3 whilst competition between fatty acids and other compounds will be covered in chapter 4.

The clinical significance of drug competitions is not well understood, due to the many readjustments of drug kinetics which may follow displacement. A small displacement may cause a very large increase in free drug levels. If a drug which is 99% bound has a 1% displacement then the free drug levels will be almost doubled. This does not mean that the plasma free drug level is doubled however. The displaced drug is free to distribute throughout the body water and the final increase in plasma free level, and any potentiation of pharmacological effect, will depend upon what effect an elevated free drug concentration has upon metabolism and excretion. It has been suggested recently that the significance of
displacement depends largely upon the mode of elimination of the drug (Shand et al., 1975). If the rate of elimination of a drug is proportional to the free drug concentration, then displacement may result in a temporary increase in free drug, perhaps to toxic levels, but the increased rate of metabolism or excretion will soon reduce the drug concentration back to normal levels. On the other hand if drug elimination is 'first order' i.e. rapid and unrelated to free drug concentration, then displacement may result in permanently elevated plasma levels, since there is no compensating increase in metabolism or excretion. But as elimination is rapid, this type of displacement is not likely to have clinical significance.

Despite all the compensating changes, drug competition can have clinically significant or even disastrous effects. The well known displacement of warfarin by various agents can result in periods of excessive hypoprothrombinaemia which has resulted in serious and fatal hemorrhages (Koch-Weser and Sellers, 1971; see chapter 3). The displacement of bilirubin by sulphonamides was tragically demonstrated in a famous study where premature infants were given either oxytetracyclin or a penicillin/sulphisoxazole combination to protect against infection. A large percentage of those children treated with the penicillin-sulphonamide mixture succumbed to death which was associated with kernicterus (Silverman et al., 1956). A good example of displacement from tissue binding sites is provided by two drugs which have been used in the treatment of malaria: pamaquine, which suppresses the tissue form of the parasite and mepacrine, which is active against the blood form. When patients on mepacrine therapy are given pamaquine the plasma levels of the latter drug are 5 to 10 fold the expected level and in the
toxic range, due to the avid binding of mepacrine to tissue sites (Brodie, 1966; Zubrod et al., 1948).

An unusual example of drug interaction at the binding level is provided by the work of Dollery et al. (1961). Patients receiving chlorothiazide were given the antihypertensive pempidine at normal doses, the resulting plasma level of pempidine was three fold normal, but there was no increase in hypertensive action. Later studies by Breckenridge and Rosen (1971) showed that at normal dose levels pempidine was about 4% bound in untreated plasma, but about 40% bound to plasma from subjects pretreated with chlorothiazide. The mechanism of this potentiation of binding is still unknown since no similar increase could be reproduced in vitro.

The proposed mechanism of action of two groups of drugs demonstrates a possible, beneficial facet of displacement. It has been suggested that the anti-rheumatic activity of drugs such as salicylate, phenylbutazone and indomethacin may be due to a displacement of tryptophan or small peptides from plasma proteins (McArthur et al., 1971; Smith et al., 1971). Similarly, Brodie (1965) proposed that some anti-inflammatory drugs act by displacing steroids from plasma binding sites.
1:7:1 INTRODUCTION

For some applications, it may be sufficient to know only the percentage of drug which is bound in plasma at one particular concentration. This type of information is, however, of no value if an understanding of the interactions involved is desired. To typify any binding reaction the minimum requirements are an estimation of the affinity of the protein for the ligand and an idea of the number of available binding sites. These parameters are calculated, in a variety of ways (see chapter 2, section 2:2:14), from a knowledge of the total drug and normally total protein, concentrations plus an experimental estimate of one or more of the components of the following equilibrium:

\[
D_f + P \xrightleftharpoons[k_2]{k_1} DP
\]

Equation (1)

where \(D_f\) is free drug, \(P\) is uncomplexed protein, \(DP\) is the drug-protein complex and \(k_1\) and \(k_2\) are the rates of the forward and reverse reactions respectively. Protein-binding techniques can be broadly divided into non-spectroscopic methods, which are generally designed to estimate the concentration of free drug and spectroscopic methods which are more often involved in measurements of the drug-protein complex.

Several comprehensive discussions of the methodology of protein-binding have appeared, including those of Goldstein, 1949; Meyer and Gutman, 1968; Steinhardt and Reynolds, 1969; Chignell, 1971, 1972, 1973;
Equilibrium dialysis can truly be called a 'classical' technique, as it was probably the first technique used in binding studies (Osborne, 1906) and has remained popular since that time. In binding studies, ligand may start on either the protein or the buffer side of the membrane and dialysis is continued until free drug has equilibrated between the two solutions.

The technique, in its classical form, suffers from several drawbacks, which have been considered in detail by Steinhardt and Reynolds (1969). Their conclusions, many of which apply to all techniques involving a membrane, can be summarised thus: Errors may be introduced by any of the following factors -

a) Donnan inequalities (Donnan 1924) due to the presence of a membrane, unless they are suppressed by high salt concentrations.

b) Salt and buffer components (e.g. NaCl, acetate, veronal) or membrane impurities can compete for binding sites, although phosphate seems to have little effect (Klotz, 1953; Keen, 1966; Nilsen and Jacobsen, 1975).

c) The membrane may bind free drug (Agren, 1968) or the protein-drug complex (Steinhardt and Reynolds, 1969).

d) The time taken for equilibration may be slow (up to 48 hrs) with the concomitant possibility of protein denaturation and bacterial growth.

e) The method is unsuitable for plasma samples, since low
molecular weight components will equilibrate across the membrane.

f) Dilution of the protein phase by osmosis may occur.

The development of purpose built apparatus and membranes has minimised several of these problems. The miniature dialysis cells designed by Weder and Bickel (1970) are achieving widespread popularity, due largely to dialysis times of one or two hours only. An extension of equilibrium dialysis, dynamic dialysis is also proving popular and is based upon the fact that the rate of diffusion of a drug out of the protein/drug compartment is proportional to the concentration of the dialysable species (Bush and Alvin, 1973; Chignell, 1971). This approach has been used to study the binding of warfarin, phenol red and methyl orange (Meyer and Gutman, 1970) and also tryptophan (Fuller and Roush, 1974) to albumin. Since the rate of dialysis is slow, a very sensitive assay for free ligand is required, radioactively labelled ligand of high specific activity has been used (for instance Fuller and Roush employed $^{14}$C tryptophan, sp.act. 0.5 Ci.mM$^{-1}$). A technique with similarities to dynamic dialysis is that of dynamic permiation, where the rate of permiation of ligand into a suitable medium (nylon has been used – Bottari et al., 1975) is taken to be proportional to free ligand concentration.

1:7:3 ULTRAFILTRATION AND DIAFILTRATION

Ultrafiltration describes the process of forcing a small proportion of a protein-ligand solution through a membrane to form a protein-free ultrafiltrate. The concentration of ligand in the ultrafiltrate is assumed to
correspond closely to the free drug concentration of the original solution. This technique is fast, but care must be taken not to remove a large percentage of the initial solution, otherwise the equilibrium may be disturbed because of the effective reduction in total ligand concentration and increase in protein concentration. The membrane employed in many ultrafiltration methods is the simple regenerated cellulose tubing and pressure is most often provided in the form of centrifugal force. Those sources of error of dialysis associated with the membrane will also be apparent in ultrafiltration methods, with the exception of Donnan inequalities. An additional problem with ultrafiltration is the possible formation of a concentration gradient of macromolecule above the membrane due to the forces used for filtration. This is not likely to affect purified albumin binding studies, since albumin will not migrate significantly under the relatively weak forces used (normally 1,000 g - 2,000 g) but problems may occur with particulate samples such as subcellular fractions. In this respect the standard, Toribara type ultrafiltration apparatus (Toribara et al., 1957; see fig. 2:1) is termed 'dead-ended', that is to say, there is no agitation of sample above the membrane.

The continuous ultrafiltration or diafiltration technique does not suffer from being dead-ended, nor can there be an upset in equilibrium with removal of filtrate. The technique is described in detail elsewhere (chapter 2) but briefly the method is to place the protein solution into an ultrafiltration cell which is bounded by a membrane. The cell is part of a closed system and pressure is applied by pumping drug solution into the cell, ultrafiltrate is continuously formed at the same rate as drug solution is introduced. Analysis of ultrafiltrate for drug, plus a knowledge of the stock concentration and the flow rate, make possible the estimation of bound drug over a wide range of
total drug concentrations. A complete binding isotherm can be produced from a single experiment using just one protein sample. Diafiltration was introduced by Blatt and his colleagues (Blatt et al., 1966) and has been used in drug binding studies by a few workers (Ryan and Hanna, 1971; Damon and Sapira, 1972; Crawford et al., 1972; Thompson, 1973).

A commercial modification of ultrafiltration named 'Centriflo' (Amicon Corp., High Wycombe, Bucks.) has recently been introduced and was evaluated as part of this project (see chapter 3). The technique is typified by ease and speed of operation and has been used by other workers to study drug-protein interactions (Wallace and Whiting, 1974; Booker and Darcy, 1973).

1:7:4 ULTRACENTRIFUGATION

The separation of free ligand from bound by sedimentation of the drug-protein complex in a strong gravitational field offers the attractive proposition of no membrane being necessary. In the most commonly used approach to ultracentrifugal studies of drug-protein interactions (for example Schachman and Edelstein, 1966), a protein gradient is produced in a protein ligand solution by high speed centrifugation. Determination of protein and ligand concentrations at various points along the centrifuge tube allows an extrapolation to zero protein concentration and thus an estimate of free drug concentration. The calculations involved may be complex and lengthy, but the availability of optical scanning of samples during centrifugation for instant assessment of concentrations may make the technique more convenient. This approach has been used to study the binding of various dyes to albumin...
with good agreement of the results with those obtained with equilibrium
dialysis (Steinberg and Schachman, 1966).

The ultracentrifuge has also been used in a simpler series of binding
experiments by Bickel and Steele (1974). They studied the binding of various
drugs to subcellular fractions by incubating the fraction and drug together
followed by complete sedimentation in the centrifuge. The drug concentration
in the particle-free supernatant was taken to represent the free drug concen-
tration. This approach was explored during the present project and is
considered further in chapter 3.

1:7:5' GEL FILTRATION

There have been several approaches to the use of cross-linked dextran
gels (most commonly 'Sephadex' gels) in protein-ligand studies, but all are
based upon the assumption that free ligand can penetrate into the matrix
of the gel whilst the protein fraction is excluded. The simplest method is
that introduced by Scholtan (1964) in which dry gel is added to a drug-protein
mixture. After equilibration, the gel matrix is assumed to have a drug
concentration equal to that of free drug. The gel is isolated and free drug
is estimated after elution. This batch method, using Sephadex, has been
utilised in several studies (Ahtee et al., 1967; Pearlman and Crepy, 1967)
and recently, using polyacrylamide gel, by Himberg et al. (1976). Wilson
(1974) found the batch technique unsuitable for the measurement of sulphon-
amide binding to albumin.

Hydrated gel, packed into columns, can be used in several modes to
estimate protein binding. In the 'zonal' method, a small volume of drug-
protein mixture is applied to the column then eluted with buffer. This results in a separation of the mixture into two zones, one of free drug the other being protein-drug complex. It has been suggested, however, that the rate of dissociation of the drug-protein complex is normally more rapid than the rate of elution and that the zonal method may yield misleading results because of this (DeMoor et al., 1962; Chignell, 1971). A method which attempts to overcome this drawback was introduced by Hummel and Dryer (1962) and involves the pre-equilibration of a column with drug solution before the application of a small volume of drug-protein mixture. The elution is then carried out using drug solution. This method has been used by some groups (Fairclough and Frutton, 1966; Crawford et al., 1971; Nambu and Nagai, 1972) but is extravagant in the use of drug solution. The method should yield very accurate results, since the free drug concentration is set by the experimental conditions rather than being determined by analysis (Steinhardt and Reynolds, 1969).

In a comparison of several gel chromatographic methods, Wilson (1974) found that 'frontal analysis' gave the most reliable results. For this technique a large volume of drug-protein mixture is applied continuously to a column with subsequent elution using buffer solution. From the drug concentrations in the leading and trailing boundaries of the elution profile the free drug may be estimated. Several drug-albumin interactions have been studied in this way (Cooper and Wood, 1968; Kriegelstein and Kuschinski, 1968; Müller and Wollert, 1976).

1:7:6 OTHER NON-SPECTROSCOPIC TECHNIQUES

Thermodynamic parameters associated with a drug-protein interaction
can give information on the types of bond which are involved in the binding and are thus useful quantities to determine. Experiments performed over a range of temperatures allow calculation of the enthalpy and entropy changes of an interaction but these parameters can be determined directly using heat burst microcalorimetry (Chignell, 1971; Chignell and Benzinger, 1972). The technique gives a measure of the temperature change when drug and protein solutions are mixed and has, for instance, been used to determine the thermodynamic parameters for the warfarin-HSA interaction (O'Reilly et al., 1969).

Fast reaction techniques must be used if an estimate of the kinetics (i.e. the rates of association and dissociation) of a drug-protein interaction is desired. This is an area where there is a surprising paucity of data, considering the importance of the dissociation rate in determining the clinical significance of protein binding (Meyer and Guttman, 1968). The kinetics of binding may be measured by observing the interaction either following rapid mixing of the components (continuous flow and stopped flow methods), or after a perturbation of the complex (Relaxation methods, e.g. temperature jump (T-jump)). Taylor (1972) has reviewed fast reaction techniques with relevance to pharmacological reactions. T-jump has been used in a study of dye binding to BSA (Froese et al., 1962), stopped flow in an examination of the bilirubin-BSA complex (Chen, 1974) and continuous flow in a later study of the same interaction (Faerch and Jacobsen, 1975).

Affinity chromatography has been largely restricted to the purification of various biological materials (see Cuatrecasas and Anfinsen, 1971), but protein-ligand interactions may be studied if either protein or drug can be
immobilised on an affinity column. Dunn and Chaiken (1975) have examined the binding of Staphlococal nuclease to a nucleotide ligand which was covalently linked to Sepharose, whilst the opposite approach of complexing the protein with agarose was used in studies of the albumin binding of various compounds including bilirubin, fatty acids, tryptophan and cortisol (Reed et al., 1975).

Electrophoresis has been the standard technique used in the separation of plasma proteins for several years. The method can also be carried out in the presence of ligand and the results give valuable qualitative information on which proteins are involved in binding and can also yield some quantitative estimation of the relative binding to different proteins (Goldstein, 1949; Desgres and De Traverse, 1966; Meyer and Gutman, 1963).

Many drugs are typified by high lipophilicity and low water solubility. The partition of a drug between organic phase and aqueous, protein containing phase has been used to estimate binding of such compounds, where ligand is initially dissolved in the organic solvent. Binding may be estimated from partition co-efficients in the presence and absence of protein. The interaction of BSA with various organic ions (Karush, 1951), antibiotics (Scholtan, 1963), steroids (Schellman et al., 1954; Alfsen, 1963), and fatty acids (Goodman, 1958a, b; Ashbrook et al., 1975; Spector et al., 1969, 1971) has been examined in this way. Some caution ought to be exercised before placing great confidence on results from partition experiments since the organic phase may interact with the protein, causing a change in conformation (Alfsen, 1963) and protein is very susceptible to unfolding or denaturation at organic/aqueous interfaces (Steinhardt and Reynolds, 1969). A dynamic partition technique has been introduced recently (Robertson and Madsen,
1974) which is related to normal partition methods in the same way that
dynamic and equilibrium dialysis techniques are related. The partition
of ligand between a liquid, protein phase and an agar medium, containing
growing bacteria, has been used to study binding (Javidan and Mrtek, 1973a, b,
1975) with apparently better precision than an equivalent chemical method.

For the analysis of drug binding in vivo several methods have been
suggested to give good estimations of plasma free drug concentration.
For drugs which are not actively transported, a good index of free drug is
the concentration found in protein-poor body fluids such as cerebrospinal
fluid (CSF) or saliva (Koch-Weser and Sellers, 1976a). A good agreement
has been found between unbound diphenylhydantoin in plasma and the
concentration in CSF (Lund et al., 1972) or saliva (Bochner et al., 1974)
in man. An in vivo dialysis method, where a small cellulose dialysis
sac was implanted in the peritoneal cavity of rats, has been described by
McQueen (1968). It was found, for the long acting drug sulphormethoxine, that
the equilibrium sac concentration was close to the plasma free concentration.
The method has been used to study the displacement of sulphomethoxine by
phenylbutazone (McQueen, 1969) and the binding of salicylate, with its
effect upon plasma tryptophan (Wilson, 1974).

1:7:7 SPECTROSCOPY - GENERAL

The preceding methods can supply accurate estimations of the concen-
tration of bound or free ligand, but generally these techniques do not give
any information on the nature of the ligand–protein complexes. The
characteristic interactions of light with ligand or protein may well be altered
by binding, and thus spectroscopic techniques can provide detailed information concerning the character of the complex. Quantitative measurements of binding can also be made using optical methods, but calculations generally involve several assumptions which may lead to greater errors than are present in data from non-spectroscopic methods. The following authors have contributed review type articles of the role of optical methods in drug-protein interactions: Steinhardt and Reynolds (1969), Chignell (1969, 1971, 1972 a, b, 1973, 1974) and Bridges and Wilson (1976).

1:7:8 ABSORPTION SPECTROSCOPY

The absorption of light due to the electronic transitions of proteins and drugs is typically in the ultraviolet (UV) region of the spectra (200 nm - 380 nm), although some drugs do absorb visible light. The characteristic absorption of free drug or protein may be changed profoundly when bound, but the absorption of the protein is from such a complex mixture of transitions that interpretation of changes may be difficult. Gross changes in protein UV spectra are generally thought to reflect major conformational changes, unfolding or denaturation (Polet and Steinhardt, 1968; Steinhardt and Reynolds, 1969). Changes in ligand absorption may be more easily understood and have been most informative in many protein binding studies.

Several types of electronic transition give rise to absorption in the UV region; saturated hydrocarbons contain only strongly bound \( \sigma \) electrons which require a large input of energy for excitation to \( \sigma^* \) antibonding orbitals, equivalent to absorption in the far UV. Unsaturated compounds containing double or triple bonds or an aromatic system contain \( \pi \) electrons
which may be excited to a $\pi^*$ anti-bonding orbital. $N - \pi^*$ transitions
are of an electron from a non-bonding atomic orbital (lone pair) into a $\pi$
 antibonding orbital, which occurs in molecules containing a double bond
between heteroatoms. The final type of transition is less important, since
it only occurs in saturated systems with bromine, sulphur or nitrogen atoms,
that is the $n - \sigma^*$ transition from non-bonding to $\sigma$ antibonding. If portions
of the absorption spectra of a ligand can be ascribed to one of these transitions
then changes with binding may indicate which parts of the molecule are most
involved in the interaction. By measuring the extinction coefficients of
bound and free ligand quantitative data can be generated, providing the
extinctions can be proven to obey Beer's law (Steinhardt and Reynolds, 1969).
Klotz (1960 and Klotz et al. 1946) has used this approach to estimate the
binding of various azo dyes to BSA, which is typified by a marked reduction
in dye absorption around 500 nm.

Studies of changes in ligand absorption with binding are often accompanied
by measurement of the ligand’s spectra in solvents of various polarity in
order to mimic the movement of ligand into the hydrophobic binding site
(Laurence, 1952). In this way, it has been shown that HABA (2-[4'hydroxy-
phenyazo] benzoic acid) binds to a hydrophobic area of BSA (Baxter, 1964)
and the dye has subsequently been used as a 'spectrophotometric probe'
in binding studies with sulphonamides (Morigushi et al., 1968), sulphonyl-
ureas and phenothiazines (Zia and Price, 1975), tetracyclines (Zia and Price,
1976) and L-thyroxine (Morigushi et al., 1974).

Most of the ligands mentioned thus far have absorption maxima in the
visible region, far from the absorption of the protein. Many drugs, however,
absorb light in the same region as proteins. In these cases spectral modifications may be very small in comparison to the total absorption. Under such circumstances difference spectroscopy, using tandem cells, has been employed. Herskovits (1967) pioneered the use of tandem cells in which protein and ligand are separated in the reference cell, but combined in the sample cell. The difference spectra produced are often multiphasic reflecting modification of both protein and ligand spectra with binding. Tandem measurements have been used in investigations of the albumin binding of phenylbutazone (Chignell, 1969a), flufenamic acid (Chignell, 1969b), phenothiazines (Huang and Gabay, 1974), bilirubin (Krasner, 1973) and steroids (Ryan, 1968).

1:7:9 FLUORESCENCE SPECTROSCOPY

The loss of energy from an electron, excited due to the absorption of light, may occur by any one of several possible mechanisms (see fig. 1:5). Fluorescence is the phenomenon which is observed when light is re-emitted by a molecule when an electron returns to the ground state from the first excited singlet state. Absorption of UV light normally results in the excitation of an electron to one of the vibrational sub levels of the first excited state, the vibrational energy being rapidly lost by radiation-less processes before light is re-emitted. Since energy is lost previous to emission the fluorescence is always at a longer wavelength than absorption. The fluorescence of any molecule is typified by the wavelengths of maximum excitation and emission, the quantum yield, the fluorescence lifetime and the fluorescence polarisation. These parameters are exquisitely sensitive to the environment of the fluophor which makes
Figure 1:5

Schematic state energy level diagram: S in singlet, T is triplet.
The $S_0$ state is the ground state and the subscript numbers identify individual states. Below is a sequential outline of the processes of concern with approximate lifetimes where appropriate.

- **Absorption**: $S_0 \rightarrow S_n$ (absorption)
- **Internal Conversion**: $S_n \rightarrow S_1$ (internal conversion)
- **Fluorescence**: $S_1 \rightarrow T_n$ (fluorescence)
- **Intersystem Crossing**: $S_0 + h\nu \rightarrow T_1$ (intersystem crossing)
- **Internal Conversion**: $T_1 \rightarrow S_0$ (internal conversion)
- **Phosphorescence**: $S_0 + h\nu \rightarrow S_0$ (phosphorescence)

(Modified from Becker, 1969)
fluorescence an ideal tool to probe ligand-protein interactions. (For a more detailed account of the theory of fluorescence and biological applications of the technique see Chen, 1972).

There are several possible approaches to the use of fluorescent spectroscopy in drug-albumin studies; since albumins are mostly class B proteins (i.e. they contain tryptophan; Weber, 1960) they are themselves fluorescent, and ligand binding may modify the phenomenon. The ligand under study may be fluorescent, the parameters of which may change with binding. Finally if the ligand is non-fluorescent and does not modify the protein's fluorescence the technique may still be used if ligand binding influences the fluorescence of a 'probe' molecule which is complexed with the protein. Chignell (1970b, 1972, 1973, 1974) has authored several review articles concerning fluorescence spectroscopy and protein binding.

a) Protein Fluorescence

Human Serum Albumin has one tryptophan residue, whilst the Bovine variety has two. The fluorescence of both proteins is due almost completely to the tryptophan residues with a small contribution from tyrosine and even less from phenylalanine. Enhancement of albumin fluorescence with ligand binding is rare, but quenching is relatively common and occurs because of one or two processes. If the environment of the fluophor, protein tryptophan, is altered, then the fluorescence characteristics may also be changed. Thus, conformational change, unfolding or partial denaturation could account for ligand induced fluorescence alterations. This process is thought less common than that of energy transfer between excited protein tryptophan and bound ligand (Chignell, 1972).
The process of energy transfer may be likened to the induction of vibrations in a tuning fork by the vibrations of a nearby tuning fork of the same frequency. Forster (1951) has shown that for energy transfer to occur the emission band of the donor molecule and the absorption band of the acceptor must overlap. Energy which is transferred in this way may either be lost by radiationless processes, or re-emitted at the emission wavelength of the acceptor molecule. The efficiency of energy transfer varies as a function of the distance between the two molecules, a fact which has been used to calculate the proximity of binding sites to albumin tryptophan (Chignell, 1970a, b, 1972). Energy transfer between warfarin and albumin tryptophan will be discussed further in chapter 3.

The quenching of albumin fluorescence by energy transfer has been used to quantify the binding of bilirubin (Chen, 1971), dicoumerol (Chignell, 1970a) and thyroxine (Steiner et al., 1966) whilst the quenching of albumin with steroid binding is thought to be due to conformational changes (Attalah and Lata, 1968). The influence of fatty acids on albumin fluorescence is considered in chapter 4.

**b) Ligand Fluorescence**

The binding of a fluorescent molecule to protein may result in the modification of one or more of the parameters of fluorescence. The change in fluorescence indicates a change in environment and in this way molecules can be used as fluorescent probes of their own binding sites. Drugs which are fluorescent and which change their characteristics upon binding include chlorpromazine (Teller et al., 1968), camptothecin (Guarino et al., 1973) and warfarin (Chignell, 1970a; Wilson, 1974). The fluorescence properties
of warfarin and other coumarin derivatives will be covered more fully in subsequent chapters. Changes in ligand fluorescence can be used to obtain estimates of the various binding parameters of the interaction (Chignell, 1972) but such values will not, of course, reflect any binding which does not affect the ligand fluorescence.

c) Fluorescent Probes

Any small molecule which undergoes a large change in fluorescent properties with binding to a macromolecule can be termed a fluorescent probe, including those drugs mentioned in the previous section. Fluorescent probes can be used to monitor changes in macromolecule structure or conformation, or to reflect competition of other ligands for the probe's binding sites (see Edelman and McClure, 1968 for a general review of the field). The earliest and probably still the most popular probes are the aminonaphthalenesulphonate derivatives such as 1-anilinonaphthalene-8-sulphonic acid (ANS), 2-p-toluidinyl naphthalene-6-sulphonic acid (TNS), and 5-dimethylaminonaphthalene-1-sulphonyl (dansyl) amino acid derivatives. These compounds have an increased quantum yield and a blue-shifted emission maximum when bound to protein or in other hydrophobic environments, compared to aqueous solution. ANS, especially, has been used in a great many studies of drug-albumin interactions, being displaced by drug in order to obtain an estimate of the drug's binding parameters (for example HSU et al., 1974). The fluorescence polarisation of the ANS-BSA complex has been used to determine the orientation of the probe when bound, and to gain insight to the topography of albumin binding sites (Anderson and Weber, 1969).
Several alternative probes, which, because of their differing structures may bind to sites other than those of ANS, have been introduced recently: kynurenin (Churchich, 1972); tetracyclines (Popov et al., 1971); bilirubin (Krasner, 1973); iprindole (Wilson, 1974); acridines (Ma et al., 1974) and sulphophthaleins (Nishikimi and Yoshino, 1972).

The suitability of the fluorescent probe approach for drug–albumin studies, with particular reference to coumarins and drug displacements is discussed at length in chapter 3.

1:7:10 CIRCULAR DICHROISM AND OPTICAL ROTATORY DISPERSION

Circular dichroism and optical rotation are two phenomena which occur when plane polarised light interacts with a solution of an optically active molecule. Even a brief explanation of the phenomena must be preceded by an account of the nature of polarised light. Electromagnetic radiation is composed of electric and magnetic fields oscillating at right angles to each other and in a plane perpendicular to the direction of propagation of the beam. Light may be collimated to produce radiation of one wavelength only and polarised so that the electric and magnetic vectors oscillate in single planes only, such light is illustrated in figure 1:7a. For further explanation only the electric vector will be considered, since it is this wave with which electrons exchange energy. Plane polarised light may be considered as the sum of two waves of circularly polarised light of opposite sign, the vectors of which are of fixed magnitude, rotate in opposite direction from the same centre of rotation and are exactly in phase (figure 1:7b). When such a beam enters an optically active solution
Figure 1:7 The polarization of radiation

a. Plane-Polarized Radiation. The direction of propagation is along the x axis. E and H are the electric and magnetic vectors respectively.

b. Circularly-Polarized Radiation. Only the electric vector is shown. By convention, this is right circular polarization, since an observer at point P would see the field rotating in a clockwise direction.

c. The same circularly-polarized wave as shown above, represented as the sum of two plane-polarized waves.
the left and right components may be retarded differentially, which results in an emergent ray of the same circularly polarised components but now out of phase. The resultant of these two is a plane polarised beam emerging at an angle to the entering beam (see figure 1:8b). This type of activity is known as optical rotation (OR) and a scan over different wavelengths is termed the optical rotatory dispersion (ORD) of a sample. Note that ORD is due to different refractive indices for left and right circularly polarised light and may thus be observed at wavelengths where the molecule does not absorb light. If the two circularly polarised beams are absorbed differentially then the emergent ray will be elliptically polarised as shown in 1:8c. This second phenomenon is known as a circular dichroism (CD) and since it is an absorptive process, it can only be observed at wavelengths where the molecule absorbs light. The combination of unequal absorption (CD) and unequal velocity of transmission (OR) of left and right circularly polarised beams (as in fig. 1:8d) at the wavelength region, where an optically active molecule absorbs light is called a "cotton effect". (For a detailed treatise on the theories of optical activity see Lowry, 1964). The applications of CD and ORD to problems in pharmacology have been reviewed by Chignell (1968, 1970; Chignell and Chignetl, 1972).

The optical activities of proteins are the result of a great number of chromophores including helical portions of the structure, and thus CD and ORD spectra are complex and difficult to interpret. Any small changes which may result from ligand binding are equally difficult to interpret but such measurements have been used to examine the interaction of proteins with azodyes (Markus and Karush, 1958), detergents (see review by Perrin
A. Plane polarized light, showing the electric vector (E) as two rotating vectors (E_L and E_R).

B. The rotation (α) of light by an optically active medium. Note that E_L and E_R are the same relative magnitude.

C. The ellipticity (ψ) of light due to an optically active medium, at a wavelength where light is absorbed but rotation is zero. E_L and E_R are in phase.

D. Optical rotation and dichroism of light.
and Hart, 1970), and some small organic molecules (Helmer et al., 1968). Some drugs, especially those of natural origin, are optically active and ORD/CD may be used to study their interactions with proteins, but the vast majority of drugs are not optically active. Nevertheless the latter group of compounds form the bulk of molecules whose binding to proteins have been studied using CD/ORD.

This paradoxical situation arises because a symmetrical (non-optically active) molecule may be perturbed upon binding by an asymmetric centre of the protein to give rise to induced or extrinsic optical activity. Many small molecules, particularly those with a high affinity, produce such extrinsic effects with binding to albumin. CD measurements of these complexes can be used to quantify the binding (Ikeda and Hamaguchi, 1969; Rosen, 1970) and may provide detailed information on the nature of the complex. The binding of the following drugs to albumin has been studied using CD or ORD: phenylbutazone (Chignell, 1969a), flufenamic acid (Chignell, 1969b), dicoumerol (Chignell, 1970; Perrin and Idsvoog, 1971), warfarin (Chignell, 1970; Perrin and Nelson, 1972), sulphoethidole (Kostenbauder et al., 1971) and benzodiazepines (Muller and Wollert, 1974, 1975a, b, 1976). The use of CD to study coumarin-albumin interactions is expanded in chapters 5 and 6.

1:7:11 **MAGNETIC RESONANCE TECHNIQUES**

Nuclear magnetic resonance (NMR) and electron spin resonance (ESR; syn. electron paramagnetic resonance, EPR) are two closely related techniques which are playing an increasingly important role in studies of ligand-macro-
molecule interactions. Both techniques are based upon the fact that some charged sub-atomic particles have a spin and an associated magnetic field. In the presence of an external magnetic field such particles align in the same direction as the external field, but they can, with the absorption of characteristic quantities of energy 'flip' to be aligned in the opposite direction. It is the absorption of energy and the subsequent relaxation to the normal state which are studied in magnetic resonance techniques.

ESR is concerned with the resonance of unpaired electrons such as those in transition metals and free radicals. Since there are few natural examples of unpaired electrons the stable spin probe has been used in many studies of biological systems. Few free radicals are stable under biological conditions, but the nitroxide radical \( \text{R}^1 = \text{N} - \text{O} \) is stable in aqueous solution at neutral or alkaline pH. In studies of ligand-protein interactions the spin probe can be complexed with the protein, or may be part of the ligand. Changes in the ESR spectrum with binding are interpreted as either an increase or a decrease in the degree of immobilisation of the probe. Triazine labelled BSA (Blanchard et al., 1973, 1975) has been used in binding studies with various anionic compounds and a series of tricyclic antidepressants. The acidic drugs were thought to cause a partial unfolding of albumin, reflected by a conversion of 'strongly immobilised' probe to a 'partially immobilised' state. The basic drugs were found to produce the opposite shift with binding. A similar approach has been used to study surfactant binding to BSA (Oakes, 1973b). The alternative variation, having a labelled ligand, has been used by Lagercrantz and Setaka (1975) in a study of HSA binding of a nitroxide derivative of stearic acid.
In NMR, the particles of interest are atomic nuclei with non-zero spin. Since the nuclei $^1\text{H}$, $^2\text{H}$, $^{13}\text{C}$, $^{14}\text{N}$, $^{17}\text{O}$ and $^{31}\text{P}$ are all members of this group, NMR has a far wider application to biological problems than ESR. Proton magnetic resonance (PMR) is the most common form of NMR, at least in regard to biological studies, since the other nuclei are less abundant in nature and enrichment is necessary to yield detectable signals. The application of NMR to pharmacological problems has been well reviewed; Burgen and Metcalf, 1970; Fisher, 1971; Hollis, 1973 and Sykes and Hull, 1973. PMR is a most useful tool in ligand-protein interactions since the various protons of a ligand give characteristic signals in the spectrum and the changes upon binding can give a direct indication of which portions of the ligand are involved in the interaction. The application of PMR to drug-albumin studies was pioneered by Jardetsky and co-workers, with their studies of the interaction of BSA with Penicillin G (Fisher and Jardetsky, 1965) and sulphonamides (Jardetsky and Wade-Jardetsky, 1965). Subsequently, the interaction of albumin with diphenylhydramine (van der Vlies, 1970); acetylsalicylic acid (Sykes, 1970); parasympathicolyltics (Wiegand, 1975); surfactants (Oakes, 1973) and the renal contrast medium atrizoate (Rodrigues De Miranda and Hilberes, 1976) have been examined using the PMR technique.

Some of the disadvantages of NMR for drug-protein studies will be discussed in chapter 6.
From the preceding introduction it can be said that there are two easily discernable facets of the drug protein binding field, clinical aspects and molecular aspects, neither of which are near to being completely understood. The clinical aspects are tremendously important for the determination of correct drug dosage and regimen, and in predicting drug interactions at the binding level. Many more in vivo type experiments ought to be performed to increase our understanding of the pharmacokinetic consequences of plasma and tissue binding. It may be argued that the clinical situation will never be fully understood until the 'microscopic' nature of drug-protein interactions is well documented. Studies of the molecular character of ligand-albumin complexes may be justified on that basis alone, but these interactions are worthy of investigation in their own right, without any extenuating arguments.

Albumin is a truly remarkable protein. How is it possible for a single macromolecule to interact with a myriad of synthetic and natural compounds and yet have apparently specific binding sites for virtually each one? Clues to the answer of this question must be sought from detailed analyses of ligand-albumin complexes. It is hoped, that in this way, the present work will contribute to a final solution of the paradox.

Many of the compounds for which albumin has the highest affinity are amphiphiles, that is to say they have both hydrophilic and hydrophobic portions, and furthermore, the majority are acidic in nature. Several previous
reports of competition between various organic acids for albumin binding sites have suggested that these compounds may bind to only a few areas of the protein. With these two observations in mind the binding of a series of medium chain fatty acids and a series of coumarin drugs to albumin, and their mutual interaction have been thoroughly studied.

The fatty acids form a homologous series with constant, ionic, carboxyl groups and a range of hydrophobic, hydrocarbon chains. A good knowledge of their binding, obtained from ultrafiltration experiments at differing temperatures, should provide some insight into the relative contribution of ionic and hydrophobic interactions in the formation of the complexes. The influence of fatty acid binding upon protein structure was studied by measurement of protein fluorescence.

The coumarin anticoagulants are a clinically important group of drugs which are typical of the organic acid type compounds avidly bound by albumin. The binding of a series of coumarins was examined using the circular dichroism technique which is highly sensitive to molecular geometry and ideally suited to studies of the relationship between ligand and binding site. The enantiomers of two coumarins, warfarin and phenprocoumon, and their binding to albumin were studied in order to assess the influence of steric factors upon binding.

Previous studies in our laboratories (Wilson, 1974) have shown that warfarin is a suitable fluorescent probe for albumin binding sites. This approach was extended and phenprocoumon was also examined as a possible probe. The competition of fatty acids and coumarins for binding sites was
examined using both the fluorescence and ultrafiltration techniques.

