A STUDY OF SMALL MOLECULE-PROTEIN INTERACTIONS:
WITH SPECIAL REFERENCE TO THE PLASMA PROTEIN
BINDING OF DRUGS

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in the
University of Surrey

by

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ABSTRACT

Equilibrium dialysis, ultrafiltration and frontal analysis gel chromatography have been shown to produce comparable results for the binding, to bovine serum albumin, of a number of drugs with markedly different degrees of binding and physico-chemical properties. The binding of sodium salicylate to rat serum has been studied by in vivo dialysis and the results shown to be in good agreement with those obtained by in vitro ultrafiltration.

The binding of a series of aliphatic carbamates to bovine serum albumin has been used to study the importance of hydrophobic bonding in small molecule-protein interactions. Binding was found to be directly correlated with the lipophilic character of the carbamates and associated with negative free energy and positive entropy changes. A model is proposed to account for the fluorescence observations on the binding of the carbamates to albumin.

Warfarin has been shown to be a fluorescent probe and this property used to investigate the nature of its interaction with different species and preparations of albumin. The binding site of warfarin on albumin was shown to be located in a hydrophobic region of the protein and its binding suggested to involve the amino acid residues; lysine, tyrosine and histidine. Differences in the affinity and nature of the binding sites were apparent for the interaction of warfarin with rat, human and bovine (crystalline and fraction V) albumins. Binding was found to occur through one primary binding site for all species, except perhaps for bovine albumin (fraction V). Approximately eight secondary sites were apparent for all albumins. Good
agreement was found for the fluorescence of warfarin between rat and human albumins and serums. The potential of the fluorescent probe technique to evaluate drug displacement from protein binding sites was demonstrated.

1-Anilino-8-naphthalenesulphonate and the novel fluorescent probes warfarin (R+ and S- isomers) and benzidine, together with difference spectra, have been used to investigate the nature of the hepatic Microsomal cytochrome P-450 binding sites (type I, type II and Reverse type I). The type I and Reverse type I sites were found to be closely associated and appear to be in a hydrophobic environment, whereas the type II was hydrophilic in nature. The binding of R(+) warfarin presents an interesting species difference being type I in the hamster, but Reverse type I in the rat. The binding of these fluorescent probes to albumin and microsomal protein has been compared and the binding sites found to be located in similarly hydrophilic or hydrophobic environments.
To my Parents
and
Mary
ACKNOWLEDGMENTS

I am greatly indebted to Dr. J.W. Bridges for his constant interest, encouragement and invaluable guidance during the course of this work. I would like to express my thanks to Professor D.V. Parke for his help and encouragement, and also for the facilities made available to me in his Department. My gratitude also goes to members of staff, especially Dr. W.E. Lindup, and my colleagues, for their help and many valuable discussions. My thanks are also due to the secretarial and technical staff.

I am especially grateful to Roche Products Ltd., for financial support and to Dr. R.F. Long for his helpful interest and advice during this study.

Finally, I should like to thank Mrs. J. King and Miss K. Ratcliffe for typing this thesis.
The Road goes ever on and on
   Down from the door where it began.
Now far ahead the Road has gone,
   And I must follow, if I can,
Pursuing it with weary feet,
   Until it joins some larger way,
Where many paths and errands meet.
   And wither then? I cannot say.

J.R.R. Tolkien.
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CHAPTER ONE

INTRODUCTION
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9. Introduction to Present Work.
1. General Introduction to Protein-Drug Interactions

The effects a drug produces in a biological system are ultimately consequences of physico-chemical interactions between the drug and 'receptor' molecules. This concept was first proposed by Ehrlich (1909) 'corpora non agunt nisi fixata'. The capacity of a drug to enter into specific combinations with constituents of the living organism, especially proteins, poses for pharmacology one of its most fundamental tasks - to elucidate the nature of drug action in terms of specific molecular interactions. It is likely that the intensity of the pharmacological response elicited by many drugs depends on the extent of receptor occupation. This is largely related to their affinity towards the receptors and to the concentration of drug reaching the environment of the receptors, i.e. the receptor compartment. It is not usually possible to measure this concentration directly, but it is anticipated to be related to drug blood levels, which are measurable, based on the assumption that an apparent distribution equilibrium is established between drug in the plasma and the receptor compartments. The factors modifying the drug concentration at the receptor site are shown in Fig. I, 1.
In 1938, Bennhold suggested that the plasma proteins were a transport mechanism for the regulated distribution of naturally occurring and medicinal substances, though this may be something of an overstatement, plasma proteins do interact with a diversity of ions and small molecules, including drugs. Goldstein's (1949) excellent and farsighted review emphasised the importance of the binding of drugs to plasma proteins. His review contained a summary of the known plasma protein-drug interactions and also set forth the concept of a rational approach to the problem of drug-plasma protein interactions. He further suggested that such interactions could serve as useful models for the general phenomenon of drug-receptor interactions. With the increased awareness of the significance of protein-binding a large number of studies have appeared since Goldstein's review. Meyer and Guttman (1968) recently summarised, in a similar tabular manner to that of Goldstein, the experimental observations on the protein binding of drugs, dyes, hormones, vitamins and other small molecules made since 1949, together with a discussion of recent developments in this field. A number of general articles and reviews on this subject have also appeared:- Scatchard (1949); Brodie and Hogben (1957); Thorpe (1964); Brodie (1965); Desgrez and DeTraverse (1966); Goldstein et al. (1968);
Prescott (1969); Kreiglstein (1969); Settle et al. (1971); Cohen (1971); Davison (1971); Benny and Hamlin (1971); Thorp (1972); Hasselblatt (1972); Hamlin and Shah (1972); Westphal and Knoefel (1972); Shah and Herd (1973). The present introduction to the subject is concerned mainly with the protein binding of drugs.
The interaction of a drug (D) with the unoccupied binding sites of a protein (P) may normally be considered as a reversible reaction obeying the law of mass action (equation 1).