In addition to the continued examination of fluorescence measurements for their suitability in drug-albumin studies, several other binding methods have been evaluated. The commercially available versions of ultrafiltration (Centriflo), diafiltration and equilibrium dialysis (Dianorm) are compared with other well-tested techniques.
CHAPTER TWO

MATERIALS AND METHODS
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2:1 MATERIALS

2:1:1 CHEMICALS

Albumins

Unless otherwise specified, Bovine Serum Albumin refers to Cohn fraction V BSA, powdered, from Sigma Chem. Co., St. Louis, Mo., U.S.A., lot 24C - 1740, 96 - 99% albumin, remainder mostly Globulins, 15.6% nitrogen content. Similarly, Human Serum Albumin refers to Albumin Kabi, lyophilized powder from ABKabi, Stockholm, Sweden, lots 30299 and 49641, 98% of total protein as albumin. In certain spectroscopic studies BSA and HSA were purchased from Behringwerke AG, Marburg, Germany, 100% pure by electrophoresis, batch No's A821 and 4729 respectively.

Drugs

The drugs used were obtained from the following sources: Warfarin (anhydrous), Sigma Chemical Co.; Phenylbutazone, Biorex Laboratories Ltd., London; Phenprocoumon, Roche Products, Welwyn Garden City, Herts; Acenocoumarin and Coumachlor were gifts from Professor E. Jähnchen, Dept. Pharmacology, University of Mainz, Germany; Sulphamethoxazole was donated by Burroughs-Wellcome, Beckenham; the enantiomers of warfarin were gifts of Dr. A. Breckenridge, University of Liverpool, England; Salicylic acid, (> 99.5%) from BDH Ltd.

All fatty acids were purchased from B.D.H. Ltd., Poole, Dorset either standard grade, 98 - 101% by acidimetric assay, or specially pure, 99.5%
where available. All fatty acids were re-distilled before use. Ethyl carbamate was obtained from BDH, octyl carbamate was synthesised by the Chemistry Division of the Chemical Defence Establishment, Porton Down, Wiltshire. Radiolabelled fatty acids were purchased from The Radiochemical Centre, Amersham as the \(^{1-14}\text{C}\) labelled sodium salts. Radiolabelled carbamates were kind gifts of Mr. S. Wood and were produced by custom synthesis at the Chemical Defence Establishment.

Scintillant materials were purchased from Packard Instrument International, Zurich, Switzerland. All other reagents were of general or analytical grade, normally from BDH Ltd. Regenerated cellulose Visking Tubing, 18/32" was obtained from The Scientific Instrument Centre Ltd., London. Spectrapore membrane tubing was donated by M.S.E. Ltd., Crawley, Sussex. Diaflo membranes were purchased from Amicon Ltd., High Wycombe, Bucks.

2:1:2 ANIMALS

Hepatic microsomes were prepared for binding experiments from male Wistar-albino rats (Porton strain) and male Syrian hamsters. The source of the animals, their diet and housing conditions were all as described by Wilson (1974).
2:2 METHODS

2:2:1 GENERAL

Unless otherwise stated, experiments were carried out using phosphate buffer 1/15 M (67 mM) at pH 7.4, which was prepared from solutions of NaH$_2$PO$_4$:2H$_2$O and Na$_2$HPO$_4$:12H$_2$O in glass distilled water. Albumin solutions were prepared by allowing the protein to dissolve overnight at a temperature of 4°C. Both BSA and HSA were assumed to have a molecular weight of 69,000, an overestimation by some 3% (M.W. calculated from amino acid composition BSA = 66,210 HSA = 66,248, Peters, 1975) which compensated for the approximate 3% impurities in the various albumin preparations.

2:2:2 ULTRAFILTRATION

In all studies, ultrafiltration was achieved by centrifugation, using one of two techniques. Routinely, ultrafiltration was performed through dialysis tubing membranes supported in a Toribara type apparatus (Toribara et al., 1957). For comparative purposes the commercial ultrafiltration system Centriflo (Amicon Corp., High Wycombe, Bucks.) was also used.

a) Toribara Apparatus

As illustrated in figure 2:1 the Toribara-type ultrafiltration apparatus consisted of a 50 ml polypropylene centrifuge tube (M.S.E. Ltd., London, England) into which a glass tube with scintered disc was placed. The glass tubes contained a scinter of porosity grade one and were manufactured
Figure 2: The two types of ultrafiltration apparatus used in this work.

Toribara apparatus

- Rubber cap
- Cellulose bag
- Glass Toribara tube
- Incorporating Scinter disc (Grade 1)
- Polypropylene centrifuge tube (50 ml)

Centriflo apparatus

- Diaflo membrane, laminated to paper support
- Polyethylene support
- Centrifuge tube (50 ml)
by the University glassblowers. Scinters which became discoloured or were suspected of being contaminated in any way were cleaned using the violent oxidative reaction between fuming nitric acid and methonal.

Visking (regenerated cellulose) dialysis tubing (18/32" Scientific Supplies Co. Ltd., London, England) was used throughout all experiments and was prepared in the following fashion: surface contamination was removed by several washes of the visking strips (~22 cm) in tap water. Strips were then boiled in distilled water for one hour followed by several rinses with distilled water. This process was found to be sufficient to remove the sulphorous compounds which contaminate visking tubing. During some portions of the work an organic wash was incorporated at this stage, but since the process was wasteful of methanol and gave no observable benefit it was discontinued in later studies. Following rinsing, visking strips were stored overnight in distilled water then transferred to phosphate buffer (1/15 M, pH 7.4) prior to use. The ultrafiltration procedure took the following routine: drug/albumin solutions (6.1 ml) were transferred using either a syringe or a disposable pasteur pipette, to a visking bag, formed by careful knotting of one end of a 22 cm visking strip. As much air as possible was excluded from the bag then the bag was sealed with a second knot. The bag was folded into a 'U' shape and placed on the scintere disc of the glass tube inside a centrifuge tube. The centrifuge tube was capped to prevent drying of the visking bag, and was ready for centrifugation. Gentle treatment of the cellulose tubing was necessary at all times to prevent the formation of minute holes in the bag. The assembled centrifuge tube was centrifuged in an MSE Mistral 6L centrifuge (using the 6 x 1L swing out rotor with 4 x 50 ml rubber adaptors) at 2,000 g
(av) (2,200 r.p.m.) for one hour at thermostatically controlled temperatures from 4°C to 37°C. After centrifugation, glass tubes were removed from the apparatus, bags were checked for signs of leakage and duplicate samples (0.1 ml) of ultrafiltrate were taken for drug analysis.

In all experiments, tubes were included which contained only drug solution, in order to check for loss in the system. An overall recovery of 95% was deemed the minimum acceptable level.

b) Centriflo

The components of the Centriflo system are shown in figure 2:1. A Diaflo membrane (Amicon Ltd., High Wycombe, England) is formulated into a cone which is supported by a plastic support, this in turn fits into a graduated centrifuge tube. Diaflo membranes were soaked overnight in distilled water, then after removal of excess liquid using paper tissues, the membranes were centrifuged for ten minutes in an MSE Mistral 6L centrifuge (using 6 x 1L swing out rotor with 4 x 50 ml rubber adaptors) at 1,000 r.p.m. in order to remove further liquid. Drug-albumin mixtures (3 ml to 5 ml) were pipetted into the dried cones and centrifugation as before at 1,000 r.p.m. at 20°C or 37°C, for 5 minutes was found to be enough to remove ~ 10% total volume as filtrate. Samples of filtrate were then taken for drug analysis. Cones were cleaned for re-use using the following procedure: after emptying, the cones were rinsed several times with 5% saline solution followed by washing for 3-4 hours in circulating tap water. The cones were then rinsed thoroughly in distilled water before storage in 10% ethanol.
Figure 2:2 The arrangement of apparatus for the continuous ultrafiltration procedure.
DIAFILTRATION

The diafiltration (syn. continuous ultrafiltration) apparatus used in this study is illustrated in figure 2:2. The binding of Sodium Salicylate and Sulphamethoxazole to BSA was examined using this technique. A brief consideration of the theory of continuous ultrafiltration will aid the understanding of the practical routines.

A diafiltration cell is, simply, a cell bounded by a membrane which is permeable to solvent and ligand but impermeable to macromolecules. A solution of ligand is passed into the constant volume cell at such a speed that it equilibrates with the macromolecule. Since the cell has a constant volume, continuous addition of ligand solution results in the production of an ultrafiltrate through the membrane, which is macromolecule free and may be collected in fractions and analysed for free ligand concentration.

The ultrafiltration of a well stirred, constant volume sample is readily amenable to mathematical analysis. Blatt (1968) has provided a comprehensive series of mathematical models to describe such situations. The treatment below best suits the conditions used in this work.

Consider the following system:-
The amount of ligand inside the cell, \( A = C \cdot V \). If the cell contains no binding macromolecule then \( C_1 = C \) and for a small addition from the stock:

\[
dA = C_0 \cdot dv - C \cdot dv
\]

but \( A = C \cdot V \), so:

\[
VdC = dv (C_0 - C)
\]

rearranging and integrating:

\[
\int_0^C \frac{dC}{C_0 - C} = \int_0^V \frac{dv}{V}
\]

\[
[- \ln (C_0 - C)]_0^V = \left[ \frac{V}{V} \right]_0^V
\]

putting in the limits:

\[
- \ln \frac{C_0 - C}{C_0} = -\frac{V}{V} \quad \text{EQU 1}
\]

or:

\[
\frac{C_0 - C}{C_0} = e^{-\frac{V}{V}}
\]

\[
\therefore \quad C = C_0 (1 - e^{-\frac{V}{V}}) \quad \text{EQU 2}
\]

From equation 1 it is apparent that a graph of \( \ln C_0 - C \) against \( v \) (volume of ultrafiltrate) will have a slope of \( -\frac{1}{V} \) an intercept of \( C_0 \) and will, of course, be a straight line. The linearity of such a graph and the value of the experimental cell volume \( (V) \) are useful checks that there are no anomalies in the system. Binding of ligand to the apparatus, for example would yield an experimental cell volume larger than the actual cell volume. (Note that this graph is constructed from an experiment with no macromolecule present).

It is reasonable that if the ligand concentration is zero at the start of the experiment, then:
Amount Added = Amount Inside + Amount Eliminated

or: \( C_0 \cdot v = CV + \int_0^v C \cdot dv \) EQU 3

This is fully compatible with previous calculations and may be checked as follows:

From equ. 2
\[
\int_0^v C \cdot dv = \int_0^v C_0 \left( 1 - e^{-\frac{v}{V}} \right) \cdot dv
\]
or: \( \int_0^v C \cdot dv = \left[ C_0 \cdot v + C_0 \cdot V \cdot e^{-\frac{v}{V}} \right]_0^v \)
i.e. \( \int_0^v C \cdot dv = C_0 \cdot v + C_0 \cdot V \cdot \left( e^{-\frac{v}{V}} - 1 \right) \)

but from equ. 2 again:
\( C_0 \left( e^{-\frac{v}{V}} - 1 \right) = -C \)

\( \int_0^v C \cdot dv = C_0 \cdot v - C \cdot v \)
or: \( C_0 \cdot v = C \cdot V + \int_0^v C \cdot dv \) i.e. EQU 3

If some drug is bound, equ. 3 is modified

\( C_0 \cdot v = V(C_f + C_b) + \int_0^v C_f \cdot dv \)

which rearranges to:
\[
C_b = \frac{C_0 \cdot v}{V} - C_f - \frac{1}{V} \int_0^v C_f \cdot dv \quad \text{EQU 5}
\]

\( C_b \) is the value one wishes to calculate, \( \frac{C_0 \cdot v}{V} \), \( C_f \) and \( \frac{1}{V} \) are all known, the final term may therefore be calculated from a graph of \( C_f \) against \( v \):

\[ \text{The lag volume only applies to the volume collected, not added.} \]
\[ \text{i.e. } V_i = \text{volume collected minus the lag volume.} \]
by the trapezoidal rule, if \( x \) = no. of fractions collected

\[
\text{Area} = \sum_{i=0}^{x} \frac{1}{2} (V_{i} + 1 - V_{i}) \cdot (C_{fi} + C_{f(i+1)}) = \int_{0}^{V} CF \cdot d
\]

Thus, from a knowledge of the stock ligand concentration \( C_{o} \), the cell volume \( V \), the ligand flow rate and the free ligand conc. \( C_{f} \) in each fraction of ultrafiltrate the concentration of bound ligand \( C_{b} \) may be calculated.

From this theory it should be obvious that two types of experiment are performed for each ligand under study, one with protein in the cell and one without. Both experiments follow the same procedure.

A stirred ultrafiltration cell (Model 12 - Amicon Ltd., High Wycombe, England) was used with a 'Diaflo' polyolefine membrane (PM 10, Amicon). Ligand was pumped with a constant flow pump (Labatron, Munich, Germany) and fractions were collected using an L.K.B. Ultrorack 7000 fraction collector (L.K.B. Instruments Ltd., South Croydon, Surrey). The apparatus was arranged as in figure 2:2 with all connecting tubing in Teflon.

Preliminary experiments were performed to determine the exact internal cell volume, the protein retentivity of the membrane and to check the accuracy of the pump and fraction collector. All solutions were degassed before use in order to prevent air bubbles forming in the pump or cell. The pump was equilibrated with stock ligand solution for 30 mins before each experiment. Membranes were soaked overnight in distilled water to remove the glycerol protective coating, then equilibrated with buffer by gentle stirring for one hour prior to use.
The cell was assembled ensuring that the supporting disc below the membrane and the outlet tubing were full with buffer to eliminate any dead space. The cell was then completely filled with buffer or protein solution and connected to the pump. The cell was stirred whilst ligand was pumped into the cell at a rate of $\sim 3$ ml/hr$^{-1}$ and fractions of ultrafiltrate were collected each 10 mins. Experiments were continued for 8 - 10 hrs, allowing about two cell volumes of ligand solution to be added. Aliquots of each fraction, the cell contents and the stock ligand solution were taken for drug analysis.

2:2:4 EQUILIBRIUM DIALYSIS

The binding of Sodium Salicylate to BSA was studied using the Dianorm equilibrium dialysis system (MSE, Crawley, England) in order to evaluate the apparatus. The system is based upon the design of Weder (Weder and Bickel, 1970; Weder et al., 1971), and consists of up to 20 dialysis cells which can be rotated at a constant speed in a temperature controlled water bath.

The cells are made in two halves which fit together, the membrane being sandwiched between them, slightly tensioned and clamped in the process. The shallow sample cavity is at the centre of each half cell and is separated from the neighbouring cavity by the membrane. Holes around the edges of the cells facilitate filling the cavities after assembly. The cells and stoppers are made of PTFE (Teflon) to minimise binding of ligands, and have an internal volume of 1.36 ml per half cell.
The apparatus was used in the following manner: Spectrapor 2 (M.W. cut off 12,000-14,000 - MSE Pub.No. 520) regenerated cellulose membrane was prepared as described in section 2:2:2 for Visking tubing. The membrane was dried with paper tissue before assembly of the cells. To one half cell 1 ml phosphate buffer (pH 7.4, 67 mM) was added using an automatic pipette (Oxford Labs.), whilst to the other half of the cell 1 ml of drug-protein or drug-buffer solution was added. Cells were stoppered and dialysis was carried out at 20°C with the cells rotating at 10 rpm. At the end of the dialysis period aliquots were removed from each half cell for drug determinations.

2:2:5 ULTRACENTRIFUGATION

Ultracentrifugation was used in an attempt to quantify the reversible binding of ligands to the microsomal fraction of liver homogenates. The method of centrifuge sedimentation used was basically that of Bickel and Steel (1974) which was in turn a modification of that of Ernster and Orrenius (1965). Ligand was added to a microsomal preparation (about 1.5 mg protein.ml⁻¹, prepared as described in section 2:2:13) at various concentrations and allowed to equilibrate for ten minutes at 4°C. Suspensions were then transferred to centrifuge tubes (10 ml - MSE) and centrifuged at 100,000 g.av. for 1 hr at 4°C (in the MSE Super Speed 50, at 40,000 rpm using a 10 x 10 ml fixed angle rotor). After centrifugation aliquots of the particle free supernatant were taken for drug analysis.
2:2:6 ABSORPTION SPECTROPHOTOMETRY

The visible and ultraviolet absorption spectra of various coumarins and their complexes with albumin were recorded using a Perkin-Elmer 356 double beam/double wavelength spectrophotometer (Perkin Elmer Ltd., Beaconsfield, England) coupled with an A3, flat-bed, X-Y recorder. The wavelengths of the apparatus were calibrated using an Holmium Oxide filter, and were found to be accurate to ± 0.2 nm over the total wavelength range. Spectra were recorded using conventional stoppered quartz cells (Helma Ltd., Westcliff-on-sea, Essex) at 25°C and 0 - 1 A units full scale. With final concentrations of albumins (BSA or HSA, Behringwerke, Marburg, Germany) at 3 x 10^{-5} M and coumarins 2 x 10^{-5} M in phosphate buffer (pH 7.4, 67 mM), the following spectra were recorded: 1, Buffer in sample and reference - if baseline not flat ± 0.01 A units material discarded; 2, Coumarin - Sample, Buffer-Reference-coumarin absorption spectra; 3, Albumin - Sample, Buffer-Reference-albumin absorption spectra; 4, Coumarin/Albumin - Sample, Buffer-Reference-Spectra of coumarin/Albumin complex; 5, Coumarin/albumin - Sample, albumin-Reference-spectra of bound coumarin; 6, Coumarin/albumin - Sample coumarin-Reference, - difference between bound and free coumarin, plus spectra of liganded albumin. (See figure 5:4 for an example of these spectra).

2:2:7 FLUORESCENCE SPECTROPHOTOMETRY

All fluorescence measurements were made using 1 cm quartz cells (Helma Ltd) in a Perkin-Elmer MPF 3 spectrofluorometer coupled to a
Perkin-Elmer QPD-33 Recorder (Perkin-Elmer Ltd., Beaconsfield, England) at a controlled temperature of $30^\circ\text{C} \pm 1^\circ\text{C}$ unless otherwise specified. Activation and emission slit widths of 5mm were used in all measurements. It should be emphasised that the fluorescence intensities and maxima reported in this work are uncorrected, except for dilution where applicable. Thus, because of lamp and photomultiplier variations and the phenomenon of self-absorption (inner-filter effect) results in this work are comparable with those obtained under similar conditions using the same equipment but not necessarily comparable with other data. Corrections which could have been employed, and the reasons for not doing so will be discussed in Chapter 3.

All results were obtained by measuring luminescence at an angle of 90° to the activation beam, spectra were recorded using a wavelength scan speed of 120 nm.min$^{-1}$ and a chart recorder speed of 12 cm.min$^{-1}$.

Fluorescence titrations took the following general procedure:-

2 ml of solvent (either buffer or protein solution) were pipetted into a 1 cm cuvette which was stopped using a suba seal stopper, mixed, and allowed to equilibrate to the cuvette holder temperature ($30^\circ\text{C} \pm 1^\circ\text{C}$). An initial spectra was recorded at the desired excitation wavelength. Fluorescence or quenching agent was then added through the Suba Seal using a 10 $\mu$l microsyringe (Terumo-Shandon, Camberley, Surrey or S.G.E., N.Melbourne, Australia) and a second spectra was recorded, superimposed upon the first. Subsequent additions were made in the same way, mixing being achieved by several inversions of the cuvette. Total additions were never more than 40 $\mu$l, which constitutes a 2% dilution. Addition
of up to 40 μl of buffer to 2 ml of fluorescent protein or probe solution produced changes in fluorescence insignificant in relation to the overall experimental error.

Fluorescence titrations of three types were performed:

a) Quenching of albumin fluorescence, in this case the fluorescence of 2 ml HSA or BSA (5 x 10^{-6} M to 5 x 10^{-5} M) was measured using an excitation wavelength of 290 nm and the effects of μl additions of quencher (1 x 10^{-3} M - 1 x 10^{-1} M) were recorded.

b) Fluorescence of probe molecules, for these experiments 2 ml of buffer (phosphate pH 7.4, 67 mM) or albumin (HSA or BSA 5 x 10^{-6} to 5 x 10^{-5} M) were titrated with probe solution (1 x 10^{-3} M - 5 x 10^{-3} M). The fluorescence of the probe was recorded with an activation wavelength of 320 nm. If applicable, the quenching of protein fluorescence could be monitored using the same sample.

c) Displacement of a fluorescent probe, in this instance a probe-protein mixture (generally a ratio of 1:1 and about 1 x 10^{-5} M) was titrated with increasing amounts of displacing agent. The fluorescence of the probe (excitation 320 nm) and, in some cases the protein (excitation 290 nm) were monitored.

2:2:8 CIRCULAR DICHROISM

Circular Dichroism measurements were made at 27°C with a Cary 61 CD spectropolarimeter (Cary, Monrovia, Calif., U.S.A.) which was calibrated using d-camphorsulphonic acid. Spectra were recorded using cylindrical quartz cells of 10 mm pathlength (Helma Ltd., Westcliff-on-Sea, Essex) with a full scale deflection of 0.05° and a spectral band width of 2 nm.
The CD spectra of albumin alone (3 x 10^{-5} M, HSA or BSA) or albumin plus ligand (6 x 10^{-5} M) were recorded in phosphate buffer (pH 6.6 to 8.2, 67 mM). A baseline of phosphate buffer alone was recorded regularly to check for changes in machine response. At least 3 different spectra were recorded and a mean spectra was produced manually by superimposition and drawing a mean by eye. Only very small deviations were seen between different spectra of the same sample. Difference spectra were obtained by subtracting the ellipticity for albumin from the drug-albumin spectra. This was performed from measurements every 5nm. In the case of enantiomers bound to albumin, the intrinsic optical activity of the isomer was also subtracted to give a 'double difference spectra'. The results are expressed as molar ellipticities \( \theta \) (deg.cm^{-2}.dmole^{-1}) which are calculated with respect to albumin concentration (3 x 10^{-5} M), total drug concentration (6 x 10^{-5} M) or bound drug concentration. In some cases anisotropy factors (g-values) were calculated, following the method of Chignell (1969), using the equation:

\[
g = \frac{[\theta]}{3300 \times \epsilon}
\]

where \([\theta]\) is the molar ellipticity of the drug bound (or in buffer for enantiomers) and \(\epsilon\) is the molar extinction coefficient of the drug bound (or in buffer for enantiomers).

2:2:9 **NUCLEAR MAGNETIC RESONANCE**

In a limited study of the binding of warfarin and phenprocoumon to BSA, the following conditions were used: drug (1 x 10^{-2} M) was dissolved initially in NaOD (30% in D_2O) then made up to volume in D_2O. Solutions of the same drug concentration were also prepared with BSA concentrations
of 1,2,3 and 4% (6.77 x 10^{-5} \text{ M} - 2.89 x 10^{-4} \text{ M}). NMR spectra were obtained at 40^\circ\text{C} using a Varian XL-100 instrument equipped with Fourier Transform (F.T.) facilities. Spectra were recorded from 50 transients using the F.T. system.

2.2.10 LIQUID SCINTILLATION COUNTING

Carbon-14 labelled ligands were used extensively throughout the work reported here, particularly in ultrafiltration experiments. In the early sections of the project the following counting conditions were used:- 0.1 ml of sample (aqueous) was added to 10 ml Bray's scintillant (Naphthalene 60 g.L^{-1}; PPO, 4 g.L^{-1}; dimethyl POPOP, 0.2 g.L^{-1}; Analar Methanol, 100 ml.L^{-1}; ethane diol, 20 ml.L^{-1} in 1:4 Dioxan) in glass scintillation vials. Radioactivity was measured using a Packard TriCarb 3320 instrument (Packard Ltd., Reading, Berks.). The bulk of the scintillation counting was, however, carried out as follows: 0.1 ml samples were transferred to polypropylene 'mini vials' and diluted with 0.4 ml distilled water. 4.0 ml of Toluene/Synperonic (BDH) scintillant was added (2:1 Toluene:Synperonic, 4 g.L^{-1} PPO) and shaken to form a clear gel. Vials were 'counted' using an LKB Wallac 1210 Ultrobeta liquid scintillation counter. A total of 20,000 counts were recorded (S.D. of counting 1.4%) or in the case of low activity samples 30 minutes counting time was used. The scintillation counter was programmed to automatically correct for quenching by the external standard channels ratio method. Efficiency of counting was normally 87-90%.
2:2:11 DRUG ASSAYS

a) Salicylate.

Sodium Salicylate was determined by a fluorometric method based on that of Oie (1971). 0.1 ml of Sodium Salicylate standard solution (10^-5 M) or sample was mixed with 4.9 ml 5N NaOH. Solutions were allowed to stand for 10 min before fluorescence was determined at an emission wavelength 395 nm with excitation at 320 nm.

b) Phenprocoumon and Warfarin.

Phenprocoumon and Warfarin concentrations in samples from dialysis binding experiments were measured using a modification of the method of Seiler and Duckert (1968) as described by Jähnchen et al. (1976). The method is based upon the fluorescence of the drugs in 0.5 M NaOH.

c) Sulphamethoxazole.

Sulphamethoxazole concentrations were measured using a modification of the Sulphonamide assay of Bratton and Marshall (1939). The procedure followed was as described by Rieder (1970).

2:2:12 RESOLUTION OF PHENPROCOUMON

Racemic Phenprocoumon was resolved into its enantiomers by fractional crystallisation using a modification of the method of West and Link (1965).

Quinidine (5.2 g, 16 mMoles) and rac.Phenprocoumon (8.9 g, 33 mMoles) were dissolved in a warmed mixture of chloroform (25ml) and acetone (50 ml). A first crop of phenprocoumon quinidine salt (5.2 g) was collected after
crystallisation had proceeded for 16 hours at 22°C. ([α]_D^20 + 92.2° C1 Butan-2-one). After holding the filtrate at -15°C for 16 hours a further 2.8 g of salt were collected. Both crops were re-crystallised twice from 1:4 (v/v) absolute ethanol-chloroform, yielding 3.71 g of phenprocoumon quinidine salt ([α]_D^20 + 76° C1 Butan-2-one).

S(-) phenprocoumon was recovered by shaking the quinidine salt with 25 ml each of chloroform and 5% NaOH, then acidifying the aqueous layer with HCl. The solid thus formed was re-crystallised twice from 70% ethanol-water to yield 1.21 g S(-) phenprocoumon. ([α]_D^25 - 123.7° C1 Ethanol). Overall yield 27%, m.p. 176-180°C.

The filtrates from the fractional crystallisation, plus those from the first recrystallisations of the two crops of S(-) phenprocoumon quinidine salt were combined then reduced to a glassy residue by rotary evaporation. Crude R(+) phenprocoumon was formed by alkali-chloroform partitioning and acidification as described previously. The crude R(+) phenprocoumon (5 g) and quinine were dissolved ethanol (90 ml), then warm water (35 ml) was added. This solution was held at -12°C for 48 hours yielding crude R(+) phenprocoumon quinine salt. This was recrystallised three times from 70% ethanol to give 3.4 g R(+) phenprocoumon quinidine salt. The R (+) phenprocoumon was recovered exactly as described for the other enantiomer. The final yield was 1.19 g (26%) R(+) phenprocoumon ([α]_D^25 + 124° C1 Ethanol, m.p. 176-180°C).
Hepatic microsomal fraction was prepared for studies using centrifuge sedimentation and for use in the examination of the binding spectra of coumarins. Microsomes were prepared as follows: male rats (~150 g) or hamsters (~50 g) were killed by cervical dislocation, livers were immediately excised, weighed and transferred to ice cold KCl (1.15% w/v). The gall bladder was removed in the case of the hamster. Livers were quickly and thoroughly scissor chopped, then well washed with several changes of ice cold KCl. All subsequent operations were carried out between 0°C and 4°C. Chopped livers were homogenised in Tris buffered KCl (66 mM Tris-HCl pH 7.4, 1.15% KCl - 3 ml per g liver) using a Potter-Elvejhem type, teflon-glass homogeniser at a speed of 2400 r.p.m. with four return strokes. The homogenate was centrifuged at 15,000 g av. (MSE High Speed 18, 8 x 5 ml rotor, 13,500 r.p.m.) in polypropylene centrifuge tubes for 20 min. The resulting supernatant was transferred to 25 ml polycarbonate centrifuge tubes and centrifuged at 105,000 g av (MSE Super Speed 50, 8 x 25 ml rotor, 40,000 r.p.m.) for 1 hour. The supernatant was decanted off, the surface of the pellet was washed with Tris buffered KCl and the microsomes were homogenised in 25 ml Tris buffered KCl for centrifugation as described above. The final pellet was either re-suspended in Tris buffer (66 mM, pH 7.4) for immediate use or suspended at about 10-20 mg protein.ml⁻¹ in SVT (0.25 M Sucrose, 0.4 mM EDTA, 20 mM Tris/HCl pH 7.4) and frozen at -40°C for storage.

Microsomal protein was determined by the method of Lowry et al. (1951)
and cytochrome p 450 concentrations by the method of Omura and Sato (1964).

Cytochrome p 450 - substrate induced spectra (binding spectra) were determined by a modification of the method of Schenkman et al. (1967).

4 ml or 5 ml of a microsomal suspension (1 - 2 mg protein.ml⁻¹) in Tris buffer (66 mM Tris-HCl pH 7.4) was equally divided between two quartz 10 mm cuvettes which were then stopped with Suba Seals. Using the primary cell compartment of the Perkin Elmer 356 spectrophotometer at 25°C and in the split beam mode a difference spectra between the two cuvettes over the range 350 - 500 nm was recorded. Any adjustments necessary to give a flat baseline were made. Substrate (normally 250 mM in DMF-dimethylformamide) was introduced into the sample cuvette using a 10 μl microsyringe (Terumo - Shandon) and equal amount of solvent was added to the reference cuvette. The resulting difference spectra was recorded superimposed upon the baseline. Further additions of substrate and solvent as described above were made in order to determine the kinetics of the cytochrome p 450 spectral interaction.

The spectral dissociation constant, Kₛ, and the maximal change A_max, were found using Lineweaver-Burk plots, as described by Al-Gallany (1975).

2:2:14 DATA ANALYSIS, STATISTICS, THERMODYNAMICS AND COMPUTER ANALYSIS

A study of the interaction of a ligand with a macromolecule will normally lead to a knowledge of the following terms: the concentration of the macromolecule (some conclusions can be made without this value), the total concentration of ligand, the concentration of bound and/or free ligand.
The problem still remains as to the way in which the data is analysed and presented.

The simplest, and still popular, method is to simply report the percentage of total drug or ligand bound. Without any further information such a figure is useless and often misleading, even if reported together with the total drug and protein concentrations, 'percentage bound' tells little of the nature of the interaction. A graph of percentage bound against total ligand (see fig. 2.3), still presented in some reports, shows merely that more drug is bound at lower concentrations. More useful parameters may be derived by considering the drug-protein interaction to obey the law of mass action. (Drug protein interactions are generally considered to be dynamic equilibria which can be treated in this way; Goldstein, 1949; Edsall and Wyman, 1958; Klotz and Hunston, 1971).

Consider the interaction of a drug molecule with a single site on the protein:

\[ P + D_f \xrightleftharpoons[k_2]{k_1} PD \]

where \( P \) = unliganded protein \( D_f \) = free drug \( PD \) = drug protein complex, \( k_1 \) and \( k_2 \) are the rate constants for the forward and reverse reactions

\[ K_A = \frac{[PD]}{[P][D_f]} = \frac{k_1}{k_2} \] EQU 1

\( K_A \) is the association constant, or more correctly apparent association constant since concentrations are always used rather than activities.

\[ K_D = \frac{[P][D_f]}{[PD]} = \frac{k_2}{k_1} = \frac{1}{K_A} \] EQU 2

\( K_D \) is the dissociation constant. Both the association and dissociation constants are referred to as equilibrium constants. It is convenient to
Figure 2:3 Graphical presentation of binding data. Two less useful plots.

These plots and those in figure 2:4 are generated from the following hypothetical binding data: (modified from Davison 1971).

<table>
<thead>
<tr>
<th>Free drug Df M</th>
<th>Total drug M</th>
<th>% Bound</th>
<th>r</th>
<th>$\frac{1}{r}$</th>
<th>$\frac{1}{D_f}$</th>
<th>$\frac{D_f}{r}$</th>
<th>$\frac{r}{D_f}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1 \times 10^{-5}$</td>
<td>$1.99 \times 10^{-5}$</td>
<td>50</td>
<td>0.099</td>
<td>10</td>
<td>$1 \times 10^5$</td>
<td>$1.01 \times 10^{-4}$</td>
<td>$1 \times 10^4$</td>
</tr>
<tr>
<td>$1 \times 10^{-4}$</td>
<td>$1.90 \times 10^{-4}$</td>
<td>47</td>
<td>0.9</td>
<td>1.1</td>
<td>$1 \times 10^4$</td>
<td>$1.11 \times 10^{-4}$</td>
<td>$9 \times 10^3$</td>
</tr>
<tr>
<td>$1 \times 10^{-3}$</td>
<td>$1.50 \times 10^{-3}$</td>
<td>33</td>
<td>5.0</td>
<td>0.2</td>
<td>$1 \times 10^3$</td>
<td>$2.00 \times 10^{-4}$</td>
<td>$5 \times 10^3$</td>
</tr>
<tr>
<td>$1 \times 10^{-2}$</td>
<td>$1.09 \times 10^{-3}$</td>
<td>8</td>
<td>9.0</td>
<td>0.11</td>
<td>$1 \times 10^2$</td>
<td>$1.11 \times 10$</td>
<td>$9 \times 10^2$</td>
</tr>
<tr>
<td>$1 \times 10^{-1}$</td>
<td>$1.0099 \times 10^{-1}$</td>
<td>1</td>
<td>9.9</td>
<td>0.1</td>
<td>$1 \times 10^1$</td>
<td>$1.01 \times 10^{-3}$</td>
<td>$9.9 \times 10^1$</td>
</tr>
</tbody>
</table>

Abbreviations explained in the text.
rearrange equation 1 in terms of $r$, the ratio of drug bound to total protein,

$$\frac{\text{moles drug bound}}{\text{total moles protein}} = \frac{[PD]}{[PD] + [P]}$$

from Eq 1

$$K_A [P][D_f] = [PD]$$

$$r = \frac{K_A [P][D_f]}{K_A [P][D_f] + [P]} = \frac{K_A [D_f]}{K_A ([D_f] + 1)}$$ \text{EQU 3}

Consideration of the equilibria between protein and ligand can take two forms from this point, either a site-oriented approach or a stoichiometric approach.

**Site Binding Constants**

The most common approach to binding equilibria assumes that the protein has a certain number of pre-existing binding sites to which a ligand can bind, this is the type of analysis used in this work. Equation 3 describes the interaction of drug with a single site. If there are $n$ of these sites on the protein, then the following equation will apply:

$$r = \frac{n K_A [D_f]}{K_A ([D_f] + 1)}$$ \text{EQU 4}

A simple graphical plot of $r$ against $D_f$ (fig. 2.3) is a hyperbola and is difficult to fit, however there are several transformations of equation 4 which will give a linear plot provided that: all the 'n' sites are equal in binding affinity and that the binding of one molecule does not affect the binding of subsequent molecules i.e. there is no co-operation or interactions between binding sites.

The reciprocal or Klotz (1946) plot is based upon the following translation of equation 4

$$\frac{1}{r} = \frac{1}{n} + \frac{1}{nK_A [D_f]}$$ \text{EQU 5}
Figure 2:4  **Graphical presentation of binding data**

Klotz or reciprocal plot  
\[ \frac{1}{r} \]

Scatchard plot  
\[ \frac{r}{D_f} \times 10^3 \]

Modified reciprocal plot  
\[ \frac{D_f}{r} \]

Three popular, linear translations of the hypothetical binding data shown in figure 2:3.
A graphical plot of \( \frac{1}{r} \) vs \( \frac{1}{[D_f]} \) has an intercept on the y axis of \( \frac{1}{n} \) a slope of \( \frac{1}{nK_A} \) and an x intercept of \( -K_A \). This plot suffers from the disadvantage of spreading low values of \( \frac{1}{r} \) very poorly, a similar plot, with the same limitations has been used in which \( \frac{[D_f]}{r} \) is plotted against \( D_f \) (Klotz, 1953 - see fig. 2.4).

The Scatchard (1949) plot is based upon the following form of equation 4:

\[
\frac{r}{[D_f]} = nK_A - rK_A \quad \text{EQU 6}
\]

where \( \frac{r}{[D_f]} \) is plotted as a function of \( r \), giving a y intercept of \( nK_A \), slope \( K_A \) and x intercept \( n \). (see fig.2.4).

More often than not, however, experimental data plotted as in fig.2.4 will not give a straight line. This could be the result of one or more of the following causes a) The binding sites are not independent e.g. there may be co-operativity in binding b) binding of ligand may induce a conformational change in the protein c) the ligand may bind in several different ways to the protein or finally d) binding sites are not equivalent.

Whilst the first three possibilities are very plausible reasons for curvature of a Scatchard or Klotz plot it is the fourth (d) which is generally thought to be responsible i.e. there exist two or more classes of site which each have their own \( n \) and \( K_A \) values. Thus considering the Scatchard plot only, equation 4 must be modified:

\[
r = \frac{n_1K_A[D_f]}{1 + K_A[D_f]} + \frac{n_2K_A[D_f]}{1 + K_A[D_f]} + \ldots + \frac{n_iK_A[D_f]}{1 + K_A[D_f]} \quad \text{EQU 7}
\]

up to the 'ith' class of site.
A theoretical Scatchard plot for a ligand with two classes of binding site.

The curve is a hyperbola with the following binding parameters: $n_1 = 2$, $n_2 = 7$, $K_1 = 4 \times 10^4$, $K_2 = 3 \times 10^3$. 
It is obvious then that the intercepts of a curved Scatchard Plot will not have the same simple values as the linear variety. If it is assumed that a curved Scatchard plot such as that in fig. 2.5 is due to the presence of two sets of binding sites then the curve may be resolved into two partial plots (straight lines) each representing one single set of binding sites which are the asymptotes of the curved line. The ways in which a curve may be fitted to experimental points and the subsequent derivation of binding parameters will be dealt with in a later section. Curved reciprocal and modified reciprocal plots will not be considered since the Scatchard plot was the method of choice for this work, however, Klotz and Hunston (1971) have dealt with those situations.

Stoichiometric Binding Constants

In contrast to the site binding concept, a stoichiometric approach considers the addition of each molecule of ligand to protein in a sequential manner. Thus the protein P is considered to interact with ligand D to form a species PD\textsubscript{1} which can then bind a second ligand molecule to form PD\textsubscript{2} etc. i.e.

\[
P + D \rightleftharpoons PD_1
\]
\[
PD_1 + D \rightleftharpoons PD_2
\]
\[
PD_2 + D \rightleftharpoons PD_3
\]
\[
PD_{1-1} + D \rightleftharpoons PD_1
\]

For these series of equilibria a series of stoichiometric equilibrium constants may be defined:

\[
K_1 = \frac{[PD_1]}{[P][D]} \quad [PD_1] = K_1 [P][D]
\]
The analysis of ligand-macromolecule equilibria in this way was first proposed by Klotz (1946 and Klotz et al., 1946) and has been used recently by several groups especially that of Spector, Ashbrook and Fletcher (see Spector et al., 1971; Fletcher et al., 1970) for the analysis of long chain fatty acid anion binding to albumin. Klotz (1974; Klotz and Hunston, 1975) has discussed at length the relationship between the site and stoichiometric approaches.

The stoichiometric analysis of binding data undoubtedly has certain advantages over the site orientated models. In particular, it does not assume a certain number of pre-existing sites and is therefore compatible with the formation or destruction of sites upon binding of ligand. The stepwise model is also sufficiently general to account for other types of co-operativity, electrostatic interactions and other phenomena which may be associated with drug-protein binding. As has been stated already the Scatchard model assumes that these phenomena are negligible. The Scatchard plot, however, produces parameters which are accurate enough for most purposes and generally these parameters may be compared with those from the literature since the Scatchard model is the most popular. Moreover the concept of, say, one primary class of sites and one secondary class, is easy to visualise and it is simple to equate displacement phenomena with such a situation.

In addition a stepwise analysis of data may require the calculation of

\[
K_2 = \frac{[PD_2]}{[PD_1][D]} \quad [PD_2] = K_1K_2 [P] [D]^2
\]

eetc..
ten or more separate equilibrium constants which must assume that the experimental data is highly accurate. In binding studies, where experimental error can be quite high, it seems more reasonable to restrict computations to the estimation of one, two or three association constants and numbers of sites. It is interesting to note that the calculation of stepwise equilibrium constants by the method of Fletcher et al. (1973) requires firstly a fit of the data to the Scatchard model to obtain initial estimates of the binding parameters.

From the various arguments presented above, coupled with the need for sophisticated computer facilities for convenient estimation of Stoichiometric equilibrium constants, it was decided to analyse the data of this work by the method of Scatchard. The limitations of this approach are, however, acknowledged.

**CURVE FITTING AND PARAMETER ESTIMATION**

Having chosen the Scatchard model to fit experimental binding data, it is necessary to consider how best to estimate the parameters from the data. This process can be carried out in two ways, either by plotting the data and physically determining the parameters from the curve, or by a statistical method, many of which are now computerised. The derivation of data from Scatchard plots by graphical means has been considered by several authors: Pennock (1973); Rosenthal (1967); Weder et al. (1974). Whilst statistical methods have been covered by Feldman (1972); Weder et al. (1973); Priore and Rosenthal (1976).

It is perhaps pertinent to point out a common misconception; consider the Scatchard plot as follows:-
Intercept 1

\[ r \div [D_f] \]

Intercept 4

Intercept 3

Intercept 2

It is common practice to draw in asymptotes at the extremities of the curve, as has been done here, to give the intercepts the following values:

1 = \( n_1 K_{A1} + n_2 K_{A2} \)  
2 = \( n_1 + n_2 \)  
3 = \( n_1 \)  
4 = \( n_2 K_2 \),

and to calculate the binding parameters from these quantities. This is, however, incorrect. Klotz and Hunston (1971) have derived the actual values, it is sufficient to say that whilst intercept 1 is \( n_1 K_{1} + n_2 K_{2} \) and 2 is \( n_1 + n_2 \), intercepts 3 and 4 do not represent simple values. It is necessary to shift these asymptotes, keeping the slope the same, until they are in a position such as illustrated in fig. 2.5 before a full estimation of the binding parameters can be made. It is the mechanism of this shift which is the crux of the graphic parameter estimation. Where computer analysis was not used in this work a graphic method based upon that of Berson and Yalow (1959) was employed.

The routine analysis of data was performed using a computer program devised by Dr. John Francis of Roche Products, to whom I am most indebted. The computer program is a general fitting program which uses a non-linear, weighted, least-squares regression procedure. This process minimises:

\[ \sum w_i e^2 = \sum \left( \frac{e^2}{\text{var}_e} \right) \]

where \( e = (y \text{ observed} - y \text{ calculated}) \) and \( \frac{1}{w} = \text{var}(y_{\text{obs.}} - y_{\text{calc.}}) \).

i.e. \( \sum \left( \frac{e^2}{\text{var}_e} \right) \) is minimised.
For the protein binding analyses, data is fitted to the Scatchard (1949)
equation:

\[ r = \frac{n \cdot K \cdot [D_f]}{K ([D_f] + 1)} \]

Since \( r = \frac{\text{concentration of bound ligand}}{\text{concentration of protein ([P])}} \) and bound = Total ([T]) - free ([D_f]),

the equation may be re-written:

\[ [T] = [D_f] + P \cdot \frac{n \cdot K \cdot [D_f]}{K ([D_f] + 1)} \]

Thus, for \( m \) classes of binding site, the program fits to the equation:

\[ [T] = [D_f] + \sum_{i=1}^{m} \frac{n_i \cdot K_i \cdot [D_f]}{K_i ([D_f] + 1)} + e \]

where \([T]\) is the dependent variable and \([D_f]\) is the independent variable.

\( \text{var}(y_{\text{obs}} - y_{\text{calc}}) \) is calculated each iteration from the current estimates of \( n_i \) and \( K_i \) and the entered values of S.D. \( (y) \) and S.D. \( (x_j) \) by means of:

\[ \text{var}(y_{\text{obs}} - y_{\text{calc}}) = \text{var}(y_{\text{obs}}) + \sum_{j=1}^{n} \left( \frac{A}{\Delta x_j} \right)^2 \cdot \text{var}(x_j) \]

where \( x_j \) is the independent variable \([D_f]\) and \( y \) is the dependent variable \([T]\).

This procedure has the most important advantage of weighting that region of \( n \) dimensional space according to the accuracy with which it is defined by the experimental observations \((y \text{ and } x_j)\). There is some doubt, however, concerning the derived S.D.'s of the coefficients. These may not have their normal statistical meaning since the errors in a least-squares procedure should only be associated with the dependent variable \((y)\). This in no way affects the accuracy of the fit, however.
STATISTICS

Where applicable results are expressed in the form: \( \bar{x} \pm \text{S.E.M.} (n) \)

i.e. mean ± standard error of the mean, with the number of observations.

Differences between groups of observations were determined using the
students 't' test with \((n_1 + n_2) - 1\) degrees of freedom. Statistical
manouevres were performed using either an Olivetti programma 101 or a
Wang programmable calculator.

THERMODYNAMICS

Thermodynamic parameters of the interaction of fatty acid anions with
BSA were determined using the following well known relationships:

The free energy of binding (\( \Delta G \)) was calculated from \( \Delta G = -RT \ln K \)
where \( R \) is the gas constant, \( T \) is in °K and for \( K \), the equilibrium constant,
the apparent association constant was used. The enthalpy of binding (\( \Delta H \))
was assumed to be constant over the temperature range 283°K to 310°K and
was thus calculated from:

\[
\ln \frac{K_2}{K_1} = -\frac{\Delta H}{R} \left( \frac{1}{T_2} - \frac{1}{T_1} \right)
\]

The entropy of binding (\( \Delta S \)) was calculated using the values found
for \( \Delta G \) and \( \Delta H \), and the relationship:

\[
\Delta S = \frac{\Delta H \cdot T}{T_2}
\]
CHAPTER THREE

THE EVALUATION OF SEVERAL TECHNIQUES FOR USE

IN DRUG-PROTEIN BINDING STUDIES
There is a multitude of techniques available to quantify and characterise the equilibrium between a ligand and its binding sites. Many of these techniques are well established and thoroughly tested. These 'classical' methods have been discussed in chapter one and have been reviewed by several authors: Goldstein, 1949; Edsall and Wyman, 1958; Rosenberg and Klotz, 1960; Steinhardt and Reynolds, 1969; Chignell, 1971; Bridges and Wilson, 1976. In recent years, several new or modified techniques have been introduced which are designed for the observation of a particular facet of the binding equilibrium or are supposed to be quicker, easier or cheaper alternatives to existing methods. Three such techniques, in the form of commercially available equipment, have been evaluated as part of this project. The conditions of the evaluations were such that comparisons may be made with previous work from these laboratories, concerning the merits of several, well known protein binding techniques (Wilson, 1974). A wide range of ligands was used in this study, with differing physico-chemical properties.

Warfarin has been shown to undergo a marked change in fluorescence properties upon binding to albumin (Chignell, 1971). This phenomenon has been used in our laboratories to probe the albumin binding sites for warfarin and to monitor the displacement of warfarin by competing ligands (Wilson, 1974). This approach has been studied further, and the related coumarin, phenprocoumon (PPC), which is a widely prescribed anticoagulant in Northern Europe, has been evaluated as a possible fluorescent probe.
Monitoring the displacement of warfarin using the fluorescent probe approach was used to study the competition of sulphamethoxazole for the coumarin binding sites. Sulphamethoxazole (SMX) is the sulphonamide portion of the antibacterial combination co-trimoxazole, which has been implicated in the potentiation of hypoprothrombinaemia when co-administered with warfarin (Hassall et al., 1975; Tilstone et al., 1976).

The surprising paucity of data concerning drug binding to extravascular tissue has already been mentioned in this thesis. It is possible that the scarcity of information is due to the lack of suitable methods for the study of binding to tissue preparations. With this in mind, the technique of centrifuge sedimentation was included in our schedule of methods for evaluation. The interaction of fatty acids with hepatic microsomal suspensions was chosen as a model system for this investigation.
3:2 EXPERIMENTAL APPROACH

3:2:1 FLUORESCENCE MEASUREMENTS

All fluorescence measurements were made with a Perkin-Elmer MPF 3 spectrophotofluorimeter, using solutions prepared in 67 mM sodium phosphate buffer, pH 7.4. Fluorescence titrations of buffer or albumin solutions (BSA was Conn fraction V from Sigma, HSA was crystalline from Kabi or Behringwerke) with coumarin drugs were performed by a modification of the method of Daniel and Weber (1966) as described in section 2:2:7. The cuvette normally contained 2 ml of buffer or albumin solution (5 - 20 x 10^{-6} M) to which was added microlitre volumes of warfarin or phenprocoumon (1 - 5 x 10^{-3} M). Displacement experiments involved the addition of microlitre quantities of competitor (stock concentration up to 2 x 10^{-2} M) to 2 ml of drug-albumin solution. The type of competition involved was determined from Dixon (1953) plots.

3:2:2 EVALUATION OF NOVEL BINDING TECHNIQUES

Sodium phosphate buffer (67 mM, pH 7.4) and Bovine Serum Albumin (Conn fraction V, Sigma) were used throughout the evaluations. All experiments were performed at 20°, except the centrifugal sedimentation, which was carried out at 4° to preserve the viability of the microsomes.

Continuous ultrafiltration was performed using an Amicon ultrafiltration cell (Standard model, size 12) fitted with a 'Diaflo' membrane (type PM 10, Amicon). The binding of sodium salicylate (stock concentration 1.0 x
10^{-3} M) and sulphanethoxazole (stock concentration 1.18 \times 10^{-3} M) to BSA (2.9 \times 10^{-4} M) was determined. Binding parameters were computed from this data using the Modfit 27 fitting programme as described in section 2:2:14. Continuous ultrafiltration does not permit the estimation of free or bound drug at pre-determined total drug concentrations. For this reason, the percentage bound values in tables 3:1 and 3:2 do not correspond exactly to the total drug concentrations presented. Table 3:3 shows the actual total drug concentrations at which the percentage bound was determined.

'Centriflo' ultrafiltration cones (Amicon) were used in the estimation of BSA (2.9 \times 10^{-4} M) binding of sodium salicylate (3.75 - 90 \times 10^{-5} M), ethyl carbamate (1 - 7.5 \times 10^{-3} M), hexyl carbamate (8.5 - 50 \times 10^{-4} M), hexanoic acid (1.67 - 5000 \times 10^{-5} M) and valeric acid (1.67 - 5000 \times 10^{-5} M). Trace amounts of 14C labelled compounds were used for the estimation of carbamates and fatty acids.

'Dianorm' equilibrium dialysis cells (MSE) were employed to examine the interaction of BSA (2.9 \times 10^{-4} M) with sodium salicylate (3.75 - 90 \times 10^{-5} M). Spectrapor 2 purified cellulose membrane (Spectrum Medical Industries), which has a molecular weight cut-off of 12,000 to 14,000, was used in the dialysis cells.

Binding estimates from the above techniques are compared with those obtained using the standard, Toribara ultrafiltration apparatus (see section 2:2:2) and with values reported by Wilson (1974).
3:2:3 TISSUE BINDING MEASUREMENTS

Centrifuge sedimentation was employed in attempts to measure the binding of lauric acid \((0.01 - 3.75 \times 10^{-6} \text{ M})\) and octanoic acid \((0.06 - 5000 \times 10^{-6} \text{ M})\) to rat hepatic microsomal suspensions \((1.5 \text{ mg protein.ml}^{-1})\). Trace amounts of \(^{14}\text{C}\) labelled fatty acids were used to estimate the ligand concentrations.
3 : 3  FIGURES AND TABLES
The stock salicylate concentration was 160 µg.ml\(^{-1}\). The cell was initially filled with sodium phosphate buffer, 67 mM, pH 7.4 (+) or 0.29 mM BSA in phosphate buffer (o).
Scatchard plot of the interaction of sodium salicylate with BSA at 21°C, as measured by the continuous ultrafiltration technique.

The solid symbols and the line refer to two normal experiments. The open symbols refer to data obtained after eight hours pre-stirring of the protein sample.
Figure 3: Scatchard plot of the interaction of sulphamethoxazole with BSA, as measured by the continuous ultrafiltration technique.

The circles and crosses refer to two different experiments.
Figure 3:4  Semi logarithmic plot of \( C_0 - C \) against volume of eluate for the continuous ultrafiltration of sulphamethoxazole.

The original sulphamethoxazole concentration \( C_0 \) was 300 \( \mu \text{g.m} \text{l}^{-1} \).

The concentration of sulphamethoxazole in the eluate \( C \) was estimated for each 0.966 ml fraction.
The regression equation of the lower points is:

$$y = 0.0528 - 0.000506 \times r = 0.998$$

from which the following binding parameters were calculated:

$$K_a = 5.06 \times 10^{-2} \text{ M}$$

Total binding capacity = 69.6 mmole.mg$^{-1}$

The experiment was carried out at a protein concentration of 1.5 mg/ml in phosphate buffer pH 7.4, 67 mM at $4^\circ$ C.
Figure 3:6 The titration of phosphate buffer and HSA with phenprocoumon or warfarin

The HSA concentration was $1 \times 10^{-5}$ M. Fluorescence was measured at the wavelength of maximum emission, using an excitation wavelength of 320 nm. 

- $\bullet$ = phenprocoumon
- $\Delta$ = warfarin

The graph shows the fluorescence intensity (arbitrary units) plotted against the coumarin concentration ($\times 10^{-6}$ M). The fluorescence increases with increasing coumarin concentration, indicating a binding interaction with HSA.
Figure 3:7  The titration of HSA with phenprocoumon or warfarin. Complex excited at 290 nm.

The HSA concentration was $1 \times 10^{-5}$ M. Emission was measured at the wavelength of maximum fluorescence.

- $\bullet$ = phenprocoumon
- $\triangle$ = warfarin
Figure 3: Uncorrected fluorescence emission spectra of phenprocoumon-albumin complexes

(a) BSA

Ex. 290

Ex. 320

Wavelength (nm)

(b) HSA

Ex. 290

Ex. 320

Wavelength (nm)

Albumin $1 \times 10^{-5}$ M, phenprocoumon concentrations indicated in $\mu$M units.
Figure 3:9 Uncorrected fluorescence emission spectra of warfarin - albumin complexes

(a) HSA

(b) BSA

The albumin concentration was $1 \times 10^{-5}$ M in all cases. The concentration of warfarin is indicated in micromolar units.
The effects of sulphamethoxazole on warfarin/HSA fluorescence

Warfarin ($2.5 \times 10^{-6}$ M)/HSA ($5.0 \times 10^{-6}$ M) solutions were excited at 320 nm. Fluorescence was monitored at 378 nm. Mean ± SEM ($n = 3$)
Figure 3: Dixon plot of the displacement of warfarin from Human Serum Albumin (5 x 10^-6 M) by sulphamethoxazole as measured by the fluorescent probe technique.
TABLE 3:1  A comparison of the percentage binding of sodium salicylate to Bovine Serum Albumin using various techniques.

<table>
<thead>
<tr>
<th>Initial Salicylate concentration x 10^5 M</th>
<th>Ultrafiltration</th>
<th>Continuous Ultrafiltration</th>
<th>Frontal Analysis</th>
<th>Equilibrium Dialysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Toribara</td>
<td>Centriflo</td>
<td></td>
<td>Kontron</td>
</tr>
<tr>
<td>3.75</td>
<td>99.1 ± 0.5</td>
<td>99.6 ± 0.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7.5</td>
<td>96.2 ± 0.9</td>
<td>97.9 ± 0.7</td>
<td>-</td>
<td>97.2 ± 1.3</td>
</tr>
<tr>
<td>15.0</td>
<td>93.3 ± 0.4</td>
<td>92.3 ± 0.5</td>
<td>96.9</td>
<td>94.5 ± 1.4</td>
</tr>
<tr>
<td>30.0</td>
<td>88.0 ± 0.3</td>
<td>88.4 ± 0.3</td>
<td>94.7</td>
<td>80.4 ± 0.5</td>
</tr>
<tr>
<td>45.0</td>
<td>83.3 ± 0.5</td>
<td>82.7 ± 0.4</td>
<td>92.0</td>
<td>80.4 ± 0.5</td>
</tr>
<tr>
<td>60.0</td>
<td>78.4 ± 0.5</td>
<td>78.0 ± 0.5</td>
<td>86.4</td>
<td>77.2 ± 0.3</td>
</tr>
<tr>
<td>75.0</td>
<td>75.8 ± 0.2</td>
<td>76.5 ± 0.2</td>
<td>81.9</td>
<td>75.1 ± 0.2</td>
</tr>
<tr>
<td>90.0</td>
<td>74.7 ± 0.3</td>
<td>74.4 ± 0.4</td>
<td>77.3</td>
<td>55.2 ± 0.7</td>
</tr>
</tbody>
</table>

All experiments were performed at 20° in sodium phosphate buffer, 67 mM, pH 7.4 with BSA at a concentration of 2.9 x 10^-4 M. Statistical comparisons by the students 't' test are with those values obtained with the Toribara apparatus.

n.s. = p > 0.05, * = p < 0.05, *** = p < 0.001.
<table>
<thead>
<tr>
<th>x 10^-5 M Total Drug Concentration</th>
<th>PERCENTAGE BOUND</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frontal analysis a</td>
</tr>
<tr>
<td>15</td>
<td>68.4 ± 1.4</td>
</tr>
<tr>
<td>30</td>
<td>65.3 ± 0.6</td>
</tr>
<tr>
<td>45</td>
<td>57.3 ± 0.9</td>
</tr>
<tr>
<td>60</td>
<td>54.8 ± 0.7</td>
</tr>
</tbody>
</table>

All experiments performed at 20° with a BSA concentration of 29.9 x 10^-5 M in phosphate buffer (67 mM, pH 7.4).

a - Data from Wilson (1974). Mean ± S.E.M. (n = 2).

b - Mean of two determinations - see Table 3:3 for full data.
<table>
<thead>
<tr>
<th>Nominal drug concentration $x 10^{-5}$ M</th>
<th>SODIUM SALICYLATE</th>
<th>SULPHAMETHOXAZOLE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EXP. No.1.</td>
<td>EXP. No.2.</td>
</tr>
<tr>
<td></td>
<td>Actual total drug conc $n^{\text{th}}$ x $10^{-5}$ M</td>
<td>% Bound</td>
</tr>
<tr>
<td>15.0</td>
<td>13.84</td>
<td>96.53</td>
</tr>
<tr>
<td>30.0</td>
<td>30.99</td>
<td>94.56</td>
</tr>
<tr>
<td>45.0</td>
<td>44.52</td>
<td>92.26</td>
</tr>
<tr>
<td>60.0</td>
<td>60.88</td>
<td>86.72</td>
</tr>
<tr>
<td>75.0</td>
<td>76.33</td>
<td>82.00</td>
</tr>
<tr>
<td>90.0</td>
<td>90.85</td>
<td>78.76</td>
</tr>
</tbody>
</table>

In all experiments BSA ($29 \times 10^{-5}$ M) in phosphate buffer (67 mM, pH 7.4) at 20°C was used.
Table 3.4: A comparison of binding constants obtained from the literature with those derived in the present study for the interaction of sodium salicylate and sulphamethoxazole with bovine serum albumin.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>( n_1 )</th>
<th>( K_1 \times 10^4 ) M(^{-1} )</th>
<th>( n_1 K_1 )</th>
<th>( n_2 )</th>
<th>( K_2 \times 10^2 ) M(^{-1} )</th>
<th>( n_2 K_2 )</th>
<th>Conditions and source of measurements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salicylate</td>
<td>1.75 ± 0.14</td>
<td>7.62 ± 0.22</td>
<td>13.3 x 10(^4)</td>
<td>9.29 ± 2.15</td>
<td>3.88 ± 1.06</td>
<td>36.0 x 10(^2)</td>
<td>Continuous ultrafiltration (20(^\circ)) this work.</td>
</tr>
<tr>
<td>Salicylate</td>
<td>1.64 ± 0.38</td>
<td>7.71 ± 0.24</td>
<td>12.6 x 10(^4)</td>
<td>4.30 ± 2.11</td>
<td>6.36 ± 4.03</td>
<td>27.0 x 10(^2)</td>
<td>Pre-stirred continuous ultrafiltration (20(^\circ)), this work.</td>
</tr>
<tr>
<td>Salicylate</td>
<td>1.04</td>
<td>5.00</td>
<td>5.2 x 10(^4)</td>
<td>6.27</td>
<td>2.98</td>
<td>18.7 x 10(^2)</td>
<td>Dynamic dialysis (37(^\circ)). Cruze and Meyer (1976)</td>
</tr>
<tr>
<td>Salicylate</td>
<td>1.01</td>
<td>6.54</td>
<td>6.6 x 10(^4)</td>
<td>7.85</td>
<td>2.50</td>
<td>19.6 x 10(^2)</td>
<td>Dynamic dialysis (37(^\circ)) of bovine plasma. Cruze and Meyer (1976)</td>
</tr>
<tr>
<td>Salicylate</td>
<td>0.37</td>
<td>3.00</td>
<td>1.11 x 10(^4)</td>
<td>3.5</td>
<td>1.0</td>
<td>3.5 x 10(^2)</td>
<td>Equilibrium dialysis (4(^\circ)). Davison and Smith (1961)</td>
</tr>
<tr>
<td>Salicylate</td>
<td>1.00</td>
<td>20.00</td>
<td>20.0 x 10(^4)</td>
<td>5.0</td>
<td>17.5</td>
<td>87.5 x 10(^2)</td>
<td>Dynamic dialysis (25(^\circ)). Meyer and Gutman (1970)</td>
</tr>
<tr>
<td>Sulphamethoxazole</td>
<td>0.9</td>
<td>3.83</td>
<td>3.45 x 10(^4)</td>
<td>2.2</td>
<td>4.09</td>
<td>9.0 x 10(^2)</td>
<td>Continuous ultrafiltration (20(^\circ)) this work.</td>
</tr>
</tbody>
</table>

a. Parameters computed using the fitting programme Modfit 27. b. Parameters calculated from figure 3.3 by the method of Rosenthal (1967).
**TABLE 3: A comparison of the percentage binding of ethyl and hexyl carbamates to bovine serum albumin using two ultrafiltration techniques**

<table>
<thead>
<tr>
<th>Total carbamate concentration x 10^{-3} M</th>
<th>ETHYL CARBAMATE</th>
<th>HEXYL CARBAMATE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Toribara method</td>
<td>Centriflo method</td>
</tr>
<tr>
<td>0.42</td>
<td>84.3 ± 0.4</td>
<td>n.s.</td>
</tr>
<tr>
<td>0.625</td>
<td>78.2 ± 0.8</td>
<td>79.7 ± 0.9</td>
</tr>
<tr>
<td>0.83</td>
<td>73.4 ± 0.9</td>
<td>71.6 ± 1.1</td>
</tr>
<tr>
<td>1.25</td>
<td>60.9 ± 0.6</td>
<td>57.2 ± 0.8</td>
</tr>
<tr>
<td>2.00</td>
<td>48.1 ± 0.5</td>
<td>49.3 ± 0.6</td>
</tr>
<tr>
<td>2.50</td>
<td>34.9 ± 0.3</td>
<td>35.3 ± 0.7</td>
</tr>
<tr>
<td>3.00</td>
<td>32.7 ± 0.6</td>
<td>34.1 ± 0.9</td>
</tr>
<tr>
<td>4.00</td>
<td>32.5 ± 0.4</td>
<td>33.2 ± 0.6</td>
</tr>
<tr>
<td>5.00</td>
<td>30.4 ± 0.5</td>
<td>33.8 ± 0.4</td>
</tr>
</tbody>
</table>

All experiments performed at 25°C with BSA at a concentration of 29.0 x 10^{-5} M in phosphate buffer (67 mM, pH 7.4)

a - Mean ± S.E.M. (n = 5)

Statistical tests of significance by the students 't' method

n.s. = p > 0.05, * = p < 0.05, ** = p < 0.01, *** = p < 0.001
TABLE 3:6  The binding of Lauric and Octanoic acids to hepatic microsomal suspensions as measured by the centrifuge sedimentation technique

<table>
<thead>
<tr>
<th>Total fatty acid concentration x 10^{-6} M</th>
<th>'Free' fatty acid in supernatant after sedimentation</th>
<th>OCTANOATE</th>
<th>IAURATE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>conc^n . x 10^{-6} M</td>
<td>Mean %</td>
<td>conc^n . x 10^{-6} M</td>
</tr>
<tr>
<td>5000</td>
<td>4945, 4905</td>
<td>98.5</td>
<td>-</td>
</tr>
<tr>
<td>500</td>
<td>477.5, 482.5</td>
<td>96.0</td>
<td>-</td>
</tr>
<tr>
<td>50</td>
<td>47.25, 47.75</td>
<td>95.0</td>
<td>-</td>
</tr>
<tr>
<td>5.0</td>
<td>4.27, 4.25</td>
<td>85.2</td>
<td>-</td>
</tr>
<tr>
<td>3.75</td>
<td>-</td>
<td>-</td>
<td>2.84 ± 0.29</td>
</tr>
<tr>
<td>2.50</td>
<td>-</td>
<td>-</td>
<td>1.85 ± 0.025</td>
</tr>
<tr>
<td>1.25</td>
<td>-</td>
<td>-</td>
<td>0.904 ± 0.019</td>
</tr>
<tr>
<td>0.5</td>
<td>0.429, 0.422</td>
<td>85.1</td>
<td>-</td>
</tr>
<tr>
<td>0.375</td>
<td>0.317, 0.329</td>
<td>86.1</td>
<td>0.267 ± 0.009</td>
</tr>
<tr>
<td>0.25</td>
<td>0.206, 0.212</td>
<td>83.6</td>
<td>0.178 ± 0.002</td>
</tr>
<tr>
<td>0.125</td>
<td>0.102, 0.106</td>
<td>83.4</td>
<td>0.0914 ± 0.0018</td>
</tr>
<tr>
<td>0.0625</td>
<td>0.0529, 0.055</td>
<td>86.3</td>
<td>0.0447 ± 0.0004</td>
</tr>
<tr>
<td>0.0125</td>
<td>-</td>
<td>-</td>
<td>0.0087 ± 0.00009</td>
</tr>
</tbody>
</table>

Experiments were performed at 4°C with rat hepatic microsomes at a final concentration of 1.5 mg.ml^{-1}. Results are presented as Mean ± S.E.M. (n = 4) or as duplicate determinations.
TABLE 3:7 The influence of sulphamethoxazole on the binding of warfarin to HSA. Literature derived values compared with those obtained using the fluorescence technique.

<table>
<thead>
<tr>
<th>Sulphamethoxazole conc$^n \times 10^{-6}$ M</th>
<th>sulphamethoxazole /warfarin ratio</th>
<th>Source of data, warf. conc$^n$ and albumin conc$^n$</th>
<th>Percentage of Warfarin bound, Mean ± S.E.M (n)</th>
<th>Percentage of Warfarin free. Mean</th>
<th>Percentage increase in free warfarin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td></td>
<td>91.00 ± 0.96 (3)</td>
<td>9.00</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
<td>This work, fluorescence</td>
<td>90.52 ± 0.19 n.s. (3)</td>
<td>9.48</td>
<td>5.3</td>
</tr>
<tr>
<td>20</td>
<td>8</td>
<td>measurements.</td>
<td>89.73 ± 0.37 n.s. (3)</td>
<td>10.27</td>
<td>14.1</td>
</tr>
<tr>
<td>40</td>
<td>16</td>
<td></td>
<td>87.37 ± 0.20 * (3)</td>
<td>12.63</td>
<td>40.3</td>
</tr>
<tr>
<td>60</td>
<td>24</td>
<td></td>
<td>85.97 ± 0.61 ** (3)</td>
<td>14.03</td>
<td>55.6</td>
</tr>
<tr>
<td>80</td>
<td>32</td>
<td></td>
<td>84.19 ± 0.94 ** (3)</td>
<td>15.81</td>
<td>75.7</td>
</tr>
<tr>
<td>100</td>
<td>40</td>
<td>Warfarin</td>
<td>82.76 ± 0.26 *** (3)</td>
<td>17.24</td>
<td>91.6</td>
</tr>
<tr>
<td>150</td>
<td>60</td>
<td>2.5 x 10$^{-6}$ M</td>
<td>79.21 ± 0.70 *** (3)</td>
<td>20.79</td>
<td>131.0</td>
</tr>
<tr>
<td>200</td>
<td>80</td>
<td>HSA</td>
<td>77.08 ± 0.49 *** (3)</td>
<td>22.92</td>
<td>155.8</td>
</tr>
<tr>
<td>250</td>
<td>100</td>
<td>5.0 x 10$^{-6}$ M</td>
<td>74.88 ± 0.66 *** (3)</td>
<td>25.12</td>
<td>180.4</td>
</tr>
<tr>
<td>300</td>
<td>120</td>
<td></td>
<td>72.14 ± 0.16 *** (3)</td>
<td>27.86</td>
<td>210.9</td>
</tr>
<tr>
<td>400</td>
<td>160</td>
<td></td>
<td>67.20 ± 0.48 *** (3)</td>
<td>31.80</td>
<td>254.9</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td></td>
<td>86.5 ± 0.06 (3)</td>
<td>13.5</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>12.5</td>
<td></td>
<td>84.0 ± 0.21 ** (3)</td>
<td>16.0</td>
<td>19.0</td>
</tr>
<tr>
<td>Hassall et al. (1975) Warf.</td>
<td>79.4 number of samples not quoted</td>
<td></td>
<td>20.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td></td>
<td>79.4</td>
<td>23.4</td>
<td></td>
</tr>
<tr>
<td>197</td>
<td>20.3</td>
<td></td>
<td>76.6</td>
<td>13.6</td>
<td></td>
</tr>
</tbody>
</table>

Statistical comparisons to those values at 0 SMX concentration by the students 't' test. n.s. = $p > 0.05$, * = $p < 0.02$, ** = $p < 0.01$, *** = $p < 0.001$. 
3: 4 RESULTS AND DISCUSSION

3: 4: 1 CONTINUOUS ULTRAFILTRATION

Preliminary studies of the continuous ultrafiltration (diafiltration) apparatus suggested that it was well suited to the type of binding experiments which we wished to perform. The PM 10 Diaflo membranes, which are hydrous gels of the complex interaction of polyanions and polycations supported by an inert, porous backing (Blatt et al., 1965, 1967), seem to be ideal for this technique. They have a very high solvent permeability (unlike conventional regenerated cellulose) and a published retentivity for albumin of >98% (Amicon Publication No. 426). When buffer was ultrafiltered through the cell containing 2% w/v BSA for several hours, no protein could be detected in the ultrafiltrate. In addition, the membranes probably do not bind ligands extensively. Membrane binding was checked by comparing the experimental cell volumes, derived from plots of \( \log C_0 - C \) against (see figure 3:4 for an example and section 2:2:3 for the theory), with the known cell volume, determined by careful weighing of the cell empty and full of a liquid of known density. The actual cell volume was found to be 14.05 ml, whilst experimental cell volumes of 14.13 and 14.05 ml for sulphamethoxazole and 14.46 and 14.86 ml for sodium salicylate were recorded. The values for sulphamethoxazole are within experimental error of the actual value and suggest no binding of this compound to the membrane or other portions of the apparatus. The slightly higher values for sodium salicylate suggest that there may be some loss of drug, but only a small amount.
Typical eluate drug concentration curves are shown in figure 3:1, for the ultrafiltration of sodium salicylate \((1 \times 10^{-3} \text{ M})\) through buffer and BSA \((2\% \text{ w/v, } 2.9 \times 10^{-4} \text{ M})\) solutions. The shapes of the curves are as expected, that in the presence of protein showing lower eluate drug concentrations, especially at the beginning of the experiment where total drug concentration is low and the percentage bound is high. The total and bound concentrations of drug for each fraction were calculated as described in section 2:2:3 and are displayed in the form of Scatchard (1949) plots in figure 3:2 for salicylate and figure 3:3 for sulphamethoxazole. It is obvious from figures 3:1 - 3:4 that diafiltration is capable of yielding a great many data points with good consistency between experiments and very little experimental scatter. It is very tempting to assume that the binding parameters calculated from such data must be highly accurate. An examination of tables 3:1 and 3:2 will show that such an assumption would be invalid, at least for these experiments. The percentage of drug bound, at all concentrations and for both drugs, is substantially higher when derived from diafiltration experiments than the equivalent values from any of the other techniques. The detection of apparently greater binding with diafiltration is also exhibited in table 3:4 where the binding parameters calculated from this technique are compared with published values derived using other techniques. The diafiltration parameters are greater than all the previous published values with the exception of the early values from Meyer and Gutman (1970), which have been superseded by later and lower values of \(K_1\) and \(K_2\) in a recent publication (Cruze and Meyer, 1976). Before seeking an explanation for this anomalously high binding, it must be stated that other workers using the diafiltration technique have encountered
similar discrepancies. Ryan and Hanna (1971) studied the binding of testosterone to BSA by diafiltration and equilibrium dialysis methods. They found that diafiltration gave consistently higher $nK$ values than equilibrium dialysis. In a comprehensive study, Blatt and co-workers (1968) found disparate results for the binding of both methyl orange and $\text{Ca}^{++}$ to HSA from continuous ultrafiltration and direct ultrafiltration experiments.