\[
\text{D}_f + P \xrightleftharpoons[k_2]{k_1} \text{DP} \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad (1)
\]

where DP is the protein-drug complex and \(k_1\) and \(k_2\) are the rate constants of the forward and reverse reactions.

At equilibrium

\[
\frac{\text{D}_f}{\text{DP}} = K = \frac{k_2}{k_1}
\]

where \(\text{D}_f\) is the concentration of unbound drug, \(P\) the molar concentration of protein and \(\text{DP}\) the molar concentrations of the drug-protein complex.

\(K\) is the equilibrium or dissociation constant.

This is the simplest form of the equation and assumes a single binding protein with a sole binding site. In the case of several binding sites or proteins a succession of such equilibria will be required to describe the overall interaction (Thorpe, 1964). \(1/K\) is termed the association constant and is a measure of the so-called affinity of the protein for the drug. Because of the use of concentrations rather than activity coefficients the constant is more accurately termed the 'apparent association constant' (k).

At equilibrium, for any given concentration of free drug, the molar concentration of bound drug depends on:- the protein concentration (P); the association constant (k) and the number of binding sites per protein molecule (n). The relationship between these values and the fraction of drug bound is expressed in equation 2, (Goldstein, 1949).
\[
\beta = \frac{1}{1 + \frac{1}{nkP} + \frac{D}{nP}} 
\]

It can be seen that the fraction bound (\(\beta\)) depends directly on \(P\), \(n\), \(k\) and inversely with the drug concentration \(D\). Goldstein (1949) has stated that reports on the percentage or fraction of drug bound should always specify the total or 'unbound' drug concentration; \(k\) and \(n\) can be affected by pH, temperature, ionic strength and dielectric constant of the solution (Goldstein, 1949; Meyer and Guttman, 1968; Thorp, 1964; Agren, et al, 1970; Cohen, 1971). Graphical methods of determining \(n\) and \(k\) are discussed in Chapter Two.

2. The Plasma Proteins

The plasma contains large quantities of proteins which are able to bind a considerable number and variety of ions and small molecules (Fig. I, 2). This is perhaps not surprising for Bennhold (1966) has calculated that 5 litres of blood contains plasma proteins having a minimum surface area of 130,000 sq. miles. Several detailed treatise of the composition, structure and function of the plasma proteins have appeared (Schultze and Heremans, 1966; Putnam, 1960; Neurath, 1965; Turner and Hulme, 1971; Marks, 1972). As well as several accounts of particular plasma proteins, e.g. lipoproteins, immunoglobulins, etc., (Dettlebach and Ritzmann, 1968; Tria and Scanu, 1969; Haaf, 1969; Scanu, 1972; Jonas, 1972; Mateau, et al, 1972; Scanu and Wisdom, 1972).

Albumin, which is the principal plasma protein (\(\approx 50\%\) of the total) makes the predominant contribution to the plasma protein binding of most drugs, and many endogenous materials such as fatty acids and bilirubin (Goldstein, et al, 1969). The albumin molecule is a single
Fig. I, 2  Examples of Interactions with Plasma Proteins
(Goldstein et al., 1968)

- Zn$^{2+}$, Lipids
- Cholesterol
- Vitamins A, K, D, E
- Cu$^{2+}$ (Ceruloplasmin)
- Lithium carmine
- Hemoglobin (Haptoglobin)
- Ca$^{2+}$, Cu$^{2+}$, Zn$^{2+}$
- Bilirubin
- Uric acid
- Vitamin C
- Adenosine
- Tetracyclines
- Chloramphenicol
- Digitonin
- Fatty acids
- Suramin
- Quinacrine
- Penicillin
- Salicylate
- p-aminosalicylate
- Sulphonamides
- Streptomycin
- Acid dyes
- Phenol red
- Histamine
- Triodothyronine
- Thyroxine
- Barbiturates