The differing results from diafiltration and other techniques have no obvious explanation, but are probably due to a combination of the following possibilities: a) The binding of ligand to the cell or membrane would give apparently high binding. Whilst there was little difference between experimental and actual cell volumes, this is not a definitive test for loss of ligand. In addition, this test is carried out in the absence of protein and the cell constituents may have different properties in the presence of protein. b) It is possible, but unlikely, that there is some weakness in the theory of ultrafiltration and thus in the mathematical manipulations used to calculate the binding data. c) Again, it is conceivable, but even more unlikely, that the parameters from the other methods are falsely low. The facts that there is good agreement between the other methods, which are based upon differing physico-chemical principles, makes this explanation difficult to believe. d) Another explanation is that the protein's binding characteristics are changed by the continuous stirring which is inherent in the technique. It would be ironic if stirring were the source of error, since it is one of the supposed advantages of diafiltration that stirring prevents concentration polarisation (see section 1:7:3).

To investigate the possibility that stirring may denature or alter the conformation of the protein, a sample of albumin was stirred for eight hours
prior to the start of a normal experiment. The binding of sodium salicylate to this preparation was measured in the usual way and the results are shown in tables 3:3 and 3:4 and figure 3:2. The percentage salicylate bound at low concentrations was not significantly different from the values obtained from freshly prepared albumin solutions, but at higher total ligand concentrations there was a consistent reduction in salicylate bound. This trend is reflected in the binding parameters, where the $n_1K_1$ values from normal and pre-stirred samples are very similar ($13.3 \times 10^4$ M$^{-1}$ and $12.6 \times 10^4$ M$^{-1}$ respectively) but the pre-stirred sample showed fewer secondary sites and a lower total binding constant, $n_2K_2$ ($27 \times 10^2$ M$^{-1}$ compared to $36 \times 10^2$ M$^{-1}$ for untreated albumin). The conclusion from this study is that prolonged stirring does seem to have an effect on albumin but the result is a reduced, not enhanced, binding. Ryan and Hanna (1971) also reported a reduced binding affinity for the testosterone–BSA complex when the BSA was subjected to diafiltration conditions for 12 hours.

Theoretically, diafiltration has some outstanding attributes. During the course of a standard ultrafiltration experiment, the volume of drug-protein solution is reduced by the removal of the ultrafiltrate. This results in an effective concentration of the protein which may alter the equilibrium position of the ligand–protein interaction (probably higher binding would be observed because of the increased protein concentration). Continuous ultrafiltration cannot suffer from such inaccuracies since the sample volume is kept constant throughout the experiment and protein cannot escape the cell. Diafiltration allows for the determination of a complete binding curve in a single experiment. This may save both time and labour, but probably more important is that only one protein sample is
used. This could be most valuable if the protein source is limited, when atypical plasma or tissue is under study, for example. The technique offers considerable possibilities for automation, especially if the eluate ligand concentration can be continuously monitored by spectroscopy. Drug displacement studies can also be performed using diafiltration. In this case, the primary ligand is ultrafiltrated through the cell until equilibrium is reached. Further ultrafiltration, with a stock solution of both primary and displacing ligand, provides a constant primary ligand concentration, with increasing displacer concentration. This approach has been used to study the salicylate displacement of sodium urate and methyl orange from HSA binding sites (Campion and Olsen, 1974).

This impressive series of advantages is of no value, however, if the source of the anomalously high binding cannot be located and eradicated. Until this is achieved, it must be concluded that continuous ultrafiltration yields inaccurate binding parameters and is therefore not to be recommended.

3:4:2 CENTRIFLO ULTRAFILTRATION

In a study of protein binding techniques, Wilson (1974) concluded that ultrafiltration, using a modified Toribara apparatus, was the method of choice for a wide variety of ligands. Toribara type ultrafiltration has been used extensively in this project, but whilst the actual experimental time is short, preparation of the membranes and the manipulations to form filled bags are time consuming and require delicate handling. The method of Centriflo ultrafiltration (Amicon), in which the membrane is laminated to a preformed cone requiring the minimum of preparation, was
seen as a welcome addition to the arsenal of protein binding techniques and has been thoroughly investigated in our laboratories.

The binding of salicylate to BSA, measured using Centriflo and Toribara ultrafiltration techniques and several other methods, is displayed in Table 3:1. The BSA binding of ethyl and hexyl carbamates, estimated by the two ultrafiltration methods, is shown in Table 3:5. In addition, the suitability of Centriflo to measure albumin binding of fatty acids and the plasma free levels of cortisol and calcium was examined.

The percentage of salicylate bound to BSA, determined by Centriflo, over the range of drug concentrations used (3.75 - 90.0 x 10^-5 M), agreed well with those from Toribara type ultrafiltration. A similar comparison for the binding of ethyl and hexyl carbamates also showed good agreement. The slightly higher values obtained from Centriflo for carbamate binding are probably due to loss of ligand by membrane binding. The ultrafiltration of protein free 14C carbamate solutions through Centriflo membranes gave ~ 95% recovery of radioactivity in the ultrafiltrate. In contrast to this good level of recovery, the same experiments with fatty acids showed very large losses of ligand. Using hexanoate and valerate at concentrations of 50 mM to 16.7 µM the loss of fatty acid was as great as 80% and consistent losses were found over the entire range. The method was, then, considered unsuitable for the determination of fatty acid binding.

In a study of the suitability of the Centriflo method for the determination of plasma free concentrations of endogenous substances, the levels of cortisol and calcium in the ultrafiltrates of human plasma samples were determined. The estimated percentage of calcium free in plasma, 54.8%
(Total Ca$^{++}$ 10.4 ± 0.5 mg/100 ml, free Ca$^{++}$ 5.7 ± 0.6 mg/100 ml) agrees well with values obtained by other methods and previous values for ultra-filterable calcium obtained from Centriflo experiments (Farese et al., 1970; Halver, 1972). Free cortisol levels were, however, much lower than the expected 5 - 15%. The binding of cortisol to Centriflo membranes was examined in the absence of protein. A maximum recovery of only 64% (at a total cortisol concentration of 1 x 10^{-5} g. L^{-1}) was obtained. Farnsworth (1974) has used Centriflo to measure free plasma cortisol levels and reported a value of 1.1% free, which is lower than would be expected, probably due to binding of the steroid to the membrane.

Centriflo cones have been used in studies of the human plasma binding of digoxin (Wallace and Whiting, 1974), diphenylhydantoin (Booker and Darcey, 1973) and a series of disopyramide derivatives (Chien et al., 1975). In these literature studies, digoxin was not found to bind to Centriflo cones, but only 88% of diphenylhydantoin and as little as 40% of disopyramide was recovered upon filtration of aqueous solutions through the cones. These losses were supposedly compensated for by using correction factors, but this ignores the fact that the membrane may not bind the same quantity of ligand in the absence and presence of protein.

It seems that Centriflo cones are particularly efficient at binding lipophilic small molecules. The membrane of the cone is of the same family (Diaflo) as that used in the continuous ultrafiltration experiments but is a different formulation. (The Centriflo membrane is named CFSOA and has a molecular weight cut off at 50,000, the membrane in diafiltration was PM 10 which has a cut off value of 10,000). It must be concluded that Centriflo is not
suitable for the study of lipophilic ligand binding to protein unless a good recovery of the ligand can be proven. Provided that the ligand under study does not bind significantly to the membrane cones, Centriflo ultrafiltration is a good, quick method for the determination of free ligand in a protein solution. The technique is very simple to perform, especially if research resources allow disposal of the membrane cones after a single experiment. The cones can be cleaned for re-use up to seven or eight times, but this procedure is lengthy and involved if complete removal of residual protein and ligand is to be ensured. It must be remembered that the cones have a molecular weight cut-off value of 50,000. This allows the filtration of small proteins or polypeptides, which may be undesirable in experiments involving plasma or tissue suspensions.

3:4:3 DIALYSIS CELLS

Since the initial description of the phenomenon of dialysis (Graham, 1861) and the first use of equilibrium dialysis in ligand-macromolecule studies (Osborne, 1906) this technique has remained popular in drug-protein binding investigations, largely because of its physical simplicity. The theoretical disadvantages of equilibrium dialysis have already been discussed (see section 1:7:2) and in addition to these, experimental problems such as leaking knots, different bag dimensions and long dialysis time all contribute to variable results. Several investigators have proposed modified dialysis apparatus to overcome these inadequacies.

Probably the most successful design to date is the half-cell system of
Weder and Bickel (1970; Weder et al., 1971). Wilson (1974) considered a commercial version of this apparatus (Kontron Diapack) to be most satisfactory for the majority of albumin binding experiments. As part of this study of protein-binding methodology, a further development of the apparatus of Weder and Bickel (1970), Dianorm (MSE Scientific Instruments), has been evaluated.

The binding of sodium salicylate to BSA studied using Dianorm is reported in figure 3:1 with equivalent values from other techniques. The percentage bound estimates are in good agreement with those from the similar Kontron system and also those from ultrafiltration experiments, which have already been discussed. The membranes which were used in the present study were of regenerated cellulose, but of a higher purity and better characterised than standard dialysis tubing. Spectrapor 2 (Spectrum Med. Ind. Inc.) was employed, which has a molecular weight cut-off of 12,000 - 14,000 and is of such a thickness to allow dialysis to proceed about twice as quickly as ordinary regenerated cellulose membrane.

The combination of the well designed Dianorm apparatus with the advanced cellulose membranes produces a technique with the admirable qualities of ease of operation, fast experimental times, good temperature control and small sample volumes which make it ideal for most ligand-macromolecule studies. In addition, no problems have been encountered with loss of ligand due to binding to the apparatus. The half cells are carved from blocks of poly-tetrafluoroethylene (PTFE) which is renowned for its inertness. Regenerated cellulose membranes seem to bind lipophilic compounds with less avidity than the synthetic polymeric membranes. Work in our laboratories has
demonstrated less than 5% loss of Cortisol in studies using cellulose
dialysis membranes, and fatty acids are recovered with similar efficiency
in ultrafiltration experiments employing Visking tubing (see chapter 4).
Half-cell equilibrium dialysis may be particularly suitable for the
examination of ligand binding to suspended tissue fractions since samples
are continuously, but gently, agitated ensuring no settling of particulate
matter and there are no forces to promote the deposition of material on the
membrane. This possibility is being investigated currently in the Department.

3:4:4 CENTRIFUGE SEDIMENTATION

The interactions of fatty acids with hepatic, microsomal suspensions
was studied both as a model system for the tissue binding of small molecules
and also to further our understanding of fatty acid-protein and fatty acid-
P450 complexes. The binding of octanoate and laurate to rat hepatic
microsomes was measured using an approach similar to that of Bickel and
Steel (1974). Table 3:5 shows the results obtained. The initial determinations
were performed over the fatty acid concentration range 5 - 0.0125 x 10^{-6} M
with a protein concentration of 1.5 mg/ml. These concentrations were
chosen to obtain a good range of fatty acid:cytochrome P450 ratios.
(Cytochrome P450 concentration was ~ 1 nmole/ml or ~ 1 x 10^{-6} M). It
was surprising to discover that there was little binding: 10% to 20%, with
no apparent variation with concentration. Since these compounds are known
to interact with hepatic microsomes, eliciting type 1 spectral changes with
K_s values of 3.45 x 10^{-4} M for octanoate and 0.79 x 10^{-4} M for laurate
(Al-Gailany, 1975), it was assumed that the method of centrifugal sedimentation
is unsuitable for studies of this type. However, further studies, using octanoic acid at higher concentrations ($5 \times 10^{-4}$ M to $5 \times 10^{-2}$ M), suggest that the technique can yield some usable binding data. The results obtained at these higher concentrations are included in table 3:5 and figure 3:5, but it must be stressed that these values are from preliminary measurements only and that conclusions drawn from them must be considered in this light.

The percentage of octanoate in the supernatant varied from 95% to 85% over the concentration range $5 \times 10^{-2}$ M to $5 \times 10^{-4}$ M. The data is presented in figure 3:5 in the form of a modified Scatchard plot. From the values at high fatty acid concentration an affinity constant of $5.06 \times 10^2$ M$^{-1}$ and a total binding capacity of $69.6 \text{ nmole.mg}^{-1}$ were calculated. These values are of the same order of magnitude as those reported by Rickel and Steel (1974) for the interaction of several acidic drugs with hepatic microsomes. If these parameters are correct, remembering that they are calculated from preliminary experiments only, then they must represent 'non-specific' binding to the proteins, lipids and membranes of the microsomal preparation. This is reflected in both the very low affinity constant and the large binding capacity.

Included in figure 3:5 are some of the data points calculated from low fatty acid concentrations. It is possible that these values form a portion of a second set of binding sites. This proposed second set of sites would be characterised by studies with fatty acid over the concentration range $5 - 50 \times 10^6$ M where, unfortunately, we have no results to date. From the available information, it seems that this second set of sites would have a very low capacity, less than $1 \text{ nmole.mg}^{-1}$, but a higher affinity, $K_a = 10^4$ M$^{-1}$.
to $10^5 \text{ M}^{-1}$. These parameters are comparable with the cytochrome P 450 concentration (0.6 nmoles. mg$^{-1}$) and the spectral dissociation constant for the octanoate - P 450 interaction ($K_s = 3.45 \times 10^{-4} \text{ M}$, Al-Gailany, 1975).

Much more work is needed before the technique of centrifuge sedimentation is fully characterised. On theoretical grounds, it seems unlikely that the method would yield good estimates of free drug in the microsomal suspension. The sedimentation of the particulate matter would be expected to disturb the initial equilibrium. The equilibrium would only be unaffected if all the macromolecules of the pellet were freely available to drug in solution or if the bound drug does not dissociate during the 60 min centrifugation. Nevertheless, Bickel and Steel (1974) did show a good correlation between binding parameters obtained from equilibrium dialysis and centrifuge sedimentation techniques.

In conclusion, whilst centrifuge sedimentation is ideal for the analysis of irreversible binding to tissue preparations (Ernster and Orrenius, 1965), the application of the technique to the study of freely reversible interactions should be considered with caution.

3:4:5 FLUORESCENT PROBES

The changes in warfarin fluorescence properties with binding to albumin were first reported by Chignell (1970) and have been well characterised in our laboratories (Wilson, 1974). In the present work, the fluorescence of warfarin and phenprocoumon complexes with human and bovine albumins have been further characterised with special reference to the nature of the
binding sites. In addition, the fluorescent probe technique has been considered as a tool to monitor drug competitions for albumin binding sites.

a) **Coumarins as fluorescent probes of their own binding sites**

The titration of albumin with either PPC or warfarin results in a quenching of the protein fluorescence and a marked enhancement of coumarin fluorescence, as illustrated in figures 3:6 to 3:9. These emission spectra are presented in the uncorrected form, as are all fluorescence data in this report. This fact must be remembered when comparisons are made with published spectra which are corrected or which were obtained using equipment or conditions other than those used here. It was felt unnecessary to correct the fluorescence spectra for the variations in illumination and photomultiplier response since our interest is mainly in modifications in spectra rather than the absolute spectra. The data is also uncorrected for any 'inner filter' effects. This is the phenomenon of absorption of exciting or emitted light by components of the solution under study. For the compounds under study, inner filter effects may occur, since the coumarins absorb light at the wavelengths of both excitation and emission for albumin and also at their own excitation wavelength (self absorption). For these (and other) reasons, coumarin concentrations were kept as low as possible during titrations to minimise changes in optical density (typical O.D. change at 320 nm for a complete titration was about 0.2). In addition, the illumination for albumin fluorescence measurements was at 290 nm where the protein absorbs substantially less light than at 270 - 280 nm.
Phenprocoumon in buffer has about a two fold higher fluorescence intensity than warfarin (see figure 3:6). This disparity, as well as the magnitude of fluorescence, is enhanced upon binding to albumin. At equimolar drug:HSA concentrations the fluorescence of warfarin is enhanced by about 7 fold and that of PPC by over 10 fold. Under the same conditions, but in the presence of BSA, warfarin fluorescence intensity is enhanced by 4 to 5 fold, PPC fluorescence enhancement is about 10 fold, the same as for HSA binding. The wavelength of maximum emission for both coumarins undergoes a blue shift of 5 to 6 nm with binding to either albumin.

The enhancement and spectral shift of ligand fluorescence with binding to protein is thought to be due to the hydrophobic nature of the binding sites. The wavelength of fluorescence emission is dependent upon the loss of energy from the excited electron before it returns to the ground state. Since the excited state has a higher dipole moment than the ground state, solvent re-orientation around the excited electron will be greater in a polar environment, producing more energy loss and emission at a longer wavelength, relative to transitions in a non-polar environment (Chen, 1972). Fluorescence yield is dependent upon the number of excited electrons which return to the ground state via radiationless processes such as internal conversion from the triplet state (see figure 1:5 for an energy level diagram). The dipole moment of the triplet state $T_1$ is less than that of the singlet state $S_1$. As before, solvent re-orientation around $S_1$ will be greater in polar environments, leading to loss in energy and a reduction in the singlet-triplet energy gap. Inter-system crossing is thus greater in polar systems and fluorescence is decreased (Brand and Gohlke, 1972). The binding sites of the coumarins
must then, be either non-polar or must maintain the solvent in a rigid structure which prevents re-orientation (Edelman and McClure, 1968).

The binding of the coumarins to albumin is also associated with a quenching of albumin fluorescence (see figures 3:7, 3:8 and 3:9). Two processes can cause a reduction of albumin fluorescence. Either the binding of coumarin results in some change in the environment of the albumin tryptophan residues (which account for almost all of the protein fluorescence) or there is a transfer of energy from the excited tryptophan molecules. From the fluorescence spectra of the warfarin-HSA or warfarin-BSA complexes excited with light of 290 nm it is difficult to decide which process is taking place. The fluorescence intensity is reduced to about 50% and there is a pronounced red shift in the emission maximum. It is possible that these effects are due to a conformational change leading to the tryptophan residues being more exposed to the polar solvent. The spectra of PPC-HSA and PPC-BSA complexes, however, show clearly that energy transfer is occurring. When the complexes are illuminated with light of 290 nm it is re-emitted at the wavelength of phenprocoumon fluorescence; 380 nm. Energy transfer probably does not contribute significantly to the enhancement of coumarin fluorescence when excited at 320 nm, since few albumin tryptophan residues will be affected by radiation of this wavelength.

Although the presence of energy transfer reactions in the warfarin-albumin complexes is not definitively shown in figures 3:8 and 3:9, the phenomenon does occur, since the excitation spectra of the complex, measured using the emission wavelength of 380 nm, shows peaks at both 290 nm and 320 nm. Chignell (1970) has used the efficiency of energy transfer between HSA tryptophan and warfarin to estimate the distance between the two fluophores
as 3.45 nm.

The greater fluorescence enhancement and the apparently more efficient energy transfer between PPC and albumin compared to warfarin and albumin might suggest that the two coumarins bind to different sites. There is, however, considerable evidence to support the view that these two very similar molecules bind to the same site. In chapter 6 it is shown that phenylbutazone competes for the binding sites of both coumarins, a fact which is supported by the reports that PPC and phenylbutazone (Seiler and Duckert, 1968) and warfarin and phenylbutazone (Aggeler et al., 1967; Solomon et al., 1968) share the same binding sites. Furthermore, recent data has shown that warfarin, phenprocoumon and acenocoumarol all bind to the same class of high affinity site on HSA (Sudlow et al., 1976). Thus, the disparate fluorescence characteristics of PPC and warfarin complexes with albumin must be due to the differing optical characteristics of the two molecules and/or differing modes of binding.

The greater fluorescence enhancement of PPC may be explained by the fact that PPC is bound more avidly by HSA and BSA than is warfarin (see chapters 5 and 6, and O'Reilly, 1971). The tighter binding probably reflects greater hydrophobic interaction between protein and ligand. A more hydrophobic environment is consistent with the greater fluorescence enhancement of PPC. The greater efficiency of energy transfer between albumin and PPC can also be partially explained by a more efficient re-emission of transferred photons due to the more hydrophobic environment of PPC. In addition, energy transfer is dependent upon the orientation of the two participating species. In chapters 5 and 6 it is proposed that the orientation of the main, coumarin ring differs in the PPC-albumin and warfarin-albumin complexes. The
orientation of the PPC coumarin ring bound to albumin may be more favourable for energy transfer than is the warfarin ring when bound.

In summary, phenprocoumon and warfarin are bound to common sites on albumin which are characterised by a hydrophobic nature and a reasonable proximity to tryptophans of bovine and human albumin. Phenprocoumon seems to be the most suitable probe for these sites since it undergoes a more pronounced fluorescence enhancement with binding. The greater fluorescence is probably due to tighter binding of the molecule and a difference in the orientation when bound, compared to warfarin.

The Fluorescent probe approach to monitoring drug interactions

All of the techniques so far described in this chapter can be used in studies of ligand competition for binding sites, provided that an assay is available which is specific for one of the ligands. Competition studies either require a change in experimental routine, as is the case with continuous ultrafiltration, or are simply carried out in the absence and presence of competitor. Standard ultrafiltration of fatty acid-albumin complexes in the presence of various concentrations of competitor has been performed as part of this project (see chapter four) and is typical of the approach normally used in displacement studies. This type of experiment is relatively time consuming and could not practicably be considered for a screening of the great number of clinically possible drug competitions for albumin binding sites. Drug interactions by displacement from albumin binding sites have been postulated to exist for several groups of drugs, including the coumarin oral anticoagulants (Wardell, 1974).
The fluorescence of a warfarin-albumin mixture, excited with light at 320 nm, is due predominantly to the fluorescence of bound drug. If a second ligand is added which competes for albumin binding sites the resulting reduction in bound warfarin will be reflected by a less intense fluorescence. In this section the use of the fluorescent probe properties of warfarin for monitoring clinically relevant drug competitions will be considered. The same approach has been used in studies of the communal binding sites of fatty acids, carbamates and warfarin, (chapter 4) and phenylbutazone and PPC (chapter 6).

In their reviews of drug interactions with coumarin oral anti-coagulants, Koch-Weser and Sellers (1971, 1976) presented data which suggested that sulphonamides may be involved in such interactions but stressed that the evidence was somewhat hypothetical. Two recent reports (Barnett and Hancock, 1975 and Hansen et al., 1975) have suggested that the antibacterial combination co-trimoxazole, which contains the sulphonamide sulphamethoxazole has been responsible for spontaneous haemorrhage in patients on continuous warfarin therapy. In a later report (Hassall et al., 1975) sulphamethoxazole was implicated as the interacting compound when it was shown to cause a small rise in free warfarin when added to a warfarin-HSA solution.

The influence of various concentrations of sulphamethoxazole on human serum albumin enhanced warfarin fluorescence is shown in figure 3:10. While sulphamethoxazole does reduce the fluorescence of warfarin the effects are quite small, even at large excesses of sulphonamide. From a Dixon-type plot of the data (figure 3:11) it can be seen that the displacement is of a 'non-competitive' type. Taken at face value this would suggest that
warfarin and sulphamethoxazole do not share the same binding sites (N.B. competitive-type displacement is expected from a single binding site), but that the displacement must be due to some steric or conformational effect. However, we must be careful not to take too literally the comparison between enzyme kinetic data and the fluorescent enhancement measurements of these experiments.

Brand and his colleagues (1967) have suggested that the percentage of bound drug (x) can be calculated from fluorescence titration data using the formula:

\[
X = \frac{(I_L/I_F) - 1}{(I_H/I_F) - 1} \quad \text{EQUATION 1}
\]

where \(I_F\) is the fluorescence intensity of the free drug (i.e. in buffer) and \(I_L\) and \(I_H\) refer to the intensities in protein-solutions of low and high concentration. Using the fluorescence of warfarin in phosphate buffer and HSA at \(20 \times 10^{-6}\) M the percentage of bound drug in \(5 \times 10^{-6}\) M HSA, with various concentrations of sulphamethoxazole present, was calculated following this equation. Table 3:7 shows the results with some literature values for comparison.

The use of the above equation, and indeed the whole fluorescent probe approach to monitoring drug displacements, requires several fundamental assumptions. Considering equation 1, \(I_H\) is taken to represent the fluorescence of the ligand when totally bound. In these experiments \(I_H\) was measured with an albumin concentration of \(2 \times 10^{-5}\) M, since higher concentrations cannot be used because of concentration quenching effects (Wilson, 1974). It is possible that warfarin is not 100% bound under these
conditions, which would explain why the fluorescent probe derived value of 90% bound (HSA 5 x 10^{-6} M, warfarin 2.5 x 10^{-6} M) is higher than comparable literature values (table 3:7). Additionally, when calculating the warfarin bound in the presence of competitor, it is assumed that the reduction in fluorescence is linearly related to reduced binding. There are several possible situations where this assumption would be false. Warfarin may be displaced, but could bind to other sites which do not enhance fluorescence. Fluorescence could be reduced by inner filter effects, charge transfer to a second ligand bound at another site or a conformational change of the protein with the binding of a second ligand all of which could occur with no modification in warfarin binding. Finally, some warfarin may be bound at sites which are not involved in fluorescence enhancement. Obviously displacement from these sites would not be observed in fluorescence measurements.

The concentrations of warfarin and SMX used in this study cover the clinically observed plasma concentrations. Continuous warfarin therapy in man leads to steady state plasma levels of 0.6 to 3.1 mg.L^{-1} (2 x 10^{-6} M to 1.0 x 10^{-5} M, Breckenridge and Orme, 1972). Following chronic oral administration of SMX a steady state plasma level of 60 mg.L^{-1} (2.3 x 10^{-4} M Nolte and Buttner, 1974) has been reported. (Note that the albumin concentration used, 0.5 x 10^{-5} M, is much lower than the plasma albumin levels of about 5 x 10^{-4} M). It is possible, then, that the plasma SMX:warfarin ratio of a patient under co-therapy could reach over 100:1. The increase in free warfarin under these conditions is very dramatic (see table 3:7). However, in common with all in vitro demonstrations of albumin displacement, it is impossible to predict if a clinically important change in anticoagulant control
would result from the SMX:warfarin competition. Factors such as redistribution and tissue binding, increased elimination and metabolism will all affect the final outcome of the displacement (Wardell, 1974).

Recently, two groups have published data concerning the use of warfarin fluorescence in displacement studies. Henry and Wosilait (1975) tested a series of compounds, at one concentration \((25 \times 10^{-6} \text{ M})\), for their ability to alter the fluorescence of warfarin \((5 \times 10^{-6} \text{ M})\) in HSA solution\((7.24 \times 10^{-6} \text{ M})\) but made no attempt to estimate changes in binding. Sudlow and colleagues (1975) studied the effects of various drugs \((about 15 \times 10^{-6} \text{ M})\) on HSA \((16.4 \times 10^{-6})\) enhanced warfarin \((2 \times 10^{-6} \text{ M})\) fluorescence. They found a good correlation between the percentage reduction in warfarin fluorescence and the reduction in binding measured by equilibrium dialysis. They did not calculate the binding of warfarin in the presence of competitors, from fluorescence measurements, nor did they consider the type of competition involved.

Fluorescent probe monitoring of drug competition for binding sites has several advantages over traditional methods. The technique can be very quick and easy to perform and it uses the minimum of drug and protein. Low concentrations of drug are used which permit clinical levels to be studied and can be useful for compounds which are poorly soluble in water. It is the concentration of bound drug which is monitored, unlike equilibrium dialysis or ultrafiltration where the much lower free concentration has to be assayed. The technique is, then, ideal for the routine screening of drugs for possible interactions with the coumarin oral anticoagulants at the binding site level. However, because of the assumptions and possible sources of error which have been outlined, strongly positive results should be confirmed with a
second technique before action is taken.
CHAPTER FOUR

INVESTIGATIONS INTO THE BINDING OF MEDIUM-CHAIN FATTY ACIDS TO SERUM ALBUMIN
Free fatty acid is the form in which fat is released, from adipose cells, into the blood. Some fatty acid (FA) is associated with lipoproteins in the blood, but the vast majority is transported bound to serum albumin. The binding of fatty acids, thus rendering them more soluble, is one of the most important, physiological roles of albumin. The interaction of human albumin with those fatty acids of physiological importance to man has been studied by several workers: Goodman, 1958; Reynolds et al., 1968; Spector et al., 1969; Ashbrook et al., 1975, and has been the subject of a recent review: Spector, 1975.

A study of the interaction of a homologous series of medium chain length fatty acids is of interest for several reasons. Such a series of molecules, with a constant charged head group and increasingly lipophilic hydrocarbon chain, provides a useful series of model compounds for investigations into the relative importance of ionic and hydrophobic interactions in ligand-protein binding. Also, medium chain length triglycerides are being increasingly used in the dietary treatment of several metabolic diseases of man, and it is important to have a thorough understanding of their interaction with albumin. Finally, several metabolic, transport and absorptive reactions are known to be chain length dependent and it was thought that this trend may be reflected in albumin binding parameters.

The fatty acids used in this study have between five and nine carbon atoms, and the IUPAC names, used throughout this report, are as follows:
pentanoic acid (C₅), hexanoic acid (C₆), heptanoic acid (C₇), octanoic acid (C₈) and nonanoic acid (C₉). The trivial names of these compounds are often used in the literature and are rather more descriptive than their systematic counterparts. Pentanoic acid has the most pungent and disagreeable smell of all fatty acids and is appropriately named valeric acid from the Latin valerum, meaning: to be strong. Hexanoic, octanoic and decanoic acids have the characteristic odour of goats and are named caproic, caprylic and capric acids, respectively, after the Latin caper: the goat. Nonanoic acid is also known as pelargonic acid, since it is present in the essential oil of Pelargonium roseum.

Ultrafiltration measurements of the binding of these fatty acids to Bovine Serum Albumin (BSA) were made under conditions which permit comparison of the results with the data of Wilson (1974) concerning the interactions of a series of aliphatic carbamates with BSA. Ultrafiltration competition measurements were made for the fatty acids with carbamates, warfarin and fatty acids of different chain length. The effects of the various fatty acids upon the fluorescence of BSA, and warfarin, when bound to albumin, were also studied.
Ultrafiltration was performed at 37° C and 10° C using the modified Toribara apparatus, as described in chapter 2. The final BSA (Conn fraction V, Sigma) concentration was always 2% w/v (2.899 x 10^{-4} M assuming a molecular weight of 69,000), whilst the final fatty acid concentration was varied between 5 x 10^{-2} M and 1.7 x 10^{-5} M. The assay of fatty acid concentration was achieved by the addition of a small volume (100 μl) of radiolabelled fatty acid (≈ 2 μCi.ml^{-1}) to each sample, before ultrafiltration, and subsequent liquid scintillation counting of the ultrafiltrate. The mass of the labelled fatty acid and the dilution of the sample were taken into account in all data calculations. The binding of fatty acid in the presence of warfarin, carbamate or other fatty acid was measured in the same manner.

The quenching of the tryptophan fluorescence of BSA (Conn fraction V) by fatty acid was investigated by titration, as described in chapter 2. The influence of fatty acids upon the fluorescence characteristics of warfarin when bound to BSA or HSA was also studied. For fluorescence measurements, the albumin concentration was normally 1 x 10^{-5} M. Microlitre additions were made from stock solutions of 1 x 10^{-3} M, 1 x 10^{-2} M or 1 x 10^{-1} M for fatty acid and 2 x 10^{-3} M for warfarin.

The FA/BSA interactions are illustrated in the form of Scatchard (1949) plots. Binding parameters were computed from the data using the Modfit 27 computer programme, as described in section 2:2:14. Two models were chosen to describe the interactions, both of which were based upon two classes of binding sites. In model 1, the coefficients n_1, n_2, K_1 and K_2
were allowed to take any value. In model 2, $n_1$ was fixed as 1, i.e. a single high affinity binding site was assumed. An estimate of the 'goodness' of the fit can be obtained from the 'S' value which describes the nearness of the computed curve to a perfect parabola (where $S = 1.0000$).

The partition coefficients of the fatty acids were calculated using the method of Leo et al. (1971). A $\Delta \log P$ value of 0.50 was assumed for each incremented change of one $\text{CH}_2$ unit and values were calculated from $\log P (\text{octanol/water}) = 1.94$ for hexanoic acid. The value for hexanoic acid is a mean of several measured and calculated estimates from Leo et al. (1971).
Figure 4:1 Scatchard plots for the interaction of Pentanoate (Valerate) with BSA at $10^\circ C$ and $37^\circ C$.

The data points represent the means of at least four experimental values at differing total pentanoate concentrations.
The lines join the computed points for the same total concentrations.
The units of the y axes are $M^{-1} \times 10^{-3}$. 
Figure 4.2 Scatchard plots of the interaction of Octanoate with BSA at 10°C and 37°C.

The data points represent the means of at least four experimental values at differing total octanoate concentrations.

The lines join the computed points for the same total concentrations.

The units of the y axes are $M^{-1} \times 10^{-3}$. 
Figure 4:3 Scatchard plots for the interaction of Hexanoate, Heptanoate and Nonanoate with BSA.

The data points represent the means of at least four experimental values at differing total fatty acid concentrations. The lines join the computed points for the same total concentrations. The units of the y axes are $M^{-1} \times 10^{-3}$. 
Figure 4.4 The relationship between the binding parameters of the fatty acid/BSA complex and the partition coefficients of the fatty acids.

The binding parameters were measured at 10°C (△) and 37°C (■).
Figure 4:5  Uncorrected spectra of BSA in the presence of warfarin and octanoate

1: BSA ($1 \times 10^{-5}$ M)
2: BSA + Warfarin ($8 \times 10^{-6}$ M)
3-9: BSA + Warfarin + Octanoate
0.4, 0.8, 1.2, 1.6, 3.2, 6.0, 10.0 $\times 10^{-4}$ M.

The excitation wavelength was 290 nm.
Table 4:1  Some physico-chemical parameters of the medium chain fatty acids

<table>
<thead>
<tr>
<th>NAME</th>
<th>Formula</th>
<th>Molecular Weight</th>
<th>m.p.</th>
<th>Water solubility g.L⁻¹ 20° C</th>
<th>Critical micelle concentration M 25° C</th>
<th>Partition coefficient as log.P</th>
<th>pKₐ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentanoic acid</td>
<td>CH₃(CH₂)₃COOH</td>
<td>102.13</td>
<td>-34.5</td>
<td>37</td>
<td>6.4</td>
<td>1.44</td>
<td>4.86</td>
</tr>
<tr>
<td>Hexanoic acid</td>
<td>CH₃(CH₂)₄COOH</td>
<td>116.15</td>
<td>-3.4</td>
<td>9.7</td>
<td>1.60</td>
<td>1.94</td>
<td>4.88</td>
</tr>
<tr>
<td>Heptanoic acid</td>
<td>CH₃(CH₂)₅COOH</td>
<td>130.18</td>
<td>-10.5</td>
<td>2.4</td>
<td>0.8</td>
<td>2.44</td>
<td>4.89</td>
</tr>
<tr>
<td>Octanoic acid</td>
<td>CH₃(CH₂)₆COOH</td>
<td>144.21</td>
<td>16.7</td>
<td>0.68</td>
<td>0.4</td>
<td>2.94</td>
<td>4.90</td>
</tr>
<tr>
<td>Nonanoic acid</td>
<td>CH₃(CH₂)₇COOH</td>
<td>158.23</td>
<td>12.5</td>
<td>0.26</td>
<td>0.2</td>
<td>3.44</td>
<td>4.95</td>
</tr>
</tbody>
</table>

Data was obtained from the following sources: C.M.C.'s: Kleven (1948), partition coefficients: see experimental approach, pKₐ's: Dippy (1938) All other data Markley (1960).
Table 4:2  Binding parameters of the Bovine Serum Albumin – fatty acid anion interaction at 10°C.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$n_1\times$10$^4$ M$^{-1}$</th>
<th>$n_2\times$10$^2$ M$^{-1}$</th>
<th>$n_3\times$10$^3$ M$^{-1}$</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentanoate</td>
<td>1.23 ± 0.10</td>
<td>2.36 ± 0.17</td>
<td>8.73 ± 0.89</td>
<td>1.039</td>
</tr>
<tr>
<td>Hexanoate</td>
<td>1.85 ± 0.27</td>
<td>2.15 ± 0.24</td>
<td>3.81 ± 0.22</td>
<td>1.039</td>
</tr>
<tr>
<td>Heptanoate</td>
<td>2.15 ± 0.27</td>
<td>3.81 ± 0.22</td>
<td>7.59 ± 0.81</td>
<td>1.039</td>
</tr>
<tr>
<td>Octanoate</td>
<td>7.31 ± 0.24</td>
<td>10.34 ± 0.73</td>
<td>43.4 ± 5.49</td>
<td>1.039</td>
</tr>
<tr>
<td>Nonanoate</td>
<td>89.5 ± 4.57</td>
<td>109.5 ± 4.57</td>
<td>43.4 ± 5.49</td>
<td>1.039</td>
</tr>
</tbody>
</table>

Determined from ultrafiltration experiments with a BSA concentration of 2.8 x 10$^{-4}$ M. Parameters were generated from the data using the Modfit 27 programme, model 2.
Table 4:3  Binding parameters of the Bovine Serum Albumin - fatty acid anion interaction at 37°C

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$n_1$</th>
<th>$K_1 \times 10^4$ M$^{-1}$</th>
<th>$n_2$</th>
<th>$K_2 \times 10^3$ M$^{-1}$</th>
<th>$n_2K_2 \times 10^3$ M$^{-1}$</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentanoate</td>
<td>1</td>
<td>1.29 ± 0.05</td>
<td>5.27 ± 1.79</td>
<td>0.388 ± 0.133</td>
<td>2.045</td>
<td>1.024</td>
</tr>
<tr>
<td>Hexanoate</td>
<td>1</td>
<td>2.39 ± 0.11</td>
<td>2.76 ± 0.37</td>
<td>1.60 ± 0.34</td>
<td>4.416</td>
<td>1.033</td>
</tr>
<tr>
<td>Heptanoate</td>
<td>1</td>
<td>4.14 ± 0.42</td>
<td>4.45 ± 0.50</td>
<td>3.08 ± 0.39</td>
<td>13.71</td>
<td>1.054</td>
</tr>
<tr>
<td>Octanoate</td>
<td>1</td>
<td>45.5 ± 1.64</td>
<td>7.45 ± 0.65</td>
<td>3.70 ± 0.41</td>
<td>27.57</td>
<td>1.022</td>
</tr>
<tr>
<td>Nonanoate</td>
<td>1</td>
<td>49.3 ± 1.52</td>
<td>9.83 ± 0.72</td>
<td>4.56 ± 0.57</td>
<td>44.82</td>
<td>1.029</td>
</tr>
</tbody>
</table>

See table 4:2 for the source of the parameters
Table 4.4  Comparison of binding models 1 and 2 for the interaction of fatty acid anions with BSA.
First class of sites, at 37°C.

<table>
<thead>
<tr>
<th>LIGAND</th>
<th>MODEL 2</th>
<th></th>
<th>MODEL 1</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( n_1 )</td>
<td>( K_1 )</td>
<td>( n_1 )</td>
<td>( K_1 \times 10^4 \text{ M}^{-1} )</td>
</tr>
<tr>
<td>Pentanoate</td>
<td>1</td>
<td>1.29 ± 0.05</td>
<td>1.21 ± 0.22</td>
<td>1.09 ± 0.18</td>
</tr>
<tr>
<td>Hexanoate</td>
<td>1</td>
<td>2.39 ± 0.11</td>
<td>1.48 ± 0.17</td>
<td>1.74 ± 0.19</td>
</tr>
<tr>
<td>Heptanoate</td>
<td>1</td>
<td>4.14 ± 0.42</td>
<td>1.67 ± 0.09</td>
<td>3.44 ± 0.19</td>
</tr>
<tr>
<td>Octanoate</td>
<td>1</td>
<td>44.5 ± 1.64</td>
<td>1.00 ± 0.09</td>
<td>45.3 ± 4.9</td>
</tr>
<tr>
<td>Nonanoate</td>
<td>1</td>
<td>49.3 ± 1.52</td>
<td>0.167 ± 0.198</td>
<td>420.0 ± 944</td>
</tr>
</tbody>
</table>
**Table 4:5** Thermodynamic parameters of the interaction of BSA with fatty acid anions

<table>
<thead>
<tr>
<th>LIGAND and class of site</th>
<th>$\Delta G$ (free energy)</th>
<th>$\Delta H$ (enthalpy)</th>
<th>$\Delta S$ (entropy)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$10^\circ C$</td>
<td>$37^\circ C$</td>
<td></td>
</tr>
<tr>
<td><strong>First class</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pentanoate</td>
<td>-5645</td>
<td>-5827</td>
<td>-3732</td>
</tr>
<tr>
<td>Hexanoate</td>
<td>-5901</td>
<td>-6207</td>
<td>-2697</td>
</tr>
<tr>
<td>Heptanoate</td>
<td>-6134</td>
<td>-6545</td>
<td>-1821</td>
</tr>
<tr>
<td>Octanoate</td>
<td>-7589</td>
<td>-8021</td>
<td>-3059</td>
</tr>
<tr>
<td>Nonanoate</td>
<td>-7702</td>
<td>-8070</td>
<td>-3848</td>
</tr>
<tr>
<td><strong>Second class</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pentanoate</td>
<td>-3616</td>
<td>-3670</td>
<td>-</td>
</tr>
<tr>
<td>Hexanoate</td>
<td>-3806</td>
<td>-4542</td>
<td>+3909</td>
</tr>
<tr>
<td>Heptanoate</td>
<td>-4409</td>
<td>-4945</td>
<td>+1219</td>
</tr>
<tr>
<td>Octanoate</td>
<td>-4507</td>
<td>-5088</td>
<td>+1268</td>
</tr>
<tr>
<td>Nonanoate</td>
<td>-4707</td>
<td>-5187</td>
<td>+319</td>
</tr>
</tbody>
</table>

The thermodynamic parameters were calculated from the association constants shown in tables 4:2 and 4:3.
Table 4.6  The effect of fatty acids on the fluorescence of Bovine Serum Albumin

<table>
<thead>
<tr>
<th>Concentration of fatty acid x 10^{-4} M</th>
<th>Percentage fluorescence of Albumin (with the wavelength of maximum emission)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pentanoate</td>
</tr>
<tr>
<td>0</td>
<td>100 (341)</td>
</tr>
<tr>
<td>0.4</td>
<td>99.5</td>
</tr>
<tr>
<td>0.8</td>
<td>-</td>
</tr>
<tr>
<td>1.2</td>
<td>97.4</td>
</tr>
<tr>
<td>1.6</td>
<td>-</td>
</tr>
<tr>
<td>2.0</td>
<td>97.2</td>
</tr>
<tr>
<td>2.4</td>
<td>-</td>
</tr>
<tr>
<td>4.0</td>
<td>96.1</td>
</tr>
<tr>
<td>6.0</td>
<td>94.4</td>
</tr>
<tr>
<td>8.0</td>
<td>-</td>
</tr>
<tr>
<td>10.0</td>
<td>-</td>
</tr>
</tbody>
</table>

The BSA concentration was 1 x 10^{-5} M. An excitation wavelength of 290 nm was used. The wavelength of maximum fluorescence is only indicated if different from the initial value of 341 nm.
Table 4:7  The effect of fatty acid on the fluorescence of warfarin bound to albumin

<table>
<thead>
<tr>
<th>Ligand concentration $\times 10^{-4}$ M</th>
<th>Pentanoate</th>
<th>Hexanoate</th>
<th>Heptanoate</th>
<th>Octanoate</th>
<th>Nonanoate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BSA</td>
<td>BSA</td>
<td>BSA</td>
<td>HSA</td>
<td>BSA</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.4</td>
<td>89.1</td>
<td>87.8</td>
<td>88.8</td>
<td>-</td>
<td>90.0</td>
</tr>
<tr>
<td>0.8</td>
<td>82.4</td>
<td>82.7</td>
<td>83.6</td>
<td>85.8</td>
<td>86.5</td>
</tr>
<tr>
<td>1.6</td>
<td>75.6</td>
<td>75.5</td>
<td>76.1</td>
<td>78.7</td>
<td>-</td>
</tr>
<tr>
<td>2.4</td>
<td>73.1</td>
<td>72.4</td>
<td>72.4</td>
<td>72.8</td>
<td>72.0</td>
</tr>
<tr>
<td>3.2</td>
<td>-</td>
<td>69.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4.0</td>
<td>67.2</td>
<td>67.3</td>
<td>67.2</td>
<td>-</td>
<td>65.5</td>
</tr>
<tr>
<td>6.0</td>
<td>63.9</td>
<td>64.8</td>
<td>62.7</td>
<td>62.7</td>
<td>58.5</td>
</tr>
<tr>
<td>8.0</td>
<td>62.2</td>
<td>62.8</td>
<td>59.7</td>
<td>59.8</td>
<td>54.0</td>
</tr>
</tbody>
</table>

Results are expressed as percentages of the fluorescence of 0.16 $\times 10^{-4}$ M warfarin in 0.1 $\times 10^{-4}$ M albumin, in the absence of fatty acid. The excitation wavelength was 320 nm, the wavelength of maximum emission was 378 nm in all cases.
<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Approximate ratios</th>
<th>FA/Albumin</th>
<th>Percentage bound</th>
<th>Warfarin</th>
<th>Octanoate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>x10^-3 M</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final concentrations</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.67</td>
<td>-</td>
<td>6:1</td>
<td>65.69 ± 0.33 ***</td>
<td>78.16 ± 0.39 ***</td>
<td></td>
</tr>
<tr>
<td>0.83</td>
<td>2:1</td>
<td>6:1</td>
<td>53.64 ± 0.44 ***</td>
<td>65.23 ± 0.47 ***</td>
<td></td>
</tr>
<tr>
<td>1.67</td>
<td>1:1</td>
<td>6:1</td>
<td>48.47 ± 0.57 ***</td>
<td>61.56 ± 0.59 ***</td>
<td></td>
</tr>
<tr>
<td>2.50</td>
<td>1:1.5</td>
<td>4:1</td>
<td>49.15 ± 1.01 ***</td>
<td>59.51 ± 0.26 ***</td>
<td></td>
</tr>
<tr>
<td>0.167</td>
<td>0:6.1</td>
<td></td>
<td>85.42 ± 0.27 ***</td>
<td>98.05 ± 0.07 ***</td>
<td></td>
</tr>
<tr>
<td>0.167</td>
<td>0:1.67</td>
<td></td>
<td>84.71 ± 0.49 n.s.</td>
<td>97.90 ± 0.22 n.s.</td>
<td></td>
</tr>
<tr>
<td>0.83</td>
<td>0:6.1</td>
<td></td>
<td>77.32 ± 1.08 ***</td>
<td>95.69 ± 0.24 ***</td>
<td></td>
</tr>
<tr>
<td>1.67</td>
<td>0:1.5</td>
<td></td>
<td>67.50 ± 0.76 ***</td>
<td>92.32 ± 0.24 ***</td>
<td></td>
</tr>
<tr>
<td>2.50</td>
<td>1:1.5</td>
<td></td>
<td>55.31 ± 0.80 ***</td>
<td>87.83 ± 0.35 ***</td>
<td></td>
</tr>
<tr>
<td>0.167</td>
<td>0:6.1</td>
<td></td>
<td>98.34 ± 0.19 ***</td>
<td>94.25 ± 0.23 ***</td>
<td></td>
</tr>
<tr>
<td>0.167</td>
<td>0:6.1</td>
<td></td>
<td>98.00 ± 0.22 n.s.</td>
<td>96.38 ± 0.39 ***</td>
<td></td>
</tr>
<tr>
<td>0.167</td>
<td>0:6.1</td>
<td></td>
<td>94.25 ± 0.23 ***</td>
<td>91.15 ± 0.47 (3) ***</td>
<td></td>
</tr>
</tbody>
</table>

**Table 4.8 The effect of warfarin on the percentage of fatty acids bound to albumin.**

**The albumin (HSA) concentration was 2.5 x 10^-4 M in phosphate buffer, 67 mM pH 7.4. Results are expressed as mean ± SEM (n = 4, unless specified). Statistical comparisons, to binding without warfarin, by the students t-test, n.s. = p > 0.05, * = p < 0.01, ** = p < 0.001, *** = p < 0.0001.
Table 4.9  The competition of carbamates and fatty acids for albumin binding sites

<table>
<thead>
<tr>
<th>Concentrations x 10^{-4} M</th>
<th>Approximate ratios</th>
<th>Percentage of ligand bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ligand</td>
<td>Competitor</td>
<td>Ligand/albumin</td>
</tr>
<tr>
<td>Pentanoate</td>
<td>nButyl-Carbamate</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>-</td>
<td>1:25</td>
</tr>
<tr>
<td>0.1</td>
<td>0.083</td>
<td>1:25</td>
</tr>
<tr>
<td>0.1</td>
<td>0.33</td>
<td>1:25</td>
</tr>
<tr>
<td>0.1</td>
<td>1.67</td>
<td>1:25</td>
</tr>
<tr>
<td>0.1</td>
<td>33.0</td>
<td>1:25</td>
</tr>
<tr>
<td>1.0</td>
<td>-</td>
<td>1:2.5</td>
</tr>
<tr>
<td>1.0</td>
<td>1.67</td>
<td>1:2.5</td>
</tr>
<tr>
<td>1.0</td>
<td>8.33</td>
<td>1:2.5</td>
</tr>
<tr>
<td>1.0</td>
<td>16.7</td>
<td>1:2.5</td>
</tr>
<tr>
<td>1.0</td>
<td>33.0</td>
<td>1:2.5</td>
</tr>
<tr>
<td>nOctyl-Nonanoate</td>
<td>Nonanoate</td>
<td>1:5</td>
</tr>
<tr>
<td>0.5</td>
<td>-</td>
<td>1:5</td>
</tr>
<tr>
<td>0.5</td>
<td>0.5</td>
<td>1:5</td>
</tr>
<tr>
<td>0.5</td>
<td>2.5</td>
<td>1:5</td>
</tr>
<tr>
<td>0.5</td>
<td>5.0</td>
<td>1:5</td>
</tr>
<tr>
<td>0.5</td>
<td>10.0</td>
<td>1:5</td>
</tr>
</tbody>
</table>

The albumin (BSA) concentration was 2.5 x 10^{-4} M in phosphate buffer, 66 mM pH 7.4 (20°C). Results are expressed as mean ± SEM (n = 4). Comparisons to binding without competitor by students 't' test. n.s. = p > 0.05, * = p < 0.05, ** = p < 0.01, *** = p < 0.001.
<table>
<thead>
<tr>
<th>Concentrations x 10⁻⁴ M</th>
<th>Approximate ratios</th>
<th>Percentage of ligand bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ligand</td>
<td>Competitor</td>
<td>Ligand/albumin</td>
</tr>
<tr>
<td>Octanoate</td>
<td>Pentanoate</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>-</td>
<td>1:25</td>
</tr>
<tr>
<td>0.1</td>
<td>0.5</td>
<td>1:25</td>
</tr>
<tr>
<td>0.1</td>
<td>10.0</td>
<td>1:25</td>
</tr>
<tr>
<td>0.1</td>
<td>100.0</td>
<td>1:25</td>
</tr>
<tr>
<td>0.1</td>
<td>1000.0</td>
<td>1:25</td>
</tr>
<tr>
<td>10.0</td>
<td>-</td>
<td>4:1</td>
</tr>
<tr>
<td>10.0</td>
<td>10.0</td>
<td>4:1</td>
</tr>
<tr>
<td>10.0</td>
<td>25.0</td>
<td>4:1</td>
</tr>
<tr>
<td>10.0</td>
<td>500.0</td>
<td>4:1</td>
</tr>
<tr>
<td>10.0</td>
<td>1000.0</td>
<td>4:1</td>
</tr>
<tr>
<td>Pentanoate</td>
<td>Octanoate</td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>-</td>
<td>1:10</td>
</tr>
<tr>
<td>0.25</td>
<td>0.125</td>
<td>1:10</td>
</tr>
<tr>
<td>0.25</td>
<td>0.25</td>
<td>1:10</td>
</tr>
<tr>
<td>0.25</td>
<td>1.25</td>
<td>1:10</td>
</tr>
<tr>
<td>0.25</td>
<td>2.50</td>
<td>1:10</td>
</tr>
<tr>
<td>0.25</td>
<td>250.0</td>
<td>1:10</td>
</tr>
<tr>
<td>25.0</td>
<td>-</td>
<td>10:1</td>
</tr>
<tr>
<td>25.0</td>
<td>2.50</td>
<td>10:1</td>
</tr>
<tr>
<td>25.0</td>
<td>25.0</td>
<td>10:1</td>
</tr>
<tr>
<td>25.0</td>
<td>125.0</td>
<td>10:1</td>
</tr>
</tbody>
</table>

The albumin (BSA) concentration was 2.5 x 10⁻⁴ M in phosphate buffer, 66 mM pH 7.4 (20°C). Results are expressed as mean ± SEM (n = 4). Comparisons to binding without competitor by students 't' test. n.s. = p > 0.05, * = p < 0.05, ** = p < 0.01, *** = p < 0.001.
4:4 RESULTS AND DISCUSSION

Much of this chapter is devoted to a study of the binding of medium chain fatty acid anions to Bovine Serum Albumin using the ultrafiltration technique. The reasons for examining the binding of a series of fatty acids have already been explained in the introduction to this chapter and in section 1.8, but no mention has yet been made of the reasons for the choice of technique or protein. Ultrafiltration and Bovine Albumin were used initially to retain continuity with previous work in the department (Wilson, 1974) but both can be justified in other ways. Ultrafiltration has the admirable qualities of being quick, relatively simple and cheap, and for these reasons is the method of choice from a practical viewpoint. Ultrafiltration has been shown to be sound theoretically and suitable for the study of a wide range of ligands (Wilson, 1974). The technique has the advantage over dialysis that there can be no Donnan inequalities, but the volume of ultrafiltrate must be kept small so as to avoid disturbing the ligand-protein equilibrium.

If the conclusions from binding studies are to be applied to clinical situations then human albumin, or better still, human plasma, should be used as the protein component. Whilst there is some interest in medium chain fatty acid binding with relevance to medium chain triglyceride diets for certain human disorders, the main aim of this investigation is to characterise the molecular nature of the interaction. The final choice of BSA is largely for convenience, and financial reasons. The protein is plentiful and inexpensive and as such has been used in the majority of in vitro binding experiments. In addition, Bovine and Human Serum Albumins are structurally very similar (see section 1.2.5) and their binding properties
are normally qualitatively, if not always quantitatively, similar. Therefore, any general conclusions which are drawn from these studies with BSA can probably be applied to HSA binding.

The BSA in this work was used as supplied (Conn fraction V, Sigma Chemical Co.) and not defatted in any way. The fatty acid content of this particular preparation is not known, but previous studies of Conn fraction V BSA have estimated the FA content as 0.2 to 0.5 moles FA/mole albumin (Chen, 1967). It may seem anomalous to study the binding of fatty acids to albumin which already has some fatty acid associated with it, but many methods of defatting are rather 'harsh'. Even the most gentle, charcoal treatment, which is claimed to have no deleterious effects upon the protein (Chen, 1967), has come in for some criticism. Steinhardt and co-workers (1972) used this method to defat BSA and concluded "the defatted protein, even after restoration of fatty acid, is irreversibly changed from the protein which has not undergone the defatting procedure". It is possible then that the advantages of removing fatty acid to produce a naked albumin are outweighed by the disadvantage of having a protein molecule which is no longer in its native form. In addition, it can be argued that albumin always has associated with it a small amount of structurally important fatty acid and that any method of removal will produce a 'non-physiological' protein molecule. The group of Soler-Argilaga have demonstrated that albumin bound fatty acid has two forms, one pool has a high turnover rate, the other a very slow turnover rate. (Soler-Argilaga et al., 1975; Soler-Argilaga and Himberg, 1976). They showed that perfused rat liver would remove 45% of fatty acid at a ratio of 1.3 : 1 with albumin, but only 0.3% when the ratio was 0.35 : 1.
ULTRAFILTRATION DATA

From ultrafiltration experiments, the concentration of free fatty acid at various total fatty acid levels, in the presence of 2% (w/v) BSA, was estimated. The data was plotted in the form of Scatchard graphs, which were non-linear in all cases. The non-linearity was taken to represent the presence of two classes of binding sites, the simplest explanation of a curved Scatchard. Initial estimates of the number of sites and the average association constant for each class were made by the method of Berson and Yallow (1959) from the Scatchard plots. The binding data was then fitted to the two-site Scatchard equation using the Modfit 27 computer program. The computer derived binding parameters are given in tables 4:2 and 4:3 for the interactions at 10°C and 37°C. These values are those derived using model 2 (see section 4:2), where \( n_1 \) is set at 1. Table 4:4 shows a comparison of some of the parameters from model 2 with the equivalent values using model 1, where all the coefficients can take any value. The product \( n_1K_1 \) is very constant, regardless of the model chosen. The parameters for nonanoate at 37°C illustrate well one of the advantages of predetermining \( n_1 \) as 1. The computer fit, in this case, gave \( n_1 = 0.17 \pm 0.2 \), which is obviously the product of a false minimum in the least squares regression. Such false estimates are removed by stipulating \( n_1 = 1 \). In addition, by setting \( n_1 \) as 1, the primary association constant \( K_1 \) can be compared with the first association constant of the stepwise approach to data analysis.

The binding data is illustrated, in the form of Scatchard diagrams, in figures 4:1 to 4:3. In these diagrams, the points represent the experimental data, each point being a mean of at least four separate determinations of
free drug at a particular total drug concentration. The line of the graph joins the best fit, computed values of $r$ and $\frac{r}{D_f}$ at the same total drug concentrations as the experimental points. It can be seen from these graphs, that the computerised best fit often 'ignores' those data points at very high $r$ values. This is particularly noticeable with the shorter chain length fatty acids (see figures 4:1a, b and 4:3a, b). The phenomenon is probably caused by two factors. Firstly, the data at high $r$ values tends to have a greater error associated with it, since the concentration of ligand bound is very small, relative to the estimated free concentration. For instance, at a total ligand concentration of 50 mM, free drug concentrations estimated as 49.50 mM and 49.75 mM give bound drug values of 500 $\mu$M and 250 $\mu$M, which is very poor reproducibility. Thus, because of the weighting system of the computer program and these relatively large errors, data at high $r$ values is given little weight in the final fit. This, possibly, shows an inadequacy of the computer program, since however variable high $r$ values may be, they are nevertheless essential components of the complete binding curve. Secondly, it is possible that two classes of sites does not adequately describe the interaction between fatty acids and BSA. Preliminary investigations have shown that a model with three sets of binding sites may more accurately fit the experimental data. However, the errors of the binding parameters of the third class of sites are so large as to make them almost useless. For example, the interaction of nonanoate with BSA fits very well ($S = 1.0000$) to a model with three sets of sites where $n_1 = 1.3 \pm 0.12$, $n_2 = 8.7 \pm 1.4$, $n_3 = 10.5 \pm 10.2$ and $k_3 = 55.2 \pm 111.9 \text{M}^{-1}$, but little significance can be attached to the coefficients $n_3$ and $k_3$. 
The binding parameters of the BSA - fatty acid complex were profoundly affected by the chain length of the ligand and by the temperature at which the equilibrium was studied. The affinity constants and number of binding sites increased with increasing chain length but the transitions were very different for the primary and secondary classes of binding. The secondary affinity constants and number of sites increased smoothly with chain length. There is a good correlation between \( \log n_2 K_2 \) and \( \log P \), as shown in figure 4:4b. In contrast, \( n_1 \) remained relatively constant with chain length, and the relationship between \( \log K_1 \) and \( \log P \) is not linear (figure 4:4a).

There is a smooth increase of \( K_1 \) from pentanoate to heptanoate, but there is a sharp increase in affinity for the octanoate - BSA complex. This sharp increase is seen regardless of the manner of representing the interactions. Thus, graphs of \( K_1 \) vs. \( P \), \( K_1 \) vs. chain length, \( \Delta G \) vs. \( \log P \) etc. all show a sharp break at octanoate, for measurements at both \( 10^0 \) C and \( 37^0 \) C.

The increased avidity of octanoate binding is probably not due to any intrinsic property of the fatty acid, since the physico-chemical characteristics of the series of fatty acids change smoothly with chain length (see table 4:1).

The interaction of fatty acids with serum albumin has been investigated by several groups. Major contributions have come from the following publications: for the binding of long chain (\( C > 12 \)) fatty acids to BSA: Spector et al., 1969, 1971; long chain fatty acids to HSA: Goodman, 1958; Ashbrook et al., 1975; medium chain fatty acids to BSA: Boyer et al., 1946; Terisi and Luck, 1952; Reynolds et al., 1968; medium chain fatty acids to HSA: Ashbrook et al., 1972. From this data several authors have correlated the chain length of the ligand with the strength of binding. Hansch (1968)
used the data of Terisi and Luck (1952), concerning medium chain fatty acid binding to BSA, and found a good correlation between binding affinity and the lipophilicity of the ligand, as represented by the octanol/water partition coefficient. This conclusion and similar findings by other authors (e.g., Reynolds et al., 1968) has led to the common conception that fatty acid binding is linearly related to chain length or lipophilicity. Our findings of a sudden increase in affinity for the transition from C7 to C8 was, therefore, a surprising one. However, thorough reading of the literature reveals that other workers have found related phenomena but have, perhaps, not expanded upon them as fully as might be expected and have certainly not offered any convincing explanations.

The substantial data of Ashbrook and his colleagues (1972, 1974) concerning FA binding to HSA has been summarised recently by Spector (1975). The relationship between log.Ka (association constants derived from the multiple equilibrium approach) and chain length was found to be sigmoidal, not linear. Increasing the chain length from C10 to C12 produced a surprisingly large increase in affinity for HSA. Spector (1975) expressed the opinion that the phenomenon is due to the 'configurational adaptability' of the binding site being greatest for lauric acid (C12) but offered no further explanation. In a survey of alkyl sulphate and sulphonate binding to BSA, Steinhardt and Reynolds (1969) considered that multiple contacts of the alkyl chain were responsible for high energy binding and that chains of less than eight carbons may be incapable of multiple contacts and thus have a reduced binding affinity. The same authors, however, concluded that carboxylates and sulphates or sulphonates probably do not have the same binding sites.
Tanford (1972) has considered the role of chain length in medium chain fatty acid binding to BSA using the data of Terlisi and Luck (1952) and Reynolds et al. (1968). A graph of the free energy of binding against chain length showed a break point at C₈, in common with our results. In this case, however, the break was associated with a less than expected increase in ΔG, the opposite finding to our results. Tanford (1972) suggested that it becomes increasingly difficult to find suitable locations for the hydrocarbon chain as the length is increased beyond seven carbons. It is probable, however, that this analysis is not based upon high affinity binding. Both groups, from which Tanford drew his data, did not examine FA:albumin ratios of less than 1:1. It is likely, then, that the high affinity sites were not fully explored. This explanation is supported by the fact that both groups reported four to seven primary binding sites, rather than the one to two found in our work. The analysis of fatty acid binding affinity, correlated with lipophilicity, by Hansch (1968), using the data of Terlisi and Luck (1952), must also be based upon secondary binding.

There have, then, been several previous observations of non-linear relationships between affinity for albumin and chain length of ligand, but no satisfactory explanation of the phenomenon has been presented. There are several theories which can explain the enhanced primary binding of octanoate relative to the shorter fatty acids. The proposals can be divided into two groups, one of which is based upon a single site binding all the fatty acids, the other postulates the existence of different sites for fatty acids of different chain lengths.

If all the fatty acids bind to the same high affinity site on the albumin molecule then the enhanced binding of octanoate could be explained by one
of the following possibilities: a) The binding site possesses at least two areas of hydrophobicity. This concept is illustrated below:

The binding site is portrayed as a cleft in the protein including a positively charged area with which the polar head group of the fatty acid is associated, and a groove of non-polar character. In the middle of the groove is an area of particularly high lipophilicity. Only fatty acids of eight carbons or more can associate with the area of high hydrophobicity. Such an arrangement would explain the recent findings of Ruf and Gratzl (1976) concerning the interaction of nitroxide spin labelled stearate to BSA. They reported that the polar head group is rigidly fixed and available to solvent, the middle of the molecule is also rigidly held but is shielded from solvent and the non-polar end of the ligand is more available to solvent and has a greater mobility. b) The binding of fatty acids with eight or more carbon atoms induces a conformational change in the protein leading to a greater stability of the complex. This is equivalent to the proposal of Spector (1975) to explain the non-linear relationship of log $K_i$ to chain length for the binding of fatty acids to HSA. The theory is also comparable with the idea of configurational adaptability of binding sites proposed by Karush (1950). c) High affinity binding may be dependent upon the contact of the ligand with several areas of the protein. Fatty acids of less than eight carbon atoms may not be able to make such multiple contacts.
This "inch worm" type of binding has been proposed by Tanford (1972) for amphiphile binding to albumin. The inability of short alkyl chains to form multiple contacts with proteins has been suggested to explain the reduced binding of sulphates and sulphonates with fewer than eight carbon atoms (Steinhardt and Reynolds, 1969).

Any alternative theory, involving two types of high affinity sites for short and medium chain length fatty acids, must include an explanation of why the short chain fatty acids do not interact with the site for the medium chain compounds and vice versa. Whilst it is easy to imagine the short chain site being too small to accommodate more than seven carbons, it is difficult to explain why short chain fatty acids could not 'fit' a larger site. It is possible that the distance between the point of attachment of the carboxyl group to the protein and the area to which the side chain binds is greater than 7 carbons in length. The area of side chain binding would have to have a very high affinity for hydrocarbon chains, since octanoate, which would just bridge the proposed gap, has a much larger association constant than the shorter chain length compounds. Further consideration will be given to these theories of fatty acid binding in the final discussion to this chapter, after competition and fluorescence data has been presented.

The temperature dependence of the binding equilibrium between FA and albumin is illustrated in the Scatchard plots of valerate/BSA and octanoate/BSA (figures 4:1 and 4:2) and in the binding parameters at 10° C (table 4:2) and 37° C (table 4:3). The effect of temperature is consistent over the range of fatty acids. The primary binding association constant (K₁) is greater at 10° C than at 37° C whilst the converse relationship holds for
secondary binding. This disparity is reflected in the thermodynamic parameters of the interactions (table 4:5) where the high affinity binding is characterised by a negative enthalpy change (ΔH) and the secondary binding by a positive ΔH.

The enthalpy change for the high affinity binding is relatively constant and is probably due to the binding of the carboxylate group to the protein. The increasingly favorable free energy change (ΔG) with increasing chain length is due to the greater, positive entropy change (ΔS). Positive entropy changes in ligand binding is normally assumed to reflect hydrophobic interactions (Tanford, 1973) but Klotz (1973) has shown that electrostatic interactions can also be entropically driven. However, it is likely that the ΔS values reflect hydrophobic interactions in this case, since they increase with chain length. Thus, fatty acid binding to the high affinity site is a combination of electrostatic interactions with the carboxyl group and hydrophobic bonding with the side chain.

The thermodynamic parameters of secondary binding are probably less reliable than the primary values, for two reasons. Firstly, the errors of K_2 are larger than K_1 for the same reasons as were previously discussed for the estimation of K_3. Secondly, K_2 is an average value for several sites, unlike K_1. The use of average K values to calculate thermodynamic parameters has led to serious errors (Spector et al., 1969, Spector, 1975). The entropy and enthalpy changes for pentanoate are not included in table 4:5 since K_2 at 37°C was atypically low, probably because of the high computed n_2 of this interaction. The binding to secondary sites is accompanied by positive enthalpy and entropy effects, which is characteristic
of hydrophobic bonding (Kauzmann, 1959). Positive enthalpy is unfavourable for binding, however, the net negative $\Delta G$ is due to the favourable entropy gain. The entropy gain occurs because the binding of the fatty acid results in the release of water molecules from the "icebergs" around the alkyl chain, and consequently, in the release of the configurational entropy of the alkyl group (Nagwekar and Kostenbauder, 1970; Nagwekar and Muangnolcharoen, 1973). Secondary fatty acid binding appears, then, to be entirely hydrophobic in nature.

4:4:2 FLUORESCENCE DATA

a) Intrinsic fluorescence of albumin

The U.V. fluorescence of albumin, like other class B proteins, is due largely to transitions of tryptophan, with only a small contribution from other aromatic residues. Bovine Serum Albumin has two tryptophan residues whilst Human Serum Albumin has only one. From the published amino acid sequences of the two proteins it is known that the tryptophan which is common to both is situated in the cleft between loops 3 and 4 and corresponds to the binding site isolated by Swaney and Klotz (1970). The second tryptophan of BSA is located within loop 3 and is thought to be buried more deeply in the globular structure of albumin. (A more detailed discussion of the location of tryptophan in BSA and HSA may be found in section 1:2:5).

The effect of medium chain fatty acids upon the intrinsic fluorescence of BSA (Conn fraction V, as used in the ultrafiltration experiments) was investigated by fluorescence titration. With an albumin concentration of $1 \times 10^{-5}$ M the addition of $\mu l$ quantities of any of the fatty acids from a
$1 \times 10^{-3}$ M stock had no effect upon the albumin fluorescence. Such a titration covers albumin/fatty acid ratios up to 1:1.5. This is in agreement with the findings of Spector and John (1968) that fatty acids did not quench BSA fluorescence at a 1:1 ratio. It is worth noting that the closely related alkyl sulphates all quench BSA fluorescence at this ratio (Steinhardt et al., 1972). Titrations performed with a stock fatty acid concentration of $1 \times 10^{-1}$ M covered the BSA:FA ratios 1:4 to 1:100, and did result in the quenching of fluorescence. The percentage reduction in BSA fluorescence at various concentrations of the five fatty acids is shown in table 4:6. At a molar ratio of 1:4 there was a small quenching of fluorescence, from 0.5% to 12% increasing with chain length. The emission maximum of fluorescence (341 nm) was shifted slightly to shorter wavelengths for nonanoate ($\sim 2$ nm) and octanoate ($\sim 1$ nm) but not for the shorter chain length fatty acids. At higher concentrations of fatty acid there was further quenching up to 35%, with a maximum blue shift in emission maximum of 14 nm to 327 nm, for nonanoate. These results are again largely similar to those of Spector and John (1968), except that they did not examine such high ratios of FA:BSA and consequently did not record as large blue shifts or quenchings.

Fluorescence titrations were also performed with HSA (crystalline) under identical conditions as for BSA. Even at high molar ratios, none of the fatty acids produced marked quenching, there was perhaps a slight enhancement, with only a small blue shift of fluorescence emission maximum. Santos and Spector (1974) have reported that palmitate produced no appreciable change in the magnitude of HSA fluorescence with only a very small blue shift in the wavelength of maximum fluorescence.
Quenching of protein fluorescence with ligand binding can occur via one of two mechanisms. Either by some kind of energy transfer from fluorophor to ligand, or by a modification of fluorophor environment. Since fatty acids do not absorb light in the near U.V. it is unlikely that any energy transfer could occur with albumin tryptophan transitions. In order for resonance energy transfer to be efficient the emission wavelengths of the donor and the absorption wavelengths of the acceptor must overlap (see section 1:7:8). Thus, fatty acid binding must cause a change in environment of the fluorophor to produce quenching. It has been suggested (Spector, 1975) that quenching may be due to an alteration in the state of ionisation of a protein – amino group, but this is based upon alkyl sulphate binding to BSA (Halfman and Nishida, 1971) and it has already been seen that alkyl sulphates and alkyl carboxylates produce dissimilar effects upon BSA fluorescence.

The facts that low molar ratios of fatty acids (where binding will be primarily to high affinity sites) do not produce any quenching of BSA fluorescence and that fatty acids produce only very small alterations in HSA fluorescence suggests that the area around the common tryptophan of BSA and HSA does not provide one of the primary sites for fatty acid binding. The reduction in BSA fluorescence at higher ratios is probably due to binding near the second tryptophan, causing a change in the environment of the fluorophor. It is possible that fatty acid also binds near the common tryptophan at high concentrations, but is is impossible to tell from the BSA data. The fact that high concentrations of fatty acid produced only small changes in HSA fluorescence suggest that the common tryptophan may be uninvolved even in secondary binding. Alternatively, the fatty acids may bind near the
tryptophan without affecting the optical properties. ANS (Santos and Spector, 1972) and iodide (Spector et al., 1973) are thought to bind to the Swaney and Klotz (1970) tryptophan site in HSA and fatty acids at high concentration do reduce the fluorescence quenching produced by these agents. The displacement of ANS and iodide may, however, be due to conformational changes produced by fatty acid binding at nearby sites. The theory that BSA fluorescence quenching is due to binding to secondary sites is consolidated by the fact that, although octanoate and nonanoate were more effective than the shorter fatty acids, there was not a dramatic increase in effect but a fairly smooth increase with chain length.

b) The fluorescence of warfarin bound to albumin

The addition of warfarin to serum albumin results in a quenching of albumin fluorescence and an enhancement of warfarin fluorescence. This phenomenon is more thoroughly considered in chapter 3, but briefly, the mechanism of quenching is thought to be partially resonance energy transfer and partially a conformational change whilst the enhancement of warfarin fluorescence is due to the less polar environment of the albumin binding site. The effect of fatty acids upon the quenched fluorescence of albumin and the enhanced fluorescence of warfarin was investigated. The results obtained with both HSA and BSA are shown in table 4:7.

The effects of fatty acid upon the fluorescence of warfarin were very similar with both HSA and BSA. In no case did the addition of one or two equivalents of fatty acid have any significant effect upon probe fluorescence. For this reason titrations were again performed at higher fatty acid concentration
using $1 \times 10^{-1}$ M fatty acid stock. Albumin concentration was $1 \times 10^{-5}$ M and warfarin concentration was either $0.8 \times 10^{-5}$ M or $1.6 \times 10^{-5}$ M. Under these conditions warfarin will be bound predominantly to high affinity sites, of which there are two, with association constants of about $1 \times 10^{-5}$ M (O'Reilly, 1973, see chapters 5 and 6). The addition of four equivalents of fatty acid produced a small reduction in fluorescence of about 8%, with a little variation between fatty acids. This data strongly suggests that warfarin and the fatty acids do not share high affinity binding sites. Both octanoate and nonanoate have larger primary association constants than does warfarin, therefore four equivalents of these fatty acids would be expected to produce a substantial reduction in the bound warfarin with a concomitant reduction in fluorescence if they shared primary binding sites. Higher concentrations of all the fatty acids reduced the fluorescence of the albumin/warfarin complex, with nonanoate being the most effective, but still producing less than 50% reduction with a 25-fold molar excess compared to warfarin. It is likely that the primary binding sites of warfarin may serve as secondary binding sites for the fatty acids. It is possible that the displacement of warfarin at high fatty acid concentrations is due to a conformational change but since the emission maximum of bound warfarin remains at 378 nm, even with excess fatty acid, this possibility is less likely.

It was suggested in the previous section that some secondary fatty acid binding was to a section near to, but not necessarily directly on, the tryptophan area of Swaney and Klotz (1970). Whilst the binding of the fluorescent probe ANS quenches albumin very efficiently (1 mole of ANS decreases HSA fluorescence by more than 95% (Santos and Spector, 1974b))
warfarin binding quenches less efficiently. This suggests that warfarin, too, is bound near, but not at, the tryptophan site. From the efficiency of warfarin - HSA energy transfer Chignell has calculated that the distance between the two fluophors must be about 3.4 nm (Chignell, 1970). This corresponds to about 70% of the "width", or minor axis, of albumin, measured by hydrodynamic methods (Peters, 1975; Chignell, 1970).

(Further evidence that warfarin and fatty acids share some binding sites will be presented in the next section).

The binding of warfarin to albumin quenches the protein fluorescence and shifts the emission maximum towards the longer wavelength of warfarin fluorescence. The effect appears relatively larger for HSA since BSA has a second tryptophan which appears unaffected by warfarin binding. The addition of fatty acid to a warfarin/albumin mixture produced interesting changes in the quenched albumin fluorescence. These changes were similar for both albumins. The magnitude of the effect increased with increasing chain length of the fatty acid used, figure 4:5 illustrates a typical titration.

The addition of FA to the warfarin/albumin complex causes only small changes in the intensity of quenched albumin fluorescence. There is, initially, a small decrease, followed by an increase of a similar magnitude. The wavelength of maximum fluorescence is more markedly affected. The maximum shifts from the longer wavelengths, associated with warfarin liganded albumin, to shorter wavelengths. This is presumably a reflection of the same conformational changes which produce the blue shift in albumin fluorescence with fatty acid binding. The tryptophan fluorescence remains quenched due to a combination of the fatty acid induced conformational
changes and the energy transfer of radiation to the residual bound warfarin.

4:4:3  COMPETITION DATA

a) Competition between fatty acids and warfarin

The binding of heptanoate, octanoate and nonanoate to HSA in the presence of various concentrations of warfarin was examined by ultrafiltration. Table 4:8 displays the results. At a fatty acid:albumin ratio of 6:1, where much of the binding will be to secondary binding sites, all the warfarin concentrations produced highly significant decreases in the percentage of fatty acid bound, even when the warfarin concentration was half that of fatty acid. However, when the fatty acid:albumin ratio was 0.6:1, where binding would be predominantly to high affinity sites only, a five fold excess of warfarin was required to produce any significant displacement. These observations agree well with those from the fluorescence measurements and give added weight to the argument that warfarin and the fatty acids do not share the same primary binding site. It is likely, however, that the primary sites can serve as secondary binding sites for the other ligand. Warfarin has a primary association constant far larger than that for secondary fatty acid binding ($\sim 10^6 \text{M}^{-1}$ compared to $\sim 10^3 \text{M}^{-1}$) so that low concentrations of warfarin displace that fatty acid bound to secondary sites. When fatty acid is bound to its high affinity sites, displacement can only occur due to secondary warfarin binding and thus a large molar excess is required.

The displacement of fatty acids by warfarin, and vice versa, has so far been considered in terms of competition for binding sites. A previous study of long chain fatty acid inhibition of warfarin binding has also shown
competition for HSA binding sites (Solomon et al., 1968). Other authors have suggested that the influence of fatty acids on warfarin binding is mediated by conformational changes, rather than direct competition (Spector et al., 1973; Laliberté et al., 1976; Chakrabarti et al., 1976). These authors cite, as evidence, the findings that fatty acids can alter the dielectric properties and viscosity of albumin solutions (Soetewey et al., 1972) but offer little further information as proof of conformational changes. Even the findings of Soetewey and colleagues (1972) may be in doubt, since their measurements were performed with defatted albumin, which may no longer hold the native conformation. There is, in short, no conclusive evidence to demonstrate that fatty acids substantially affect the conformation of drug binding sites.

It is a common failing of all in vitro displacement experiments that the physiological effect of competition cannot be predicted with certainty, since redistribution, elimination, metabolism play important roles in determining the final levels of displaced compounds. From our results it would seem that elevated plasma fatty acid concentrations could displace warfarin, only if the FA/albumin ratio was predisposed to binding to secondary sites. Increased FA levels, due to stress or fasting, do increase the prothrombin times of rats administered with warfarin (Chakrabarti et al., 1975; Laliberté et al., 1976). The theory that FA binding to secondary sites is the important factor is consolidated by the observation that an increase in free warfarin is only seen when the FA:albumin ratio is greater than 2:1 (Gugler et al., 1974).

One important lesson which may be learned from all our studies of fatty acid/warfarin interactions is that either ligand can be shown to displace the other, provided the correct concentrations are chosen. It is important, then,
to choose pharmacologically relevant levels of drugs and physiologically significant albumin and ligand concentrations for displacement studies, if clinically applicable conclusions are to be arrived at.

b) **Competition between fatty acids and carbamates**

Previous work in our laboratories has characterised the binding of a homologous series of aliphatic carbamates to BSA (Wilson, 1974). The uncharged carbamates used had the equivalent alkyl chains to the fatty acids of this work. A good correlation was found, for the carbamates, between the binding affinity (expressed as nK) and lipophilicity (measured as octanol/water partition coefficient). Since the fatty acids did not show a smooth increase in binding with chain length it was of interest to see if these two series of compounds shared the same binding sites. The effects of various concentrations of n butyl carbamate upon pentanoate binding and the influence of nonanoate on n octyl carbamate were examined. The results are displayed in table 4:9. Since Wilson (1974) did not estimate the binding of n octyl carbamate, the interaction of this compound with BSA was studied using ultrafiltration and radioactive ligand. Because of the very limited aqueous solubility of n-octyl carbamate it was not possible to determine a full binding isotherm. From the data obtained the total binding constant \( nK \) or \( n_1K_1 + n_2K_2 \) if there are two sets of sites was estimated as \( 6 \times 10^4 \, M^{-1} \). Wilson interpreted his carbamate data in terms of only one class of sites which had a decreasing 'n' value with increasing chain length. Thus octyl carbamate may be considered to have the binding parameters \( n = 2 \) or 3, \( K = 2 - 3 \times 10^4 \, M^{-1} \).
From table 4:9 it can be seen that nonanoate has no influence upon n-octyl carbamate binding at any of the concentrations used. Under the conditions of these measurements (BSA = 2.5 x 10^{-4} M, n-octyl carbamate 1 x 10^{-4} or 1 x 10^{-5} M) the carbamate will be bound primarily to the high affinity sites. Since nonanoate has a high affinity association constant about 20 fold higher than n-octyl carbamate and yet there is no displacement of carbamate these two compounds cannot have the same high affinity binding sites. At the highest nonanoate concentration used (1 x 10^{-3} M, FA:BSA ratio 4:1) there was some displacement of n-octyl carbamate which could be indicative of the carbamate binding site being a secondary fatty acid binding site. The $K_a$ for nonanoate is of the same order of magnitude as the association constant of n-octyl carbamate.

From the data in table 4:9, using similar logic as followed above it is likely that n-butyl carbamate and pentanoate do not share high affinity sites. The association constants for the two compounds are $0.7 x 10^3$ M^{-1} (Wilson, 1974) and $2.30 x 10^4$ M^{-1} respectively but even a 330 fold molar excess of n-butyl carbamate causes only a slight reduction in high affinity pentanoate binding to BSA. However at higher pentanoate concentration where some secondary fatty acid sites may be filled a 3.3 fold excess of n-butyl carbamate caused a significant reduction in fatty acid binding. Again it is probable that some secondary fatty acid binding is to the main carbamate binding sites.

In conclusion, the neutral aliphatic carbamates seem not to have any mutual, main, BSA binding sites with the charged aliphatic carbamates.

Thus the anomalous increase in binding of fatty acids of eight carbons or
more is probably a reflection of the nature of specific anion binding sites rather than a special interaction of fatty acid with a common organic ligand binding site.

c) Competition between different fatty acids

The influence of pentanoate on octanoate binding, and the effects of octanoate on pentanoate binding were examined by ultrafiltration. The effects of various concentrations of competitor at two ligand concentrations were examined. Ligand concentrations were chosen to give situations where binding was predominantly to the high affinity site and also where a significant proportion of binding was to secondary sites. Table 4:10 shows that pentanoate has little effect on primary octanoate binding. A 10,000 fold excess of pentanoate produced only a 7% reduction in octanoate bound. Similarly, octanoate had no effect upon primary pentanoate binding until 1000:1 ratio was reached, when there was a substantial reduction in the percentage bound. The secondary binding of both octanoate and pentanoate was significantly reduced in the presence of 1 equivalent, or more, of the other fatty acid.

4:4:4 FINAL DISCUSSION

Several alternative explanations were presented in section 4:4:1 to account for the higher association constant of octanoate, relative to the shorter fatty acids. The combined fluorescence and competition data suggests that there may be two different binding sites, one for fatty acids
up to seven carbons and the other accommodating longer fatty acids. The following findings lead to this conclusion: The binding of the fatty acids to their primary sites does not cause an alteration in the fluorescence properties of BSA. Therefore, the environment of both of BSA tryptophans are unaffected by binding. This suggests that there are no major conformational changes with fatty acid binding. Pentanoate and octanoate do not displace each other from their high affinity sites, except at very large molar excesses of competitor. If a single primary site were involved for both FA, then competition should be observable at much lower competitor concentrations.

A recent publication by Rodrigues de Miranda and co-workers (1976) contains some interesting parallels with our work. This group also examined the binding of fatty acid anions to BSA, and used a similar, computerised, fitting procedure to examine the data. They, also, found a non-linear relationship between chain length and binding affinity. From competition experiments, the group conclude that two primary sites exist for medium and short chain fatty acids. One site can accommodate fatty acids up to pentanoate, the other, fatty acids from six to at least eight carbons in length. The concentrations of fatty acids used in the displacement experiments was not reported, so it is not certain if they were designed to show competition for high affinity sites only. These findings are qualitatively similar to our work, but the break point appears to be C$_6$ to C$_7$ rather than C$_7$ to C$_8$. Rodrigues de Miranda et al. (1976) suggested that a limit of hydrophobic bonding area might be a general feature of proteins.

It is possible that the peculiar chain length dependence of fatty acid binding to albumin may be a general feature of the interaction of macro-
molecules with alkyl chains. Al-Gailany (1975), in our laboratories, studied the interaction of fatty acids with hepatic microsomal suspensions. The FA elicit a type 1 substrate induced binding spectra from which the spectral dissociation constants (Ks) can be calculated. Hexanoate and heptanoate gave relatively large Ks values of $85 \times 10^{-4}$ and $21 \times 10^{-4}$ M respectively, whilst octanoate, to undecanoate gave Ks values, changing smoothly with chain length, from $3.45 \times 10^{-4}$ to $0.78 \times 10^{-4}$ M. Again, the fatty acids of eight carbon atoms, or more, have different binding properties to the shorter compounds.

In a recent review of fatty acid hydroxylation in rat kidney cortex microsomes, Ellin and Orrenius (1975) showed ω-hydroxylation to be chain length specific. Hexanoate, and shorter fatty acids, yielded no hydroxylated products whilst octanoate and longer FA were hydroxylated, with increasing velocity with chain length. Ellin and Orrenius (1975) suggest that the shorter fatty acids are not long enough to bridge the gap between the binding site and the hydrophobic region of the cytochrome. This is a very similar theory to that which was proposed earlier, to explain why the short chain FA did not bind to the primary site for FA of eight carbons or more.

Many of the reactions involved in normal fatty acid metabolism and transport are highly chain length specific. The treatment of steatorrhoea, caused by a variety of conditions, is based upon the fact that fatty acids of less than ten carbon atoms have different fates in the body, relative to longer fatty acids (Scheig, 1968). Medium chain triglycerides are absorbed without hydrolysis, broken down by intramucosal lipase, and transported to the liver, via the portal vein. In contrast, long chain fatty acids are
hydrolysed in the lumen, absorbed, recombined in the mucosa, to form triglycerides, then exported as chylomicra, via the lymphatics (Bloom et al., 1951). Many of the enzyme systems involved in fatty acid synthesis and degradation are chain length specific. For example, fatty acid thiol kinase, which catalyses the formation of acyl-CoA from saturated fatty acids, exists in two forms. One of these varieties will react with fatty acids containing 4 to 12 carbon atoms, the other is capable of activating fatty acids up to 22 carbons in length (Mahler and Cordes, 1971).

To summarize; the albumin binding sites for fatty acids are chain length specific. It is likely that two primary sites exist. One is involved with the binding of fatty acids up to seven carbon atoms in length, the other with the binding of longer molecules. These primary sites can probably act as secondary sites for the fatty acids for which they are not specific. This chain length specificity appears to be a general phenomenon of the interaction of fatty acid alkyl groups with biological macromolecules.
CHAPTER FIVE

OPTICAL STUDIES OF THE INTERACTION OF SEVERAL COUMARINS WITH SERUM ALBUMIN
5:1 INTRODUCTION

In a brilliant piece of scientific detective work, Link and his colleagues (Campbell and Link, 1941; Link, 1943) discovered that the agent responsible for the hemorrhagic disorder produced in cattle by the ingestion of spoiled sweet clover is dicoumerol. Dicoumerol (bishydroxycoumarin) is a dimer of 4-hydroxy coumarin, which does not normally occur naturally but is formed when sweet clover hay spoils in silage. It is formed from the parent molecule, coumarin, the substance which imparts to new mown hay its typical sweet smell. The isolation of dicoumeral led to the synthesis of a variety of structurally related compounds possessing anticoagulant activity. Warfarin, Phenprocoumon and Acenocoumarin have found extensive use as clinically effective oral anticoagulants in man, whilst Coumachlor and also Warfarin are used as rodenticides.

It is well known that the coumarin anticoagulants are highly bound to plasma proteins, (see O'Reilly, 1973 and references therein) and interest in this field has been intense due to possible drug interactions (see chapter 3). There have, however, been only a few systematic studies of the binding of several coumarin derivatives (O'Reilly, 1971; Garten and Wosilait, 1971) and only one involving the use of circular dichroism measurements (Chignell, 1970). There have been reports concerning the binding of one or two coumarin derivatives to albumin by Perrin and Nelson, 1972; Perrin et al., 1975 and Wilting et al., 1976 for Warfarin, Perrin and Idsvoog, 1971 and Perrin et al., 1975 for dicoumeral and finally by Perrin and Nelson, 1972 for 4-hydroxy-coumarin.
In this work four closely related coumarin derivatives and their binding to Bovine and Human Serum Albumins were studied, using the techniques of UV-visible absorption spectroscopy and circular dichroism. No attempt was made to quantify the binding, although this can be done from CD data (Rosen, 1970) other methods are probably more suitable for the estimation of binding parameters and, furthermore, substantial data is available on this aspect of these interactions (see O'Reilly, 1973). Attention was focussed upon those experiments giving data which might lead to a better understanding of the mechanism of the coumarin-albumin interaction.
5:2 EXPERIMENTAL APPROACH

Both Human and Bovine Serum Albumins were used, as supplied by Behringwerke (see chapter 2). The coumarins were dissolved in a few drops of IN NaOH, to aid solution in sodium phosphate buffer, 67 mM, pH 7.4. The pH of the drug-albumin solutions was checked immediately before recording spectra.

The UV-visible spectra were recorded at 20°C in 10 mm quartz cells as described in chapter 2. Spectra were recorded from at least three separate drug-albumin solutions, from which a mean extinction coefficient and an absorbance maxima were calculated. The final albumin concentration was $3 \times 10^{-5}$ M whilst the drug concentration was $2 \times 10^{-5}$ M.

Circular Dichroism measurements were made at 27°C in 10 mm cylindrical cells, as described in chapter 2, from at least three separate drug-albumin solutions. The final albumin concentration was $3 \times 10^{-5}$ M and the drug concentration was $6 \times 10^{-5}$ M. The ellipticities reported below are difference values, having had the ellipticity for for albumin subtracted from them. Results are expressed as molar ellipticities, calculated with respect to albumin concentration, based upon a molecular weight of 69,000. Anisotropy values were calculated as described in chapter 2. CD measurements were also made at pH values of 6.6, 7.0, 7.4, 7.8 and 8.2.
5.3 FIGURES AND TABLES
Figure 5: The absorption spectra of the coumarins, (a) in phosphate buffer (67 mM, pH 7.4) and (b) in HSA solution.

The concentrations were: coumarin $2 \times 10^{-5}$ M, HSA $3 \times 10^{-5}$ M. For (b), HSA was used as a blank.
Figure 5:2  The absorption spectra of the coumarins, (a) in phosphate buffer (67 mM, pH 7.4) and (b) in BSA solution.

The concentrations were: coumarin $2 \times 10^{-5}$ M, BSA $3 \times 10^{-5}$ M. For (b), BSA was used as a blank.
Figure 5:3 The difference spectra between bound and unbound forms of the coumarin drugs.

These spectra were recorded with the sample cuvette containing albumin (3 x 10^{-5} M) and coumarin (2 x 10^{-5} M) in phosphate buffer, the reference cuvette contained coumarin (2 x 10^{-5} M) only.
Figure 5: An example of the UV spectra from which data was taken.

The samples were phenprocoumon (2 x 10^{-5} M) and Human Serum Albumin (3 x 10^{-5} M) in 67 mM sodium phosphate buffer pH 7.4.

Spectra were recorded as follows:

1. Baseline
2. Coumarin absorption
3. Albumin absorption
4. Albumin/coumarin absorption
5. Bound coumarin absorption
6. Bound/free difference

Sample: Buffer  Sample: Coumarin  Reference: Buffer
Sample: Albumin   Sample: Albumin/coumarin  Reference: Albumin
Sample: Albumin/coumarin   Reference: Coumarin
Figure 5: An example of the circular dichroism spectra from which measurements were taken.

- a = $3 \times 10^{-5}$ M HSA in sodium phosphate buffer, 67 mM, pH 7.4.
- b = $3 \times 10^{-5}$ M HSA with $6 \times 10^{-5}$ M Warfarin in phosphate buffer.
In all cases the concentrations were: BSA $3 \times 10^{-5}$ M, coumarin $6 \times 10^{-5}$ M.
Figure 5: CD Difference spectra of the coumarins bound to HSA

Acenocoumarin

![Graph showing CD difference spectrum of Acenocoumarin]

Coumachlor

![Graph showing CD difference spectrum of Coumachlor]

Phenprocoumon

![Graph showing CD difference spectrum of Phenprocoumon]

Warfarin

![Graph showing CD difference spectrum of Warfarin]

In all cases the concentrations were: coumarin $6 \times 10^{-5}$ M, HSA $3 \times 10^{-5}$ M.
Figure 5:8 CD Difference spectra of 4-hydroxycoumarin in the presence of HSA

4-Hydroxycoumarin $6 \times 10^{-5}$ M, HSA $3 \times 10^{-5}$ M.

Molar ellipticity calculated with respect to HSA concentration.
Table 5: Structures and some properties of the coumarin derivatives under study

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<th>Name and synonyms</th>
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<th>M.W.</th>
<th>Av. daily dose for therapeutic hypoprotrombinaemia mg.</th>
<th>Binding Parameters</th>
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<td><img src="image" alt="Structure" /></td>
<td>308.32</td>
<td>7</td>
<td>$K_a = 23.1 \pm 0.5 \times 10^4 M^{-1}$</td>
</tr>
<tr>
<td>4-Hydroxy-coumarin</td>
<td><img src="image" alt="Structure" /></td>
<td>162.1</td>
<td>1500</td>
<td>$n = 0.9 \pm 0.1$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$K_a = 7.9 \pm 0.3 \times 10^4 M^{-1}$</td>
</tr>
</tbody>
</table>

The values of daily dose for hypoprotrombinemia and the binding parameters (which refer to the high affinity sites only, at 27°C, pH 7.4, phosphate buffer, crystalline HSA) are taken from O'Reilly, 1973.
Table 5:2  **UV Absorption data for the coumarin derivatives free and bound to Human and Bovine Serum Albumins**

<table>
<thead>
<tr>
<th></th>
<th>Free (in phosphate buffer pH 7.4 67 mM)</th>
<th>Bound (in BSA)</th>
<th>Bound (in HSA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>maximum</td>
<td>shoulder</td>
<td>maximum</td>
</tr>
<tr>
<td><strong>wavelength</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Warfarin</td>
<td>308</td>
<td>291</td>
<td>311</td>
</tr>
<tr>
<td>extinction co-efficient</td>
<td>12.87</td>
<td>10.50</td>
<td>11.96</td>
</tr>
<tr>
<td>Acenocoumarin</td>
<td>302.5</td>
<td>291.5</td>
<td>304.5</td>
</tr>
<tr>
<td>extinction co-efficient</td>
<td>19.38</td>
<td>18.77</td>
<td>17.08</td>
</tr>
<tr>
<td>Phenprocoumon</td>
<td>309</td>
<td>292</td>
<td>313.5</td>
</tr>
<tr>
<td>extinction co-efficient</td>
<td>14.63</td>
<td>11.19</td>
<td>13.50</td>
</tr>
<tr>
<td>Coumachlor</td>
<td>307.5</td>
<td>292</td>
<td>311</td>
</tr>
<tr>
<td>extinction co-efficient</td>
<td>13.50</td>
<td>11.03</td>
<td>12.21</td>
</tr>
</tbody>
</table>

Extinction co-efficient (mM, 25°C) and absorbance maxima are from recordings of solutions of drug (2 x 10^{-5} M) in phosphate buffer with a buffer blank in the case of 'free', drug (2 x 10^{-5} M) in protein (3 x 10^{-5} M) with a protein blank (3 x 10^{-5} M) for 'bound'. 
Table 5:3  **Circular dichroism data for the coumarin - Bovine Serum Albumin complexes at pH 7.4**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Wavelength positions of CD maxima nm.</th>
<th>Molar ellipticity [θ] x 10⁻⁴</th>
<th>Anisotropy factor g x 10⁵</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Warfarin</td>
<td>305</td>
<td>+ .293 ± .009</td>
<td>+ 9.2</td>
</tr>
<tr>
<td></td>
<td>287</td>
<td>+ .531 ± .016</td>
<td>+ 21.8</td>
</tr>
<tr>
<td></td>
<td>255</td>
<td>+ 1.288 ± .058</td>
<td></td>
</tr>
<tr>
<td>Phenprocoumon</td>
<td>332</td>
<td>- .126 ± .010</td>
<td>- 6.3</td>
</tr>
<tr>
<td></td>
<td>304</td>
<td>+ .125 ± .017</td>
<td>+ 3.4</td>
</tr>
<tr>
<td></td>
<td>291</td>
<td>- .129 ± .019</td>
<td>- 5.2</td>
</tr>
<tr>
<td></td>
<td>275</td>
<td>+ .361 ± .016</td>
<td></td>
</tr>
<tr>
<td></td>
<td>256</td>
<td>+ 1.42 ± .012</td>
<td></td>
</tr>
<tr>
<td>Acenocoumarin</td>
<td>332</td>
<td>- .426 ± .007</td>
<td>- 21.6</td>
</tr>
<tr>
<td></td>
<td>286</td>
<td>+ 1.575 ± .046</td>
<td>+ 35.0</td>
</tr>
<tr>
<td></td>
<td>281</td>
<td>+ 1.908 ± .014</td>
<td>+ 32.5</td>
</tr>
<tr>
<td>Coumachlor</td>
<td>316</td>
<td>- .424 ± .014</td>
<td>- 10.9</td>
</tr>
<tr>
<td></td>
<td>292</td>
<td>- .334 ± .013</td>
<td>- 9.9</td>
</tr>
<tr>
<td></td>
<td>256</td>
<td>+ 1.405 ± .070</td>
<td></td>
</tr>
</tbody>
</table>

All figures are difference values obtained by subtraction of the corresponding value for BSA alone. Molar ellipticity calculated with reference to total drug concentration (6 x 10⁻⁵ M, BSA 3 x 10⁻⁵ M). Anisotropy values calculated with respect to drug bound. Results expressed as mean ± SEM (n = 6).
### Table 5:4  Circular dichroism data for the coumarin - Human Serum Albumin complexes at pH 7.4

<table>
<thead>
<tr>
<th>Compound</th>
<th>Wavelength positions of CD maxima nm</th>
<th>Molar ellipticity ([\theta] \times 10^{-4})</th>
<th>Anisotropy factor (g \times 10^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Warfarin</td>
<td>312</td>
<td>+ .424 ± .019</td>
<td>+ 13.6</td>
</tr>
<tr>
<td></td>
<td>268</td>
<td>- .262 ± .029</td>
<td>- 25.0</td>
</tr>
<tr>
<td></td>
<td>257</td>
<td>+ .418 ± .062</td>
<td></td>
</tr>
<tr>
<td>Phenprocoumon</td>
<td>317</td>
<td>- .572 ± .020</td>
<td>- 15.4</td>
</tr>
<tr>
<td></td>
<td>292</td>
<td>- .891 ± .014</td>
<td>- 31.2</td>
</tr>
<tr>
<td></td>
<td>268</td>
<td>- .298 ± .014</td>
<td>- 30.3</td>
</tr>
<tr>
<td></td>
<td>257</td>
<td>+ 1.031 ± .048</td>
<td></td>
</tr>
<tr>
<td>Acenocoumarin</td>
<td>330</td>
<td>- .429 ± .012</td>
<td>- 18.3</td>
</tr>
<tr>
<td></td>
<td>289</td>
<td>+ .498 ± .023</td>
<td>+ 10.0</td>
</tr>
<tr>
<td></td>
<td>(268 )</td>
<td>(&lt; .075)</td>
<td></td>
</tr>
<tr>
<td>Coumachlor</td>
<td>325</td>
<td>- .107 ± .017</td>
<td>- 3.7</td>
</tr>
<tr>
<td></td>
<td>268</td>
<td>- .262 ± .020</td>
<td>- 15.4</td>
</tr>
<tr>
<td></td>
<td>256</td>
<td>+ .771 ± .019</td>
<td></td>
</tr>
</tbody>
</table>

All figures are difference values obtained by subtraction of the corresponding value for HSA alone. Molar ellipticity calculated with respect to total drug conc\(^n\) \((6 \times 10^{-5} \text{ M}; \text{ HSA } 3 \times 10^{-5} \text{ M})\). Anisotropy values calculated with respect to drug bound. Results expressed as Mean ± SEM (n = 6).
Table 5.5  
**Influence of pH on the circular dichroism of the coumarin-BSA complexes**

<table>
<thead>
<tr>
<th>Compound and wavelength position of maxima</th>
<th>Molar Ellipticity [θ] x 10⁻³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 8.2</td>
</tr>
<tr>
<td>Acenocoumarin</td>
<td></td>
</tr>
<tr>
<td>332</td>
<td>-2.72 ± 0.098</td>
</tr>
<tr>
<td>286</td>
<td>+7.87 ± 0.377</td>
</tr>
<tr>
<td>281</td>
<td>+9.71 ± 0.459</td>
</tr>
<tr>
<td>Phenprocoumon</td>
<td></td>
</tr>
<tr>
<td>332</td>
<td>-1.69 ± 0.055</td>
</tr>
<tr>
<td>304</td>
<td>+1.08 ± 0.108</td>
</tr>
<tr>
<td>291</td>
<td>+2.46 ± 0.190</td>
</tr>
<tr>
<td>256</td>
<td>+15.03 ± 0.22</td>
</tr>
<tr>
<td>Coumachlor</td>
<td></td>
</tr>
<tr>
<td>316</td>
<td>-2.84 ± 0.056</td>
</tr>
<tr>
<td>292</td>
<td>-0.984 ± 0.108</td>
</tr>
<tr>
<td>256</td>
<td>+15.42 ± 0.190</td>
</tr>
<tr>
<td>Warfarin</td>
<td></td>
</tr>
<tr>
<td>305</td>
<td>+3.12 ± 0.125</td>
</tr>
<tr>
<td>287</td>
<td>+4.98 ± 0.358</td>
</tr>
<tr>
<td>255</td>
<td>+12.03 ± 0.22</td>
</tr>
</tbody>
</table>

The results are expressed as difference values.

Molar ellipticities calculated with respect to total drug conc (6 x 10⁻⁵ M; BSA 3 x 10⁻⁵ M).

Mean ± S.E.M. (n = 3)
Table 5: Influence of pH on the circular dichroism of the coumarin - HSA complexes

<table>
<thead>
<tr>
<th>Compound and wavelength position of maxima</th>
<th>Molar Ellipticity [8] x 10⁻³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH</td>
</tr>
<tr>
<td></td>
<td>8.2</td>
</tr>
<tr>
<td>Acenocoumarin</td>
<td></td>
</tr>
<tr>
<td>330</td>
<td>-3.44 ± 0.95</td>
</tr>
<tr>
<td>289</td>
<td>+7.11 ± 2.36</td>
</tr>
<tr>
<td>Phenprocoumon</td>
<td></td>
</tr>
<tr>
<td>320</td>
<td>-2.46 ± 0.22</td>
</tr>
<tr>
<td>292</td>
<td>-6.45 ± 0.056</td>
</tr>
<tr>
<td>268</td>
<td>-3.38 ± 0.289</td>
</tr>
<tr>
<td>257</td>
<td>+7.87 ± 0.001</td>
</tr>
<tr>
<td>Coumachlor</td>
<td></td>
</tr>
<tr>
<td>325</td>
<td>- .492 ± .164</td>
</tr>
<tr>
<td>268</td>
<td>-2.62 ± .492</td>
</tr>
<tr>
<td>256</td>
<td>+8.20 ± .328</td>
</tr>
<tr>
<td>Warfarin</td>
<td></td>
</tr>
<tr>
<td>312</td>
<td>+5.74 ± .001</td>
</tr>
<tr>
<td>268</td>
<td>-3.17 ± .190</td>
</tr>
<tr>
<td>257</td>
<td>+5.63 ± .190</td>
</tr>
</tbody>
</table>

The results are expressed as difference values.

Molar ellipticities calculated with respect to total drug concentration (6 x 10⁻⁵ M; HSA concentration 3 x 10⁻⁵ M)
Mean ± S.E.M. (n = 3)
Table 5: UV absorption and circular dichroism maxima for the coumarin derivatives bound to albumin

<table>
<thead>
<tr>
<th>Compound</th>
<th>Type of Spectra</th>
<th>Wavelength positions of maxima (nm) and signs of induced ellipticity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Warfarin</td>
<td>UV Bound to HSA</td>
<td>312 291</td>
</tr>
<tr>
<td></td>
<td>CD &quot; &quot; &quot;</td>
<td>312+ 268- 257+</td>
</tr>
<tr>
<td></td>
<td>UV &quot; &quot; BSA</td>
<td>311 291</td>
</tr>
<tr>
<td></td>
<td>CD &quot; &quot; &quot;</td>
<td>305+ 287+ 255+</td>
</tr>
<tr>
<td>Phenprocoumon</td>
<td>UV Bound to HSA</td>
<td>314 293</td>
</tr>
<tr>
<td></td>
<td>CD &quot; &quot; &quot;</td>
<td>317 292- 268- 257+</td>
</tr>
<tr>
<td></td>
<td>UV &quot; &quot; BSA</td>
<td>313.5 292.5</td>
</tr>
<tr>
<td></td>
<td>CD &quot; &quot; &quot;</td>
<td>332-,304+ 291- 275+ 256+</td>
</tr>
<tr>
<td></td>
<td>Enantiomers in buffer</td>
<td>311 290 268 262 241</td>
</tr>
<tr>
<td>Acenocoumarin</td>
<td>UV Bound to HSA</td>
<td>305 291</td>
</tr>
<tr>
<td></td>
<td>CD &quot; &quot; &quot;</td>
<td>330- 289+ (268-)</td>
</tr>
<tr>
<td></td>
<td>UV &quot; &quot; BSA</td>
<td>304.5 291.5</td>
</tr>
<tr>
<td></td>
<td>CD &quot; &quot; &quot;</td>
<td>332- 286+ 281+</td>
</tr>
<tr>
<td>Coumachlor</td>
<td>UV Bound to HSA</td>
<td>312 292</td>
</tr>
<tr>
<td></td>
<td>CD &quot; &quot; &quot;</td>
<td>325- 268- 256+</td>
</tr>
<tr>
<td></td>
<td>UV &quot; &quot; BSA</td>
<td>311 292.5</td>
</tr>
<tr>
<td></td>
<td>CD &quot; &quot; &quot;</td>
<td>316- 292- 256+</td>
</tr>
<tr>
<td>4-OH Coumarin</td>
<td>CD Bound to HSA</td>
<td>310+ 290+ 268-</td>
</tr>
</tbody>
</table>
Table 5:8  The percentage of coumarin bound to albumin and the relationship to coumarin partition coefficient

<table>
<thead>
<tr>
<th>Albumin</th>
<th>Ligand</th>
<th>Percentage bound Mean ± SEM (n=6)</th>
<th>Partition coefficient, octanol/phosphate buffer (pH 7.4, 67 mM) Mean ± SEM (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA</td>
<td>Acenocoumarin</td>
<td>77.71 ± 0.06</td>
<td>7.3 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>Coumachlor</td>
<td>86.67 ± 0.05</td>
<td>38.1 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>Phenprocoumon</td>
<td>83.19 ± 0.06</td>
<td>17.3 ± 0.35</td>
</tr>
<tr>
<td></td>
<td>Warfarin</td>
<td>77.05 ± 0.04</td>
<td>10.6 ± 0.20</td>
</tr>
<tr>
<td>RSA</td>
<td>Acenocoumarin</td>
<td>74.79 ± 0.21</td>
<td>7.3 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>Coumachlor</td>
<td>86.85 ± 0.13</td>
<td>38.1 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>Phenprocoumon</td>
<td>85.18 ± 0.08</td>
<td>17.3 ± 0.35</td>
</tr>
<tr>
<td></td>
<td>Warfarin</td>
<td>81.16 ± 0.12</td>
<td>10.6 ± 0.20</td>
</tr>
</tbody>
</table>

The percentage of coumarin (6 x 10^{-5} M) bound to albumin (3 x 10^{-5} M) was determined by half-cell equilibrium dialysis at 25°C.
5:4 RESULTS AND DISCUSSION

5:4:1 ELECTRONIC ABSORPTION SPECTRA

The electronic absorption spectra of the four coumarins were qualitatively similar, as shown in figures 5:1 and 5:2. It can be seen that these compounds exhibit a relatively intense transition in the region 300 - 310 nm with a well defined shoulder at 290 - 293 nm. In some cases a much weaker shoulder was observable in the region 275 - 280 nm. Table 5:2 lists the absorption maxima and extinction coefficients for the four compounds.

In structures as complex as these, it is difficult to ascribe transitions to specific portions of the molecule. However, reference to simpler compounds aids the assignment. Brand and Toribara (1972) have reported the absorption spectra of coumarin in phosphate buffer (pH 7.4, 0.1 M). They found two intense transitions, one at 277 nm and a second at 308 nm, but no sign of a peak at 290 nm. It is almost certain then that the absorption (in the region 300 - 310 nm) of the anti-coagulant coumarins under study is due to transitions in the main coumarin ring system, the same can be said of the weak shoulder at 275 - 280 nm but this is less important.

The intense absorption of coumarin at 308 nm is due to \( \pi \rightarrow \pi^* \) transitions in the conjugated system. The structural difference between coumarin and the derivatives under study is the addition of a substituted benzyl moiety. In the cases of Warfarin, Phenprocoumon and Coumachlor this addition has little effect upon the absorption around 308 nm (see table 5:2). This is to be expected since the electrons of the coumarin ring cannot be further
delocalised by conjugation with the electrons of the phenyl ring due to the intervening secondary methyl linkage. Not only is ordinary resonance conjugation unlikely but hyperconjugation, as proposed by Crawford (see Crawford, 1953), is also unlikely since a separation of charge involving both the coumarin and phenyl rings would require the existence of two double bonds to the linking atom (Sangster and Irvine, 1955). In addition, the substituted benzy1 moieties of these compounds would not be expected to contribute significantly to absorption in the wavelength region of 308 nm.

In contrast to the other coumarin derivatives, Acenocoumarin has a more intense transition at the lower wavelength of 302.5 nm. This change in absorption, relative to coumarin, could be due either to some effect upon the \( \pi \) electrons of the coumarin ring, or to the presence of an additional chromophore. As has been stated, it is unlikely that there can be any conjugation between the coumarin and phenyl rings, and the presence of the electron withdrawing p-nitro group will not change the situation. Thus, it is likely that there is an additional absorbing chromophore in Acenocoumarin. An isolated nitro group has only a weak \( n \rightarrow \pi^* \) transition around 270 nm, but this is masked in aromatic nitro compounds by \( \pi \rightarrow \pi^* \) transitions (Rao, 1975). Benzene normally has an absorption around 200 nm, associated with \( \pi \rightarrow \pi^* \) transitions, however large bathochromic shifts in this absorption can arise with nitro substituted derivatives (Doub and Van derbelt, 1947). For example, p-nitro phenol and p-nitro toluene have absorption maxima at 317.5 nm and 285 nm respectively. It is possible then, that the transitions of the main coumarin ring and of the substituted p-nitro benzyl portions of acenocoumarin overlap to give the intense transition observed at 302.5 nm. It is interesting to note that Doub and Van derbelt
(1947) also reported p-chloro substitution of benzene derivatives (as in coumachlor) to produce only small bathochromic shifts in absorption maxima.

As well as the main peak in the region 300 - 310 nm, all the coumarin anticoagulants studied exhibited a well defined shoulder at 291 - 292 nm. It has already been stated that coumarin itself has no absorption maxima in this region. However, 4-hydroxy coumarin, in acid solution, has an absorption spectrum quantitatively identical with that of warfarin (Brand and Toribara, 1972). It seems very likely, then, that the shoulder at 291 - 292 nm is due to the 4-hydroxy substituent on the coumarin ring. The absorption spectrum for warfarin reported here is in good agreement with published spectra (Chignell, 1970; Brand and Toribara, 1972), the absorption spectra of the other three compounds are not readily available.

Table 5:1 and figure 5:2 give details of the absorption spectra of the coumarins when bound to HSA and BSA. This data was obtained with a sample solution of albumin (3 x 10^{-5} M) with coumarin (2 x 10^{-5} M), and a reference solution of albumin (3 x 10^{-5} M) alone. The spectra thus recorded have been taken to reflect the absorption of bound coumarin. This can be justified from several stand points. The drug-albumin ratio used was 2:3, under these circumstances about 90% of the coumarin derivatives are bound to albumin. There is, then, little contribution to the spectra from free drug. The spectra must also reflect any ligand induced changes in albumin absorption. However, over much of the wavelength range of interest, albumin has no absorption. It can be seen from figure 5:4 that the absorption of albumin is, in fact, only a little perturbed by coumarin derivative binding.
Upon binding to albumin, all the derivatives showed a bathochromic (red) shift of 2 - 5 nm in the main absorption peak, with a reduction in the intensity of absorption. The shoulder at 291 - 292 nm also showed a reduction in intensity, but to a small degree and there was little, if any, change in the position of the maxima. Binding to HSA produced a greater red shift in the main peak compared to BSA but there were no other consistent differences between the two albumins.

A red shift in ligand absorption maximum, upon binding to albumin, has been reported for several drugs including phenylbutazone (Chignell, 1969a) and flufenamic acid (Chignell, 1969b), and for several dyes (Klotz et al., 1946). Chignell (1969a) suggested that the red shift is due to the movement of ligand from polar, aqueous solution, to the more hydrophobic environment of the protein binding site. This seems to be in conflict with the well known fact that $\pi \rightarrow \pi^*$ transitions shift to shorter wavelengths with decreasing polarity. This phenomena has even been used to distinguish $\pi \rightarrow \pi^*$ transitions from other types. There are, however, many exceptions to the rule, with benzene being the most notable. The blue shift in protein UV absorption upon unfolding or denaturation (Beaven and Holiday, 1952) has been attributed to the movement of aromatic residues from the hydrophobic interior of the protein to the polar solvent (Yanari and Bovey, 1960). It seems logical that the binding of an aromatic compound to a protein would produce the opposite, i.e. red, shift, which is indeed what is observed with these coumarin derivatives. It is interesting to note that coumarin itself has an absorption maximum at 308 nm in aqueous media (Brand and Toribara, 1972) whilst in methanol, which has a lower dielectric constant (24.3 compared to 78.5 for water), the maximum is at 313 nm (Lang, 1964).
The reduction in intensity of the coumarin's absorption upon binding is not, perhaps, what would be expected. The red shifts of phenylbutazone and flufenamic acid (Chignell, 1969a, b) are accompanied by an increase in intensity, and the movement of an aromatic, absorbing species from polar to non-polar solvents normally results in an increase in intensity of electronic transitions. Several workers have, however, reported a reduction in absorption intensity of various ligands upon binding to albumin e.g. Thyroxine, (Tritsch, 1968) and azosulphathiazole (Klotz, 1960). There does not seem to be a simple mechanistic explanation for binding induced changes in absorption intensity.

The spectral differences between BSA bound and HSA bound coumarin derivatives were relatively small. The larger red shift upon binding to HSA is possibly a reflection of a greater hydrophobicity of binding sites, but little further information can be gained from a comparison of the two sets of data.

The shoulder around 292 nm, which has been attributed to the 4-hydroxyl substituent of the coumarin ring, is relatively unaffected by binding to albumin. The simplest conclusion would be that the 4-hydroxyl group is not involved in the binding. However, the group is acidic in nature, deprotonisation being encouraged by the following:

![Chemical structure]

Such a polar group is unlikely to be situated in an area of high hydro-
phobicity upon binding, but it may be involved in the binding process via electrostatic interactions.

The preceding discussion was based upon the spectra of bound drug a second kind of difference spectra were recorded. In this case, the sample solution was coumarin \(2 \times 10^{-5}\) M with albumin \(3 \times 10^{-5}\) M and the reference was coumarin alone (see figure 5:3). As we have seen, over much of the absorbance range of the coumarins, albumin shows no absorbance and, thus, the difference spectra must reflect the changed transitions of bound and free drug. The spectra below 285 nm, where albumin absorbs significantly, will be considered later. These difference spectra between bound and free drug are comparable with those obtained using the tandem cuvette techniques of Herskovits (1967). In this system, cuvettes with two compartments are used, in the reference cell one half is filled with ligand solution, the other with protein solution, whereas the two solutions are combined in the sample cell. Differences in the absorption of bound and free ligand and/or protein are reflected in the resulting spectra, which are often multiphasic. This technique has been used to examine the binding of several compounds to albumin including phenylbutazone, (Chignell, 1969a), flufenamic acid (Chignell, 1969b), HABA (Baxter, 1964), dicoumerol (Perrin and Idsvoog, 1971) and several phenothiazines (Huang and Gabay, 1974). Comparison of spectra obtained in this way, with those in the presence of organic solvent or detergent, in place of protein, can give some information concerning the nature of the binding site, but the technique is generally more useful in quantitative studies.

The spectra obtained in this study are shown in figure 5:3. The spectra are qualitatively similar for all the derivatives, with both HSA and BSA,
being typified by a broad positive maximum in the region 310 - 350 nm and a minimum around 300 nm. The magnitude of the spectra in the presence of HSA was always greater than the equivalent for BSA. In common with previous observations, this may reflect a greater hydrophobicity of the HSA binding sites but the evidence is far from conclusive. The acenocoumarin difference spectra were atypical in having only a very small maximum around 330 nm with a large minimum at 305 nm. This discrepancy may be due to the different nature of the transitions which produce the main absorption of acenocoumarin compared to the other derivatives.

In figure 5:4 the absorbance spectra of the protein alone (scan 3) may be compared with the protein's spectra in the presence of ligand, with ligand as reference (scan 6). In the region 275 - 285 nm, where the absorbance of free and bound ligand is very similar (cf spectra 2 and 5), there is a small, but consistent, difference between the native and liganded protein absorbance. These differences are probably due to small conformational changes with binding, involving changes in the environments of some aromatic residues (which contribute most to protein absorption at these wavelengths).

5:4:2 CIRCULAR DICHOISM

The binding of the coumarin derivatives to albumin produced new cotton effects in addition to the intrinsic effects of the protein. All the coumarins produced such changes with both albumins (see figure 5:5, a typical recording from which data was taken). There have been only limited studies
of the coumarin-albumin interaction using C.D. measurements. Chignell (1970) reported that dicoumerol did produce cotton effects with binding to HSA, warfarin, acenocoumarin and 4-hydroxy coumarin did not. In a series of simple observations, Perrin and Nelson (1972) demonstrated optical activity upon binding with both warfarin and 4-hydroxy coumarin. In later work, Perrin and co-workers (Perrin et al., 1975; Wilting et al., 1975) confirmed the production of optical activity with warfarin binding to albumin, but did not examine wavelengths below 300 nm. To our knowledge, BSA-coumarin interactions and the binding of Phenprocoumon and Coumachlor to HSA have not previously been examined using C.D. techniques.

There are several possible sources for the new optical activity produced with the binding of the coumarins to albumin. The most obvious is a change in the intrinsic activity of the protein. However, this is unlikely since a large conformational change is needed to cause marked changes in protein C.D., and we have seen in the previous section that coumarin binding induces only small changes in the protein conformation, at least as far as the light absorbing residues are concerned. In addition, the wavelength maxima of the new cotton effects correspond well with the wavelengths of the coumarins absorption maxima.

Before considering a second source of the new optical activity it is pertinent to reiterate the fact that the coumarin derivatives used in this study are racemic mixtures of two enantiomers. Whilst the racemate has no optical activity, the individual enantiomers do show cotton effects (see next chapter). Thus, if the binding of racemate to albumin affected the intrinsic optical activity of one of the enantiomers then new cotton effects would be produced. However, it can be seen from figure 5:8 that 4-hydroxy-
coumarin binding to albumin produces new optical activity at wavelengths similar to the other derivatives. 4-Hydroxy coumarin does not have an asymmetric locus and thus the preceding source for optical activity is unlikely to be the correct one. The only plausible explanation of the new cotton effects is that the electronic transitions of the coumarin derivatives, when bound, are perturbed by an asymmetric locus of the protein. In other words, the cotton effects are 'extrinsic' or 'induced'.

There are many qualitative and quantitative differences between the induced spectra of the various coumarins when bound to BSA or HSA, as can be seen from figures 5:6 and 5:7. Similarities do exist, however, particularly in the wavelengths of the C.D. maxima, which are displayed in table 5:7. In the region 300 - 350 nm all the interactions induced a maximum, and from previous discussion it is reasonable to assume that this is due to a perturbation of the $\pi \rightarrow \pi^*$ transitions of the main coumarin ring. The sign and magnitude of the maximum varies with compound and protein. Acenocoumarin and Coumachlor give negative, and warfarin positive, maxima with binding to either protein. Phenprocoumon gives a negative maximum with HSA, but binding to BSA produces a biphasic spectrum with a negative maximum at 332 nm and a positive maximum at 304 nm.

The sign and magnitude of an induced cotton effect depends upon the spatial relationship between the perturbed chromophore and the asymmetric centre (Schellman, 1968). The space around a chromophore is divided into areas of positive and negative contribution to the induced activity according to well defined symmetry rules. Thus, a given asymmetric centre may
generate positive or negative effects depending upon its spatial relationship with the perturbed chromophore. The magnitude of an induced cotton effect will depend upon the distance between the chromophore and asymmetric locus (Schellman, 1968, 1969).

As well as activity above 300 nm, the binding of the coumarins to albumin generated several induced bands at shorter wavelengths. With the exceptions of the binding of warfarin and coumachlor to HSA, all the interactions produced an extrinsic C.D. maximum around 290 nm. The absorption of UV light at this wavelength has been assigned to the 4-hydroxyl portion of the coumarin ring, and it seems reasonable that the induced C.D. may arise from perturbations of the same chromophore. The magnitude and sign of the band near 290 nm again varied with the nature of the coumarin and the protein. Acenocoumarin binding produced the largest effects in this region, particularly in combination with BSA where two maxima at 286 and 281 nm can be seen.

The interaction of the coumarins with HSA produced a small band of negative ellipticity at 268 nm, a wavelength at which the enantiomers of warfarin and phenprocoumon also show a small maximum. The chromophore which is perturbed to give this activity must, again, be a portion of the main coumarin ring since 4-hydroxycoumarin also gave a cotton effect at this wavelength. The exact nature of the chromophore is unknown. The interaction of acenocoumarin with HSA was again unusual in producing a band at 268 nm too small to quantify accurately due to the small signal:noise ratio. The lack of visualisation of a 268 nm peak for the BSA-coumarin interaction may reflect some fundamental difference in the binding site or
bonding mechanism, compared to HSA. Alternatively, the lack of effect may be explained by the fact that the intrinsic spectra of BSA has a pronounced peak at this wavelength where HSA has only a shoulder. This peak of BSA may mask the small induced effects which were apparent with HSA.

The most pronounced induced effects were produced in the region 256-257 nm; with the exception of acenocoumarin, all the benzyl coumarin derivatives gave positive effects with both albumins. There is no equivalent effect in the spectra of the 4-hydroxycoumarin-HSA complex nor in the intrinsic spectra of the enantiomers, which perhaps suggests that the binding of warfarin, coumachlor and phenprocoumon induce a conformational change in albumin, which is reflected in this C.D. band. An alternative explanation is that these effects are due to perturbations of the transitions of the phenyl electrons. The absence of a band for 4-hydroxycoumarin binding is consistent with this view. The lack of a 256/7 nm effect with acenocoumarin may be explained by the profound spectral effects of a nitro substituent upon aromatic systems, which has already been discussed. The effect of the nitro group may be to shift this cotton effect to a higher wavelength, perhaps to the 286-281 nm region where acenocoumarin binding produces a large maximum.

From the foregoing discussion, several conclusions may be drawn concerning the coumarin-albumin interaction. Perhaps the most important conclusion may be gleaned from the simple fact that extrinsic cotton effects are produced with binding. Since specific areas around a chromophore contribute to negative and positive cotton effects, a simple one point attachment of ligand and protein will not produce extrinsic optical activity since the ligand is free to rotate into both areas (Chignell, 1969b). Thus, the
coumarin-albumin complex must be a rigid one with at least two points of interaction.

Several chromophores have been shown to contribute to the extrinsic cotton effects. All the derivatives show perturbations in the $\pi \rightarrow \pi^*$ transitions of the conjugated coumarin ring system on binding to albumin, and the involvement of the 4-hydroxyl region of the ring is suggested in several of the spectra. The band at 268 nm, formed by interaction with BSA is also indicative of the main coumarin ring being involved in the binding. The phenyl portion of the side chain may be responsible for induced activity in the 286 nm region, but this is not confirmed. The remaining part of the side chain (propyl in the case of phenprocoumon, butanonyl in the cases of warfarin, acenocoumarin and coumachlor) has not been considered, but that is not to say that it is uninvolved in binding. A propyl group would not be expected to show any optical properties in the wavelength region under study, and the carbonyl group of the butanonyl moiety will only have a very weak $\pi \rightarrow \pi^*$ transition around 280 nm.

Despite the general similarities of the induced optical activities, there exist many differences in the sign, intensity and shape of the spectra. The differences reflect both the nature of the electronic transitions in the ligand and the spatial arrangement of the chromophores in relation to the asymmetric centre. A cursory glance at figures 5:6 and 5:7 will show that the effects generated upon binding to BSA and HSA are profoundly different, the most obvious and consistent difference is seen in the wavelength region 260-285 nm, where binding to HSA generates negative effects and BSA binding produces positive ellipticity. These differences presumably reflect the
dissimilar natures of the two sets of binding sites.

The difference in extrinsic spectra for the various coumarin derivatives is best considered by referring to warfarin as a 'standard'. The addition of a nitro group to the phenyl ring, to produce acenocoumarin has a profound effect upon the induced optical activity. Muller and Wollert (1973a) reported that nitro substituted benzodiazepines had different induced optical activity compared to other compounds in the series. The hydrophilic nitro group of acenocoumarol gives the compound a reduced partition coefficient, compared to warfarin (table 5:8). This reduction in lipophilicity is reflected in a smaller percentage of acenocoumarin bound to BSA, than warfarin, but there is no significant difference between the two compounds for HSA binding.

The p-chloro substituent of coumachlor makes the compound more lipophilic than warfarin. This is reflected in an increased partition coefficient and an increase in the percentage of coumachlor bound to BSA or HSA (table 5:8). A greater interaction of the side chain of coumachlor with the albumin binding site might affect the arrangement of the main ring with the asymmetric locus. This would explain why warfarin and coumachlor have induced spectra of opposite sign (above 290 nm) for binding with both HSA and BSA. In a similar way, the substitution of the butanonyl group of warfarin with the less polar propyl group, as in phenprocoumon, increases lipophilicity. This shows as increased percentage bound and partition values of phenprocoumon compared to warfarin. Again, an
increase in the binding of the side chain might affect the coumarin ring binding, the induced C.D. effects above 290 nm are opposite in sign for warfarin and phenprocoumon interactions with HSA. This difference is less pronounced for the binding to BSA which might reflect the relative importance of side chain binding for the two albumins.

One important lesson which should be learnt from these results is how subtle modifications of a complex molecule can have a profound effect upon the mode of interaction with albumin. Also, the data effectively illustrates how albumins from various species can vary in their binding characteristics.

5:4:3 THE INFLUENCE OF pH

The extrinsic C.D. spectra of the coumarin-albumin complexes were examined at various pH values, over the range 6.6 to 8.2. A change in the pH of a drug-albumin solution can cause a change in the magnitude or character of the interaction for any one of several reasons. If the ligand is ionisable, with a pKₐ in the region of the pH range under study, then binding changes may result from the different properties of the ionised and unionised forms of ligand. Chemical and structural changes of the protein with varying pH may also cause changed ligand interactions. The number and ratio of positively and negatively charged amino acid residues will change with pH, and if such residues are present at the binding site, the binding characteristics of the protein may alter. Albumin is known to undergo a structural transition beginning near pH 7.0 with completion at pH 9.0. This phenomenon is known as the N→B transition (Wallerik,
1973) and has been reported to alter the binding properties of the protein (Shecter, 1969; White et al., 1973; Müller and Wollert, 1974). The better known N → F transition occurs over the pH range 4 to 2 and is thus unlikely to be important in binding studies.

The C.D. spectrum of albumin does not change over the pH range 6.6 to 8.2 (Müller and Wollert, 1974) thus, any changes in the spectra of the coumarin-albumin complexes must be extrinsic in origin. The wavelength positions of the extrinsic spectra did not change with pH. There were, however, small alterations in the intensity of the bands. These alterations are shown in tables 5:5 and 5:6. The variations with pH were small for all the interactions, which is consistent with the opinion that ionic bonds play only a small role in the coumarin-albumin complex (see Chapter 3). The induced peaks around 290 nm, which have been assigned to the 4-hydroxyl portion of the coumarin ring, were changed, in all cases, with variation in pH. There was, however, no consistency in the direction or magnitude of the change. The 4-hydroxyl group is ionisable, with a pKa of 5 (for warfarin, Lachman, personal communication cited by Perrin and Nelson, 1972). There would then be significant changes in the amounts of ionised and unionised coumarin derivatives over the pH range under study. Different binding properties of these two forms would explain the changes in the 290 nm peak.

A significant change in the induced ellipticity above 300 nm was produced with pH change, in most of the interactions. Decreasing pH shifted the bands in a negative direction, which could be due to increasing protonation of the 4-hydroxyl moiety moving the coumarin ring towards an area of negative contribution to the cotton effects. The positive band at 312 nm for the warfarin-HSA complex is an example of such a negative shift with decreasing
pH. O'Reilly (1971) has reported that warfarin has a higher affinity for HSA with decreasing pH, so that, in this case, an increase in affinity is mirrored by a decrease in ellipticity; a surprising finding. However, Perrin and Nelson (1972) reported the extrinsic C.D. spectra of the warfarin-HSA complex, at 310 nm, to be positive at pH 7.4 but negative at pH 5.0. This is consistent with the proposed movement of the coumarin ring into a negative contributing area with increasing protonation and is compatible with the reduction in positive ellipticity going from pH 8.2 to pH 6.6, which must reflect a portion of this inversion. Because of the small size and the lack of consistency of the pH induced spectral changes no further conclusions can be reasonably drawn from these results.
CHAPTER SIX

THE BINDING OF THE ENANTIOMERS OF WARFARIN

AND PHENPROCOUMON TO SERUM ALBUMIN
INTRODUCTION

The coumarin oral anticoagulants, of the general formula:

\[
\begin{array}{c}
\text{O} \\
\text{C} \\
\text{H} \\
\text{C} \\
\text{H} \\
\text{R}_1
\end{array}
\]

possess an asymmetric centre which results in the compounds having two isomeric forms, or enantiomers. Both phenprocoumon and warfarin exist in two isomeric forms namely the R (+) and S (-) forms, but the clinical formulations of the drugs are racemic. Due to their very widespread use, and because of the serious potentiation of anticoagulant effect with the co-administration of certain common therapeutic agents, the metabolism and distribution of coumarin drugs have been intensively studied.

The S(-) optical isomer of both phenprocoumon and warfarin is a more potent anticoagulant than the R (+) enantiomer in man and the rat. There are also stereoselective pathways of metabolism and elimination for the warfarin isomers in these two species and a stereospecific distribution of phenprocoumon in man (a full list of references for these facts will be given in the discussion).

We have studied the binding of the enantiomers of phenprocoumon and warfarin to bovine and human serum albumin using the equilibrium dialysis technique. In order to investigate the mechanisms of stereoselective binding, the interactions have also been studied using the techniques of
circular dichroism and fluorescence. Attempts were also made to use nuclear magnetic resonance measurements to probe the coumarin-albumin complex and although the experiments were largely unsuccessful, data is presented which illustrates well the difficulties of examining drug-protein interactions using this technique.

The binding of racemic phenprocoumon and its enantiomers to hepatic cytochrome P450, as determined by substrate induced binding spectra, was also examined. Since the coumarin drugs are thought to be metabolised largely by the mixed function oxidase system, it was hoped that any stereospecificity in biotransformation would be reflected in the binding spectra.
6:2 EXPERIMENTAL APPROACH

The enantiomers of warfarin were kindly donated by Professor A. Breckenridge (University of Liverpool, England). Phenprocoumon (PPC) enantiomers used in the dialysis and circular dichroism measurements were gifts of Hoffmann-La Roche (Basle, Switzerland), whilst those used in the fluorescence, NMR and microsomal experiments were prepared by a modification of the method of West and Link (1965) as described in chapter 2. Human and bovine serum albumins (crystalline, 100% electrophoretically pure) were obtained from Behringwerke (Marburg, Lahn, Germany) for all measurements except those involving NMR where BSA (Conn fraction V) was used from the Sigma Chemical Co.

6:2:1 DIALYSIS

The binding of PPC and warfarin ($1.5 - 6.0 \times 10^{-5}$ M) to BSA and HSA ($3 \times 10^{-5}$ M) in phosphate buffer (67 mM, pH 7.4) at 25°C was measured using the half-cell equilibrium dialysis technique (see chapter 3). Drug concentrations were determined by the fluorimetric method of Seiler and Duckert (1968) as modified by Jähnchen et al. (1976). The binding values are expressed as percentage bound at the concentrations used in the C.D. and fluorescence work. The PPC - HSA interaction was more thoroughly investigated, and the data was analysed by the method of Scatchard (1949). Those data points which contribute to the line equivalent to the high affinity binding were analysed by linear regression to obtain the $y$ intercept which was taken as the total binding constant (TBC), $n_1k_1 + n_2k_2$. 
6:2:2 CIRCULAR DICHROISM

The CD measurements were made at 27°C in a Cary 61 spectropolarimeter using 10 mm cylindrical cells. Other conditions of measurement are detailed in chapter 2. The intrinsic spectra of the enantiomers (6 x 10⁻⁵ M) were recorded in phosphate buffer (67 mM, pH 7.4) and the spectra of the racemates and enantiomers were also determined in the presence of HSA or BSA (3 x 10⁻⁵ M). Results are expressed as molar ellipticities θ (deg. cm⁻² dmole⁻¹) calculated with reference to albumin concentration, total drug concentration or bound drug concentration. All the results in the presence of albumin are reported as difference values using the ellipticity of albumin at the same wavelengths as a blank, except for those spectra in the lower halves of figures 6:2 to 6:5 which are 'double difference' spectra, i.e., the intrinsic ellipticities of both albumin and the isomers have been subtracted.

6:2:3 FLUORESCENCE

Fluorescence titration of racemic phenprocoumon and its isomers into buffer (sodium phosphate, 67 mM, pH 7.4) or albumin solution (1 x 10⁻⁵ M) was performed in the normal way, as described in chapter 2. The competition of phenylbutazone for the binding sites of racemic, R (+) and S (-) phenprocoumon and warfarin was examined by the fluorescent probe displacement technique.
The NMR spectra of racemic warfarin and racemic phenprocoumon (0.2 M in NaOD/D$_2$O) were examined using a Varian XL 100 instrument by accumulation using the Fourier Transform (FT) facilities. The NMR spectra of phenprocoumon (1 x 10$^{-3}$ M) in the presence of BSA (Conn fraction V, 1% to 5% w/v in D$_2$O) was also obtained. The effect of protein upon the various resonances of phenprocoumon was estimated from the observed line broadenings. $\frac{1}{T_2}$, the reciprocal transverse relaxation time, was taken as $\pi \Delta \nu$ when $\Delta \nu$ is the observed line width at half peak height.

BINDING SPECTRA

Hepatic microsomal suspensions were prepared from rat and hamster as outlined in chapter 2. Substrate induced binding spectra were recorded using either the Perkin-Elmer 356 double-beam, double-wavelength spectrophotometer or the equivalent machine produced by the American Instrument Company. The spectral dissociation constants ($K_s$) were calculated from Lineweaver-Burk diagrams of titrations of phenprocoumon (250 mM in Dimethylformamide, DMF) into microsomal preparations, by the method of Al-Gailany (1975).
6 : 3  FIGURES AND TABLES
Figure 6: Scatchard plots of the interactions of HSA with phenprocoumon and its enantiomers

Each point represents mean ± SEM (n = 4 or 5). The values of the closed symbols were used in regression analyses to give the following equations:

S (-) PPC: \[ \frac{r}{D_f} = 723,935 - 388,667r \ (R = 0.88, n = 20), \ TBC = 7.24 \times 10^5 \ M^{-1} \]

R (+) PPC: \[ \frac{r}{D_f} = 403,392 - 174,012r \ (R = 0.84, n = 18), \ TBC = 4.03 \times 10^5 \ M^{-1} \]

RS (±) PPC: \[ \frac{r}{D_f} = 633,915 - 297,697r \ (R = 0.84, n = 19), \ TBC = 6.34 \times 10^5 \ M^{-1} \]

The total binding constant (TBC) = \( n_1K_1 + n_2K_2 \), or the y intercept.
Figure 6.2 Circular dichroism spectra of phenprocoumon and its isomers in buffer and in the presence of HSA.

Upper half: PPC isomers (6 x 10^{-5} M) in buffer or in HSA (3 x 10^{-5} M) solution, where the effects of HSA have been subtracted.

Lower half: PPC isomers and racemate (6 x 10^{-5} M) in HSA (3 x 10^{-5} M) solution. The effects of HSA have been subtracted in all cases, plus the intrinsic effects in the case of the isomers. The molar ellipticity (θ) is calculated with respect to HSA concentration. Each point is the mean of five determinations.
Figure 6:3  Circular dichroism spectra of warfarin and its isomers in buffer and in the presence of BSA

Upper half: Warfarin isomers (6 x 10^{-5} M) in buffer or in BSA (3 x 10^{-5} M) solution, where the effects of BSA have been subtracted.

Lower half: Warfarin isomers and racemate (6 x 10^{-5} M) in BSA (3 x 10^{-5} M) solution. The effects of BSA have been subtracted in all cases, plus the intrinsic effects in the case of the isomers. Molar ellipticity (θ) is calculated with respect to BSA concentration. Each point is the mean of five determinations.
Figure 6:4  Circular dichroism spectra of phenprocoumon and its isomers in buffer and in the presence of BSA

Upper half: PPC isomers ($6 \times 10^{-5}$ M) in buffer or in BSA ($3 \times 10^{-5}$ M) solution, where the effects of BSA have been subtracted.

Lower half: PPC isomers and racemate ($6 \times 10^{-5}$ M) in BSA solution. The effects of BSA have been subtracted in all cases, plus the intrinsic effects in the cases of the isomers. The molar ellipticity ($\theta$) is calculated with respect to BSA concentration. Each point is the mean of five determinations.
Figure 6:5  Circular dichroism spectra of warfarin and its isomers in buffer and in the presence of HSA

Upper half: Warfarin isomers (6 x 10^-5 M) in buffer or in HSA (3 x 10^-5 M) solution, where the effects of HSA have been subtracted.

Lower half: Warfarin isomers and racemate (6 x 10^-5 M) in HSA (3 x 10^-5 M) solution. The effects of HSA have been subtracted in all cases, plus the intrinsic effects in the cases of the isomers. The molar ellipticity (θ) is calculated with respect to HSA concentration. Each point is the mean of five determinations.
Figure 6: The titration of phosphate buffer and HSA with phenprocoumon

Fluorescence intensity (arbitrary units)

Phenprocoumon concentration  \( \times 10^{-6} \) M.
HSA concentration 1 \( \times 10^{-5} \) M. Excitation wavelength 320 nm.
The open circles cover the values for all three forms of PPC in buffer.

\( \Delta = R,S \text{ (+) PPC} \quad \circ = S \text{ (-) PPC} \quad \square = R(+) \text{ PPC} \)
Figure 6.7 Dixon plot of the displacement of phenprocoumon from HSA by phenylbutazone

The HSA concentration was $10 \times 10^{-6}$ M, PPC concentration was $10 \times 10^{-6}$ M. Excitation wavelength 320 nm, emission monitored at 380 nm. ▲ = R(+)PPC, ■ = Racemic PPC, ○ = S(−)PPC.
The phenprocoumon concentration was $2 \times 10^{-3}$ M in NaOD/D$_2$O (pD 10). $\frac{1}{T_2}$ was taken as $\pi \Delta \nu \frac{1}{2}$, measured from those spectra in figure 6:7.

- $\bullet$ = aromatic protons,
- $\blacksquare$ = methyl protons.
The spectrum was recorded from a solution of 0.2 M PPC in 30% NaOD. The resonances are equivalent to the following protons: a and b, aromatic protons; d, the proton of the asymmetric carbon; e, f, ethyl protons. The water peak at c has been obliterated.
Figure 6:10  The influence of BSA on the NMR spectrum of phenprocoumon

+ 4% BSA

+ 3% BSA

+ 2% BSA

+ 1% BSA

$2 \times 10^{-3}$ M PPC alone
Table 6:1 The percentage binding of warfarin, phenprocoumon and their enantiomers to Bovine and Human Serum Albumins

<table>
<thead>
<tr>
<th>Ligand</th>
<th>BSA</th>
<th></th>
<th>HSA</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$1.5 \times 10^{-5}$ M</td>
<td>$6.0 \times 10^{-5}$ M</td>
<td>$1.5 \times 10^{-5}$ M</td>
<td>$3.0 \times 10^{-5}$ M</td>
</tr>
<tr>
<td>S (-) PPC</td>
<td>7.80 ± 0.17 (6)</td>
<td>13.33 ± 0.32 (4)</td>
<td>5.92 ± 0.44 (6)</td>
<td>8.06 ± 0.52 (6)</td>
</tr>
<tr>
<td>R (+) PPC</td>
<td>6.80 ± 0.36 * (5)</td>
<td>14.28 ± 0.59 (4)</td>
<td>9.34 ± 0.21 ** (6)</td>
<td>12.16 ± 1.05 ** (6)</td>
</tr>
<tr>
<td>Racemic PPC</td>
<td>-</td>
<td>14.82 ± 0.20 (6)</td>
<td>6.68 ± 1.05 (6)</td>
<td>9.08 ± 0.12 (6)</td>
</tr>
<tr>
<td>S (-) Warfarin</td>
<td>13.16 ± 0.14 (7)</td>
<td>20.09 ± 0.97 (4)</td>
<td>10.12 ± 0.36 (6)</td>
<td>-</td>
</tr>
<tr>
<td>R (+) Warfarin</td>
<td>11.05 ± 0.41 *** (7)</td>
<td>18.63 ± 0.53 n.s.</td>
<td>10.92 ± 0.61 n.s.</td>
<td>-</td>
</tr>
<tr>
<td>Racemic Warfarin</td>
<td>-</td>
<td>18.84 ± 0.30 (6)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The binding data was determined by equilibrium dialysis using an albumin concentration of $3 \times 10^{-5}$ M in phosphate buffer (67 mM, pH 7.4) at 25°C. Results are expressed as mean ± SD (number of observations). Statistical tests for differences between R (+) and S(-) performed by the students 't' method.
(n.s. = $p > 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$)
Table 6:2  Circular dichroism data of the phenprocoumon - albumin complexes

<table>
<thead>
<tr>
<th>Ligand</th>
<th>wavelength of observation (nm)</th>
<th>In phosphate buffer</th>
<th></th>
<th>In HSA 3 x 10⁻⁵ M</th>
<th></th>
<th>In BSA 3 x 10⁻⁵ M</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Molar ellipticity [θ] x 10⁻⁴</td>
<td>g x 10⁵</td>
<td>Molar ellipticity [θ] x 10⁻⁴</td>
<td>g x 10⁵</td>
<td>Molar ellipticity [θ] x 10⁻⁴</td>
<td>g x 10⁵</td>
</tr>
<tr>
<td>S (−) Phenprocoumon</td>
<td>310</td>
<td>-0.40 ± 0.03 (8)</td>
<td>-8.3</td>
<td>-0.98 ± 0.03 (8)</td>
<td>-24.5</td>
<td>-0.44 ± 0.03 (8)</td>
<td>-9.9</td>
</tr>
<tr>
<td></td>
<td>290</td>
<td>-0.99 ± 0.03 (8)</td>
<td>-27.8</td>
<td>-2.03 ± 0.09 (8)</td>
<td>-78.6</td>
<td>-0.59 ± 0.02 (8)</td>
<td>-18.7</td>
</tr>
<tr>
<td></td>
<td>270</td>
<td>-0.49 ± 0.02 (8)</td>
<td>-40.1</td>
<td>-0.76 ± 0.02 (8)</td>
<td>-70.1</td>
<td>-0.16 ± 0.04 (8)</td>
<td>-10.2</td>
</tr>
<tr>
<td>R (+) Phenprocoumon</td>
<td>310</td>
<td>+0.35 ± 0.01 (8)</td>
<td>+6.9</td>
<td>+0.19 ± 0.02 (8)</td>
<td>-10.8</td>
<td>+0.48 ± 0.03 (8)</td>
<td>+10.5</td>
</tr>
<tr>
<td></td>
<td>290</td>
<td>+0.90 ± 0.02 (8)</td>
<td>+23.9</td>
<td>+0.16 ± 0.05 (8)</td>
<td>+0.6</td>
<td>+0.44 ± 0.05 (8)</td>
<td>+11.2</td>
</tr>
<tr>
<td></td>
<td>270</td>
<td>+0.47 ± 0.01 (8)</td>
<td>+37.5</td>
<td>-0.15 ± 0.02 (8)</td>
<td>-23.8</td>
<td>+0.40 ± 0.07 (8)</td>
<td>+33.7</td>
</tr>
<tr>
<td>Racemic Phenprocoumon</td>
<td>310</td>
<td>-</td>
<td>-</td>
<td>-0.53 ± 0.01 (6)</td>
<td>-13.4</td>
<td>+0.15 ± 0.04 (6)</td>
<td>+3.3</td>
</tr>
<tr>
<td></td>
<td>290</td>
<td>-</td>
<td>-</td>
<td>-0.84 ± 0.01 (6)</td>
<td>-32.6</td>
<td>-0.03 ± 0.01 (6)</td>
<td>-0.8</td>
</tr>
<tr>
<td></td>
<td>270</td>
<td>-</td>
<td>-</td>
<td>-0.45 ± 0.03 (6)</td>
<td>-45.6</td>
<td>+0.25 ± 0.06 (6)</td>
<td>+19.3</td>
</tr>
</tbody>
</table>

[θ] = molar ellipticity, calculated with respect to total drug concentrations (6 x 10⁻⁵ M). g = anisotropy factors, calculated with respect to total drug concentration in the case of isomers in buffer and with respect to bound drug concentrations in the case of drugs in the presence of albumin. Those values in the presence of albumin are difference values using the ellipticity of albumin as the blank. Results are expressed as Mean ± SD (number of observations).
Table 6.3  Circular dichroism data of the warfarin – albumin complexes

<table>
<thead>
<tr>
<th>Ligand</th>
<th>wavelength of observation nm</th>
<th>In phosphate buffer</th>
<th>In HSA 3 x 10^-5 M</th>
<th>In BSA 3 x 10^-5 M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>[θ] x 10^-4</td>
<td>Molar ellipticity</td>
<td>Molar ellipticity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>g x 10^5</td>
<td>[θ] x 10^-4</td>
<td>g x 10^5</td>
</tr>
<tr>
<td>S (-) Warfarin</td>
<td>310</td>
<td>-0.65 ± 0.02 (6)</td>
<td>-13.8</td>
<td>-0.47 ± 0.02 (6)</td>
</tr>
<tr>
<td></td>
<td>290</td>
<td>-0.79 ± 0.02 (6)</td>
<td>-20.3</td>
<td>-1.10 ± 0.03 (6)</td>
</tr>
<tr>
<td></td>
<td>270</td>
<td>-0.44 ± 0.02 (6)</td>
<td>-29.6</td>
<td>-0.91 ± 0.05 (6)</td>
</tr>
<tr>
<td>R (+) Warfarin</td>
<td>310</td>
<td>+0.66 ± 0.02 (6)</td>
<td>+12.9</td>
<td>+1.05 ± 0.03 (6)</td>
</tr>
<tr>
<td></td>
<td>290</td>
<td>+0.80 ± 0.05 (6)</td>
<td>+18.9</td>
<td>+1.11 ± 0.02 (6)</td>
</tr>
<tr>
<td></td>
<td>270</td>
<td>+0.46 ± 0.01 (6)</td>
<td>+29.7</td>
<td>0.00 ± 0.06 (6)</td>
</tr>
<tr>
<td>Racemic Warfarin</td>
<td>310</td>
<td>-</td>
<td>-</td>
<td>+0.44 ± 0.02 (6)</td>
</tr>
<tr>
<td></td>
<td>290</td>
<td>-</td>
<td>-</td>
<td>+0.11 ± 0.01 (6)</td>
</tr>
<tr>
<td></td>
<td>270</td>
<td>-</td>
<td>-</td>
<td>-0.32 ± 0.04 (6)</td>
</tr>
</tbody>
</table>

[θ] = molar ellipticity, calculated with respect to total drug concentrations (6 x 10^-5 M). g = anisotropy factors, calculated with respect to total drug concentration in the case of isomers in buffer and with respect to bound drug concentrations in the case of drugs in the presence of albumin. Those values in the presence of albumin are difference values using the ellipticity of albumin as the blank. Results are expressed as Mean ± SD (number of observations).
### Parameters of the Substrate Induced Spectra of Warfarin and Phenprocoumon in Rat or Hamster Hepatic Microsomes

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Rat</th>
<th>Hamster</th>
</tr>
</thead>
<tbody>
<tr>
<td>S(-) Warfarin</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>R(+) Warfarin</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>RS(-) Warfarin</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>S(-) Phenprocoumon</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>R(+) Phenprocoumon</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>RS(+) Phenprocoumon</td>
<td>b</td>
<td>b</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Rat $K_s \times 10^{-5}$ M</th>
<th>Hamster $K_s \times 10^{-5}$ M</th>
<th>Type of spectrum</th>
<th>Type of spectrum</th>
</tr>
</thead>
<tbody>
<tr>
<td>S(-) Warfarin</td>
<td>$16.0 \pm 1.4$</td>
<td>$11.0 \pm 1.2$</td>
<td>R1</td>
<td>R1</td>
</tr>
<tr>
<td>R(+) Warfarin</td>
<td>$45.0 \pm 4.1$</td>
<td>$8.0 \pm 1.5$</td>
<td>R1</td>
<td>R1</td>
</tr>
<tr>
<td>RS(-) Warfarin</td>
<td>$23.0 \pm 1.8$</td>
<td>$8.8 \pm 1.0$</td>
<td>R1</td>
<td>n.d.</td>
</tr>
<tr>
<td>S(-) Phenprocoumon</td>
<td>$4.75$</td>
<td>n.d.</td>
<td>R1</td>
<td>n.d.</td>
</tr>
<tr>
<td>R(+) Phenprocoumon</td>
<td>$4.74$</td>
<td>n.d.</td>
<td>R1</td>
<td>n.d.</td>
</tr>
<tr>
<td>RS(+) Phenprocoumon</td>
<td>$4.18$</td>
<td>n.d.</td>
<td>R1</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

- Data from Wilson (1974), mean ± SEM (n = 4 animals).
- $K_s$ values from the following linear regressions of Lineweaver-Burk plots:
- S(-) PPC: $\frac{1}{\Delta A} = 54.94 + 0.00261 \frac{1}{[PPC]}$ (r = 0.992, n = 7)
- R(+) PPC: $\frac{1}{\Delta A} = 59.04 + 0.00247 \frac{1}{[PPC]}$ (r = 0.972, n = 9)
- RS(+) PPC: $\frac{1}{\Delta A} = 54.85 + 0.00260 \frac{1}{[PPC]}$ (r = 0.983, n = 7)
6:4 RESULTS AND DISCUSSION

The equilibrium dialysis studies of the binding of warfarin and phenprocoumon enantiomers to albumin yielded interesting, but confusing, results (table 6:1). Previous studies of the binding of R (+) and S (-) warfarin to HSA have shown only very small differences between the two enantiomers. O'Reilly (1971, 1973) reported the S (-) isomers to have a higher association constant than R (+) but no statistical information was provided. In a more thorough study, Sellers and Koch-Weser (1975) showed a slightly greater binding of S (-) to HSA, compared to the R (+) enantiomer. Our results are fully compatible with these findings. We found a lower free S(-) concentration at both drug levels studied (1.5 x 10^{-5} M and 6.0 x 10^{-5} M, HSA concentration 3.0 x 10^{-5} M), but there was not a statistically significant difference from the values of the R (+) isomer. That there is only a very slight difference between the binding of the two warfarin enantiomers to human albumin is confirmed by the observation that the apparent volume of distribution of the drugs, in man, are not significantly different (Hewick and McEwen, 1973).

To our knowledge, the binding of the warfarin enantiomers to BSA has not previously been reported. Table 1 shows the rather surprising finding that R (+) warfarin is bound more strongly than S (-) warfarin at both concentrations studied. The difference was only significant, however, at the lower drug concentration of 1.5 x 10^{-5} M (BSA 3 x 10^{-5} M). It would be interesting to see if the volumes of distribution for the two isomers would be different when administered to cattle. Unfortunately, even the splendid work of Link and his associates at the Wisconsin Agricultural Research Farm
(Campbell and Link, 1941; Link, 1943) did not include such an investigation. Again, it would be interesting to examine the binding of the Isomers to rat albumin, the rat being another species where the volumes of distribution of the two enantiomers of warfarin are not significantly different (Hewick, 1972; Breckenridge and Orme, 1972).

In the course of this work, more attention has been focussed upon phenprocoumon, since it is less well studied, but nevertheless a much prescribed anticoagulant (especially on the continent). In contrast to warfarin, the isomers of PPC were bound to HSA in a most disparate manner. Table 6:1 shows that more S (-) isomer is bound at all the concentrations studied and the difference is highly significant at low drug concentrations. A Scatchard plot of the PPC - HSA interaction was used to calculate the total binding constants (TBC, equivalent to $n_1K_1 + n_2K_2$) for the three forms of PPC. The total binding constant for racemic PPC ($4.03 \times 10^5 \text{ M}^{-1}$) agrees well with that reported by O'Reilly (1971, 1973) of $5.05 \times 10^5 \text{ M}^{-1}$ and by Foged et al. (1976) of $7.3 \times 10^5 \text{ M}^{-1}$. The pharmacokinetics of the enantiomers of phenprocoumon in man have been studied by Jähnchen et al. (1976) and Hewick and Shepherd (1976). Both studies involved the administration of each form of the drugs to five subjects. Jähnchen et al., found the apparent volume of distribution of the S (-) PPC isomer to be smaller in all subjects, whilst Hewick and Shepherd reported the same finding for three subjects. A smaller volume of distribution of S (-) PPC is consistent with the higher human albumin binding of this isomer.

The BSA binding of the R (+) isomer of PPC, like that of warfarin, is more avid than that of the S (-) isomer. However, unlike warfarin, where the difference between R (+) and S (-) is far greater for BSA than HSA, the
difference between R (+) PPC and S (-) PPC is significantly less for BSA than for HSA.

To summarise the binding data: at low drug concentrations (1.5 x 10^{-5} M), HSA binds the S (-) enantiomers more avidly than the R (+) enantiomers. The difference is very marked in the case of PPC, but not significant for the warfarin isomers. In contrast, BSA binds the R (+) enantiomers to a greater extent than the S (-), and the difference is more marked for warfarin than for PPC. At this drug concentration HSA binds a greater amount of drug than BSA. At higher drug concentrations (6 x 10^{-5}) there is apparently no stereospecific binding in any of the complexes. Also, BSA binds more drug than does HSA. It seems, then, that only the high affinity sites of albumin bind the coumarin drugs stereospecifically.

Stereospecific binding by albumin has been reported for only a few small molecules. The enantiomers of tryptophan (McMenamy and Oncley, 1958); noradrenaline (Powis, 1975); Oxazepam Hemisuccinate (Müller and Wollert, 1975) and two dansyl amino acids (Koga et al., 1975) have differing affinities for serum albumin, but little is known of the mechanisms of stereospecific albumin binding.

Circular dichroism measurements have proved useful in studies of optical isomer binding to albumin (Perrin, 1973; Müller and Wollert, 1975) because of the unique sensitivity of the technique to molecular geometry. The CD spectra of PPC and warfarin racemate and isomer complexes with HSA and BSA are shown in figures 6:2 to 6:5. Tables 6:2 and 6:3 show the molar ellipticities and anisotropy factors of the same complexes at the wavelengths 310 nm, 290 nm and 270 nm. These measurements were all made from spectra
recorded at drug concentrations of \(6 \times 10^{-5}\) M (albumin \(3 \times 10^{-5}\) M), qualitatively identical spectra were obtained at a concentration of \(3 \times 10^{-5}\) M. The higher concentration was used because the greater intensity of the effects allowed more accurate measurements to be made.

The intrinsic CD spectra of the R (+) and S (−) isomers of both coumarins were of equal magnitude, but opposite sign (see tables 6:2 and 6:3 and figures 6:2 and 6:3) which is confirmation of the optical purity of the enantiomers. The spectra of PPC and warfarin isomers were qualitatively similar, showing main CD maxima at about 310 nm, 290 nm and 240 nm with less well defined maxima at 268 nm and 262 nm. The racemic compounds showed no optical activity in the presence of phosphate buffer alone.

New cotton effects were produced with the albumin binding of all the enantiomers. It is worth repeating the logic which leads to the conclusion that these new effects must be extrinsic or induced. An alteration in the intrinsic spectra of albumin with binding is unlikely to be the source since large changes in protein conformation, tantamount to denaturation, are required to produce such significant changes in the CD spectra. In addition, the new CD maxima fall at the same wavelengths as the intrinsic spectra of the enantiomers. A change in the perturbations of the drug enantiomers by its own asymmetric carbon atom can be excluded since the symmetrical 4-hydroxy-coumarin exhibits similar, new Cotton effects when bound to albumin (see figure 5:8). Changed Cotton effects can arise from binding induced changes in molar extinction coefficients. However, the anisotropy values (which are calculated, taking into account absorption changes) differ in the same way as the molar ellipticities when in buffer and in albumin solution (see tables 6:2 and 6:3). The only remaining explanation is that the new Cotton
effects are due to perturbations of the electronic transitions of the drugs bound, by an asymmetric locus of the protein. It was shown in the previous chapter that the induced ellipticity in the wavelength regions 310 nm and 370 nm are due to transitions in the main coumarin ring, whilst those around 290 nm were shown to originate in transitions of the 4-hydroxyl moiety of the compounds.

Figures 6:2 to 6:5 show a gradation in the similarities between the induced spectra of the R (+) and S (-) enantiomers. For PPC bound to HSA (figure 6:2) the induced spectra are of the same sign, shape and at the same wavelength positions for each form of the drug. The same can be said of the spectra for the various forms of warfarin when bound to BSA (figure 6:3), except the very small negative effects around 290 nm and 270 nm for the R (+) enantiomer, at which wavelengths the racemic and S (-) forms have positive minima. The induced spectra of the PPC - BSA complexes (figure 6:4) are, again, of similar shape and wavelength position, but in this case, the signs of the induced ellipticities between 260 and 300 nm are positive for the S (-) isomer and negative for the R (+) isomer (above 300 nm the signs of the induced spectra are the same). Finally the interaction of S (-) warfarin and R (+) warfarin with HSA produced very different spectra. To a large extent, wavelength positions of peaks and troughs do not coincide, and the signs of the spectra are disimilar for much of the wavelength region under study. There is only one real similarity between the two spectra, at 270 nm where there is a negative peak for each enantiomer. Within experimental error (the standard errors are not shown so as not to further complicate the diagrams) the induced spectra of the racemates fall between the two isomers in all four cases, as would be expected.
This gradation in the spectral relationship between R (+) and S (−) enantiomers can be explained by referring to the binding parameters as shown in table 6:1. The largest difference between any of the enantiomers is that shown by the PPC - HSA interaction (5.92 ± 0.44% free for S (−), 9.34 ± 0.21% for R (+) at 1.5 x 10⁻⁵ M drug and 3.0 x 10⁻⁵ M HSA). This is the interaction which gave the greatest similarities between the induced CD spectra of the two enantiomers. At the other end of the scale, there are no significant differences between the percentage binding of the enantiomers of warfarin with HSA. The CD spectra for these interactions show the greatest divergence of all those recorded. The other two sets of data also fit well with this inverse relationship, the CD spectra of the warfarin enantiomers bound to BSA are very similar and these interactions show highly significant differences in binding. Finally, the PPC - BSA interaction shows a small degree of stereospecific binding but pronounced differences in the signs of the induced CD spectra of the enantiomers.

In chapter 5 it was shown that the induced CD effects in the wavelength region 350 - 270 nm are due to perturbations of the 4-hydroxy coumarin ring system. Since the induced spectra of the PPC enantiomers bound to HSA are very similar, it must be concluded that the orientation of the 4-hydroxycoumarin ring is identical in both complexes. If this is so, the orientation of the other substituents at the asymmetric carbon atom (i.e. the phenyl and ethyl groups and the single hydrogen) must be different. These substituents specifically contribute to the binding mechanism and the binding energy (see previous chapter and also O'Reilly, 1971; Chignell, 1970 and Garten and Wasilait, 1971) probably by hydrophobic interactions with the protein.
The arrangement of S (-) side chains with the binding site must allow more bonds to be formed, since this isomer exhibits the stronger binding. To continue this argument, the enantiomers of warfarin, when bound to HSA, are perturbed to give different CD spectra. This suggests that the orientation of the 4-hydroxycoumarin ring cannot be the same for the S (-)-HSA complex and the R (+)-HSA complex. If the orientation of the main ring is dissimilar, then the side groups may be able to interact with the protein in the same manner, for each isomer. This could explain why there are only very small differences in the binding parameters for the warfarin isomers with HSA. The PPC - BSA and warfarin - BSA complexes show intermediate behaviour in both CD spectra and binding parameters, which presumably reflects the partially different orientations of side chains and coumarin nuclei in these complexes.

In order to gain further insight into the mechanisms of coumarin isomer binding to albumin, the techniques of nuclear magnetic resonance and fluorescence were used. It was hoped that NMR measurements would show the relative contributions of the various portions of the coumarin molecule to the overall binding process. This approach has been used to study the albumin binding of sulphonamides (Jardetsky and Wade-Jardetsky, 1965), penicillin (Fisher and Jardetsky, 1965) acetylsalicylic acid (Sykes and Hull, 1973) and acetrizoate (Rodrigues de Miranda and Hilbers, 1976). Initial experiments were performed using racemic PPC and warfarin at high (0.2 M) concentrations, in order to record the basic NMR spectra of the compounds. Good results were obtained for PPC (see figure 6:6), but not for warfarin. This was because the compounds were dissolved in a D$_2$O solution of NaOD at a high pH (pD) in order to obtain the required concentration. Under these
conditions, the protons of the warfarin aliphatic side chain exchange freely with deuterium, due to the tautomerism of the ketone functional group, with the resulting loss of side chain resonances. Because of this reaction, no further NMR measurements were made with warfarin.

Exploratory experiments with racemic PPC in D₂O/NaOD showed that the minimum concentration of drug which gave a satisfactory NMR spectra was 2 x 10⁻³ M. Since we wished to study the binding of the drug to the high affinity sites, a protein concentration of at least 2 x 10⁻³ M (≈14% w/v albumin) would be required. Figure 6:7 shows the effects of up to 4% albumin (BSA in this case, for economic reasons. The NMR spectra of BSA and HSA are very similar) on the NMR spectra of 2 x 10⁻³ M racemic PPC. It is obvious from these results that it would be impossible to study the binding at drug–albumin ratios of about 1:1 since the spectrum of the protein would mask the drug resonances. This is a major drawback of the NMR technique for the study of small molecule–albumin interactions. In all the previous reports, small molecule has been present in about a 100 fold excess over protein. Under these conditions, any information obtained will probably reflect secondary or 'non-specific' binding which makes up the bulk of binding complexes a high ligand concentration. Figure 6:8 shows how the reciprocal transverse (spin-spin) relaxation time T₂⁻¹ of the methyl and aromatic protons of racemic PPC varied with protein concentration over the range 0 - 4% BSA. Both peaks were broadened at about the same rate, which suggests that the side chain as well as the main aromatic rings are involved with binding. Otherwise, little information can be derived from the NMR measurements. Because of the practical difficulties which arose, the NMR
measurements were discontinued. This short study does, however, illustrate that nuclear magnetic resonance techniques may be of little value if information is required on mixtures where the ligand and macromolecule concentrations are of the same order of magnitude. The technique may provide useful information on secondary binding, or primary binding where the association constant is small, when excess ligand can be used.

Phenprocoumon and warfarin are both fluorescent probes for the coumarin binding sites (see chapter 3). The interaction between the enantiomers of PPC and HSA was studied using the fluorescence measurements. Attention was focussed on this particular interaction since the isomers have been shown to bind to different degrees, but CD measurements did not show any great dissimilarities. The racemic, S (-) and R (+) forms of PPC have identical fluorescence intensities in buffer (see figure 6:9) and identical excitation and emission maxima of 317 nm and 385 nm respectively. The fluorescence enhancement with binding to HSA was also the same for each form of PPC, within the limits of experimental error (figure 6:9). The excitation and emission maxima were shifted to 325 nm and 380 nm respectively. (The blue shift in emission wavelength was explained in chapter 3, the red shift in excitation maximum is equivalent to a similar shift in absorption maximum, which was discussed in chapter 5). It is, perhaps, not surprising that there was no difference in the fluorescence enhancement of the isomers, since the coumarin rings of the two molecules are thought to lie in the same orientation. The fluorescence of PPC is probably due largely to transitions in the main coumarin ring and, thus, stereospecific fluorescence of PPC - HSA would not be expected. Following the same argument, the
enantiomers of warfarin might be expected to show different fluorescence properties when bound to HSA. Wilson (1974) studied the fluorescence of warfarin and its isomers when bound to albumin, but did not find any significant differences between the various forms of the drug. It seems, then, that whilst the coumarin rings of S (-) and R (+) warfarin may be differently orientated with respect to the asymmetric locus of the protein, they are nevertheless in areas of similar hydrophobicity.

The competition of phenylbutazone and warfarin for albumin binding sites is well known (Solomon and Schrogie, 1967). It has been shown that the fluorescent probe properties of warfarin can be used to monitor this competition (Wilson, 1974). Initial suggestions that the potentiation of the anticoagulant effect of warfarin, in man, with concomitant phenylbutazone administration could be due to displacement of the coumarin from albumin binding sites (Aggeler et al., 1967) have been modified, because of recent pharmacokinetic data. The plasma clearance of S (-) warfarin is slowed by phenylbutazone administration, whilst the clearance of the R (+) isomer is increased (Lewis et al., 1974; Schary et al., 1975). It was concluded that inhibition of the metabolism of the more potent S (-) isomer is responsible for the potentiation effect of phenylbutazone. (The different potencies and routes of metabolism of PPC and warfarin isomers will be discussed further).

The competition of phenylbutazone and PPC for HSA binding sites was examined using the fluorescent probe displacement technique. The results are expressed in the form of a Dixon plot in figure 6:10. The displacement appears to be biphasic, the two portions being equivalent to phenylbutazone concentrations of 0 - 25 x 10^{-6} M and 25 - 150 x 10^{-6} M (PPC and HSA concentrations were 10 x 10^{-6} M). The initial displacement, at low
phenylbutazone concentrations, accounts for much of the reduction in fluorescence. This is probably due to competition between phenylbutazone and PPC for primary binding sites. The reduction in fluorescence at higher phenylbutazone concentrations is probably a reflection of competition for secondary binding sites. Using low concentrations of phenylbutazone, Wilson (1974) showed a competitive inhibition of racemic warfarin binding to HSA. It seems, then, that phenylbutazone and the coumarins not only share high affinity binding sites, but also some secondary binding is also to communal sites on HSA. There was no difference between R (+) and S (-) PPC in the degree of displacement by phenylbutazone. Whether the difference between racemic PPC and the isomers, as shown in figure 6:10, is an authentic disparity or merely experimental variation remains to be proven by further investigation.

Our studies of the enantiomers of warfarin and phenprocoumon were extended by an examination of the substrate induced binding spectra which were produced on the addition of the compounds to rat or hamster hepatic microsomes. Warfarin and phenprocoumon are hydroxylated by the microsomal mixed function oxidase system and, in addition, warfarin undergoes side chain reduction of the ketonic function by a soluble enzyme system. The stereospecific metabolism of warfarin is thought to explain many of the pharmacodynamic differences between the isomers. In man, S (-) warfarin is eliminated more rapidly than R (+) (Hewick and McEwen, 1973; Breckenridge et al., 1974). The principal metabolic product of S (-) warfarin, in man, is 7 hydroxywarfarin, with minor quantities of S, S warfarin alcohol and 6 hydroxywarfarin. The R (+) isomer is metabolised mainly to the R,S warfarin alcohol, with the remainder as 6 hydroxywarfarin (Lewis et al., 1974).
In the rat, however, R (+) warfarin is eliminated more quickly than S (-) (Breckenridge and Orme, 1972; Hewick, 1972). In vitro experiments using rat hepatic tissues have shown 7 and 8 hydroxylation to be stereoselective for R (+) warfarin, this isomer was also metabolised more rapidly than S (-) warfarin. In contrast 4'hydroxylation was stereoselective for the S (-) enantiomer. Reduction of the side chain ketonic function was stereoselective for R (+) warfarin and reduction was to the S alcohol (Pohl et al., 1976). These stereospecific pathways of drug metabolism are reflected in the substrate induced binding spectra of the warfarin enantiomers. Previous work in our laboratories has shown that both R (+) and S (-) warfarin elicit a reverse type one (R1) binding spectra with rat hepatic microsomes but the spectral dissociation constants (K_s) were very dissimilar at 45.0 x 10^{-5} M and 16.0 x 10^{-5} M respectively (Wilson, 1974; see table 6:4). Experiments were also performed using hamster hepatic microsomes, where there was an even more marked stereospecificity, the R (+) isomer eliciting a type 1 spectra and the S (-) a reverse type 1. There is, however, no available data concerning the metabolism of warfarin enantiomers in this species.

The available information on phenprocoumon metabolism and elimination is far less complete than that for warfarin. The plasma clearance of S (-) PPC in man is slightly less than that of R (+) PPC (Jahnchen et al., 1976; Hewick and Shepherd, 1976) but there is no significant difference in the rate of elimination. It has already been suggested that the difference in the plasma clearance of the PPC isomers may be due to their protein binding characteristics, rather than stereospecific metabolism, but there is no metabolic evidence to support this theory. In a short study of the urinary
excretion of PPC isomers in the rat, Golding and West (1969) found R (+) PPC to be eliminated faster than S (-) PPC (as is the case for warfarin in the rat). The difference was not large, however, and the recent findings that PPC related material is excreted mainly in the faeces (Haddock et al., 1975) also casts some doubt upon the significance of the data of Golding and West (1969).

We have investigated the substrate induced binding spectra of racemic, R (+) and S (-) phenprocoumon in order that some tentative prediction of the presence of stereospecific microsomal metabolism may be made. At present, there is no information available on the metabolism of PPC isomers, but racemic PPC has been studied (Haddock et al., 1975) (obviously one of the stereoselective reactions of warfarin, the ketonic reduction, cannot take place with PPC). Racemic PPC is hydroxylated in the same positions as racemic warfarin, but the relative amounts of the hydroxylated metabolites are very different. Whilst warfarin is excreted from the rat mainly as 7-hydroxywarfarin (Link et al., 1965), the major fecal and urinary metabolites of PPC are the 6 and 4' hydroxy derivatives. The addition of each of the forms of phenprocoumon to rat or hamster hepatic microsomes elicited reverse type one (R1) spectra changes in all cases (table 6:4). From Lineweaver-Burk diagrams of the titration of rat microsomes (2 mg.ml\(^{-1}\) protein) with phenprocoumon (5 x 10\(^{-5}\) M to 4 x 10\(^{-4}\) M) spectral dissociation constants (K\(_s\)) were calculated. The K\(_s\) values were very similar for each of the three forms of phenprocoumon (table 6:4). Since the isomers elicit the same type of spectral change, and have similar dissociation constants, it seems that they interact with the mixed function oxidase system in similar fashions. This suggests that the enantiomers of phenprocoumon may not
be stereoselectively hydroxylated. The evidence is by no means conclusive, but the conclusion fits well with the known pharmacokinetic parameters of the individual isomers.

It has been suggested that the binding of coumarins to serum albumin could be used as a model for the interaction of the drugs with their receptor sites (O'Reilly, 1969, 1971, 1973). However, some of the inadequacies of this model have been illustrated by the binding and activities of the isomers of warfarin. The S (-) warfarin isomer is about five times more potent than the R (+) isomer, both in man (O'Reilly, 1971, 1974) and in the rat (Eble et al., 1966; Breckenridge and Orme, 1972). This difference is not just a function of the metabolism or the distribution of the isomers, but the intrinsic anticoagulant activity of the S (-) isomer has been shown to be at least double that of R (+) (Nagashima et al., 1969; Breckenridge and Orme, 1972). However, as has been shown in this and previous work (table 6:1; O'Reilly, 1971; Sellers and Koch Wesser, 1975) the enantiomers differ only slightly in their binding to human albumin. It seems, then, that HSA is not a good model for receptor binding. On the other hand, it is possible that the nature of the albumin-coumarin interaction should be considered, not the magnitude of the complex. We have shown that the main coumarin rings of S (-) and R (+) warfarin are in different orientation when bound to albumin. It may be that the orientation of the coumarin rings at the receptor sites accounts for the difference in potency of the two enantiomers.

In the case of phenprocoumon, the coumarin rings of the two enantiomers are thought, by us, to be bound to HSA in similar orientations. Whilst the S (-) isomer is more potent in man, (Hewick and Shepherd, 1976) it
has been suggested that this is due to differences in distribution between the two isomers (Jähnchen et al., 1976). In addition, when considering albumin binding as a receptor model, it is apparent that the protein binding data must be generated from the same species as that in which anticoagulant activity is measured. Thus, we have shown that bovine and human albumins interact very differently with the isomers of both phenprocoumon and warfarin.
CHAPTER SEVEN

FINAL DISCUSSION: THE NATURE OF LIGAND-ALBUMIN COMPLEXES
The binding sites of albumin have been the subject of considerable research and discussion, but only recently has significant progress been made towards understanding the topography of the protein. Recent elucidation of the amino acid sequences of bovine and human albumins (Brown, 1975; Behrens et al., 1975) has given substance to previously nebulous concepts of the nature of albumin binding sites. The proposed model of albumin (Brown, 1975) includes many loops which, when associated in the native protein, give rise to several clefts in the molecule. Indeed, if anion binding sites are regions of apolar character with adjacent areas of positively charged residues (Swaney and Klotz, 1970), then the structure of albumin is ideally suited for the binding of organic acids. Several binding areas have been isolated, for various compounds, and these have been discussed in chapter 1 and are illustrated in figure 1:6. Note that all the specific binding areas are concerned with ionic molecules or compounds with polar regions. Our studies with the coumarin drugs, particularly warfarin, have indicated that there is a high affinity binding site, located near the available tryptophan of albumin, which is not a primary site for the fatty acids. There appear to be two types of high affinity sites, for short and medium chain fatty acids, which are not primary binding areas for warfarin.

It seems, then, that there are several areas of the albumin molecule which provide high affinity interactions with organic acids. We have demonstrated the existence of three such areas, but there are probably several more. Thorp (1972) has suggested that there are three separate primary binding areas on human albumin, and proposed that these areas may change conformation to suit the ligand present. Sudlow and Colleagues (1975) have shown two distinct drug binding sites on albumin, one of which binds warfarin, phenyl-
butazone, iophenoxic acid, DNSA and some sulphonamides. The other is specific for flufenamic acid, CPIB, ethacrynic acid and ANS. We have also shown displacement of warfarin and phenprocoumon by phenylbutazone, thus the coumarin site which we have studied is probably equivalent to one of the sites of Sudlow et al. (1975).

Whilst these binding sites are specific, they are not exclusive. Primary sites for one type of compound can interact with a second compound, if the second compound is present in excess and has saturated its own high affinity sites. We have shown that the primary site for warfarin can serve as a secondary site for fatty acids and vice versa. The primary short chain FA and medium chain FA sites can each act as secondary sites for the other category of fatty acid.

We have not studied the binding of basic compounds to albumin and there is little information available. It is likely that there are specific areas of albumin which provide high affinity interactions with basic compounds, but probably far fewer in number than the anion binding sites. Kriegstein et al. (1972) have reported that the basic phenothiazines bind to a single site on albumin.

In addition to these specific sites, which are involved in the binding of charged molecules, albumin appears to possess areas of lipophilic character which are able to interact with many organic small molecules. There are probably many of these sites, perhaps partially overlapping, with a wide range of affinities depending upon the ligand involved. They provide both secondary sites for the binding of charged ligands, when in excess of primary site concentration, and are also involved in the interactions with uncharged ligands. The binding parameters of neutral molecules to albumin are characterised by
a greater number of 'primary' sites but lower association constants than equivalent values for ionic compounds (Steinhardt and Reynolds, 1969). The carbamates are typical of neutral ligands for albumin. Carbamates have 4 to 9 primary sites with associations constants of about $10^3$ M$^{-1}$ (Wilson, 1974). From displacement experiments, it was shown that carbamates and fatty acids do not share any primary sites. In addition, carbamate binding was only affected by fatty acid when concentrations were such that FA was bound to secondary sites (table 4:9).

The nature of a ligand-albumin complex is not only dependent upon the type of binding site but also the properties of the ligand and the types of bond which can be formed between the two species. These three determinants of binding are intimately related since the structure of ligand determines the sites to which it will preferentially bind and the bonds formed depend upon both ligand and site. The thermodynamic parameters of fatty acids binding to BSA (table 4:5) suggest that their primary binding is a combination of electrostatic and hydrophobic interactions. The electrostatic binding is roughly constant for all the fatty acids, but hydrophobic interactions, indicated by a positive entropy change, become larger with increasing chain length. Secondary binding is accompanied by positive enthalpy and entropy changes, which is typical of hydrophobic bonding. Secondary binding appears to be primarily a function of the lipophilicity of the ligand, the secondary binding of the fatty acids increased linearly with chain length or partition coefficient.

The interaction of warfarin and HSA is accompanied by negative $\Delta H$ and positive $\Delta S$ values (O'Reilly, 1973) which have been interpreted as indicating the presence of hydrogen bonding and hydrophobic bonding. The importance of hydrophobic bonding in the coumarin-albumin complex is shown by our
spectral and equilibrium dialysis studies. The lipophilicities of the four compounds, as determined by octanol/water partition, correlated well with the percentage of drug bound. There was little influence of pH on the extrinsic CD spectra of the coumarins when bound, which suggests that electrostatic interactions do not play a major role in bonding. The binding of the coumarins to albumin produced changes in the UV absorption and fluorescence of the compounds which indicate that the binding sites are hydrophobic in nature.

CD studies of the coumarin derivatives showed that the various compounds take up different orientations when bound to albumin. The lipophilicity of the side chains is thought to influence the arrangement of the molecule with respect to the asymmetric centre of the binding site. The spatial relationship between ligand and binding site can be most important, as has been shown in our studies on the enantiomers of phenprocoumon and warfarin. The isomers of these compounds bind differently to albumin, due to different orientations at the same binding site. Furthermore, the human and bovine albumins differ in their stereospecificity for the isomers. This point illustrates the fact that albumins from various species might be generally very similar in structure and binding properties, but they can exhibit quite disparate binding parameters in certain cases. This should be remembered if the interaction with albumin is to be used as a model for other binding reactions, for instance, drug-receptor interactions.

This discussion on albumin binding sites has been performed with the supposition that the sites are pre-existing on the molecule. This is the approach which was adopted by Scatchard (1949) in his mathematical model for ligand binding. All workers who use the Scatchard plot are, perhaps
unknowingly, concurring with this theory and the assumptions which are implicit in this treatment of data. The assumptions that there is no interaction between binding sites, that binding sites are not created or destroyed and that the affinities within a class of sites are identical may not be absolutely correct, but the Scatchard analysis yields parameters which are readily understandable and easy to manipulate. There is the alternative approach of Karush (1950) which proposes that the albumin molecule has a high degree of conformational adaptability and that binding sites are created and moulded to suit the ligand present. The mathematical analysis which is commensurate with this theory is that of multiple step-wise equilibria proposed by Klotz et al. (1946) and recently championed by Fletcher and his colleagues (Fletcher et al., 1970, 1973). This model makes no assumptions of pre-existing sites or independence between sites and is probably superior to the Scatchard (1949) model since it can be applied to any binding situation. However, the solution of the model requires computer analysis and the parameters are often unwieldy, being a group of 8 or 10 association constants.

All the binding equilibria studied in this project have been analysed using the Scatchard model and, although there are limitations, the data is fully compatible with this approach. For the binding of ligands to primary sites we have found no evidence for distinct conformational changes. Primary fatty acid binding had no effect upon albumin fluorescence, although very high fatty acid concentrations did quench albumin, probably because of conformational changes. The interaction of albumin with the coumarins produced only very small changes in the UV absorption spectra of the protein and apparently no changes in CD effects. Since the majority of ligand binding to albumin in vivo is to primary sites, because of the low small molecule concentrations, it seems unlikely that conformational changes play
a major role in normal binding to albumin.

The displacement of one ligand by another has also been considered, by a number of authors, to be the result of conformational changes when the two ligands do not share the same high affinity sites. For example, it has been suggested that fatty acids affect the binding of warfarin by alterations of the conformation of the drug binding sites (Chakrabati et al., 1976). However, their studies, and many other similar investigations, were carried out with high ligand concentrations relative to albumin. Under these circumstances competition for common secondary sites can result in displacement even though the two ligands do not share the same primary sites. We have shown that most bound compounds can be shown to be displaced if the correct concentrations of protein and competitor are chosen.

Our investigations with the coumarins and fatty acids have shown the usefulness of studying a series of closely related ligands in order to understand the mechanisms of binding to albumin. Further studies, using other groups of compounds should extend our knowledge of the binding sites of albumin. The study of more series of acidic compounds should lead to an estimate of the total number of anion binding sites and a better understanding of their specificity, whilst work with series of basic compounds would yield valuable information in this little studied area.
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