- Fe$^{2+}$ (Transferrin)
- Steroid hormones (Transcortin)
- Vit. B_{12}
- Sialic acid
- Thyroxine
polypeptide chain with a molecular weight of about 69,000, depending on
the species. Its high concentration in plasma and relatively low molecular
weight make it the major determinant of intravascular colloid osmotic
pressure (oncotic pressure). Albumin has an iso-electric point at about
pH 5, when it carries about 100 each of positive and negative charges.
At plasma pH (7.4) it has a net negative charge of 18 (White, et al., 1968),
but it can nevertheless interact strongly at this pH with anions as well as
cations, indeed acidic drugs are generally more avidly bound. The
circulating mass of intravascular albumin totals approximately 140 g, for
a 75 kg man, of which about ten per cent or more is displaced per hour,
mainly into the interstitial fluid, from which it is returned to the blood
via the lymphatic system. Despite the fact that the delivery of newly
synthesised albumin appears to by-pass the lymph under normal conditions
(Rothschild, et al., 1972). The protein concentration of the thoracic
duct lymph is almost half that of plasma. The extravascular albumin mass
(approx. 60-70% of the total) is in a state of very active exchange with
the plasma pool. The half-life in the body of albumin is about 10-25 days,
and approximately 13 g of albumin is normally synthesised and catabolised
per day. The liver appears to be the only site of synthesis (Miller and
Bale, 1954; Rothschild, et al., 1972), but catabolism takes place at
several sites, notably the liver, kidneys and gastrointestinal tract
(Schultz and Heremans, 1966; Turner and Hulme, 1971). A curious anomaly
of albumin synthesis in which two electrophoretically distinct albumins
are present in the plasma, occurs as an uncommon and totally asymptomatic
inborn error of metabolism, or biological variant.

It is now generally accepted that in most normal healthy adult's
albumin concentration in plasma is between 3.5 and 4.5 g/100 ml (Marks,
1972). Like all other plasma proteins, albumin shows a 10-20 per cent
change in concentration with posture; the higher levels being found within 10-30 minutes of assuming the standing position. Serum albumin levels are also known to be affected by strenuous exercise (Poortmans, 1971). Serum albumin is lower during infancy and senility than during childhood and adult life. Small fluctuations occur in the serum albumin concentration during the menstrual cycle, with maximum levels occurring during midcycle and serum albumin levels also fall progressively during pregnancy, (Marks, 1972; Stimson, 1972). No cause of hyperalbuminaemia is known apart from the general increase in plasma proteins that occurs as a result of dehydration or venistasis during blood collection. Diseases causing hypoalbuminaemia on the other hand, are common and include:- nephrosis, hepatitis, cirrhosis, carcinomas, gastrointestinal diseases, hypergammaglobulinemia, malnutrition (e.g. Kwashiorkor), heart disease, hypothyroidism. In addition, stress, injury, alcohol, carbon tetrachloride and acclimatisation to heat are known to decrease albumin production (Rothschild, et al, 1972). Analbuminaemia is a rare familial disorder in which there is a congenital defect in albumin synthesis.

The α and β globulins also form an important group of binding proteins (Westphal, 1961; Sandberg, et al, 1966; Seal and Doe, 1966), several have a high affinity, but relatively low capacity, for a number of endogenous substances and chemically related synthetic compounds. The physiological importance of these high affinity binding globulins is not clear, but they may act as transporters and regulators of biological activity (Salvatore, 1966; Keller, et al, 1969). Westphal (1971), has reviewed the extensive literature on the interaction of corticosteroids with plasma proteins, particularly the corticosteroid binding globulins. The metal-binding globulins, transferrin and ceruloplasmin, interact strongly and specifically with iron and copper, respectively, and are essential to
the transport of these ions. Binding globulins have also been identified for other endogenous compounds including testosterone (Rosner and Deakins, 1968; Clark et al. 1971; Lea and Støa, 1972), oestradiol (Clark, et al., 1971), progesterone (Ryan and Westphal, 1972); thyroxine (Rall et al. 1964; Salvatore et al. 1966); vitamin B₃₂ (Retief et al. 1967; Hippe and Olesen, 1971), vitamin A (Kanai et al., 1968) and vitamin D (Morgan et al., 1958). A review of the binding of vitamins to plasma proteins has been presented by Raoul (1966). Thyroxine displays a high affinity interaction with prealbumin (Tritsch, 1972), which appears to be greater than with thyroxine-binding globulin (Davis and Gregerman, 1971).

The plasma γ-globulins have not so far been found to interact significantly with drugs (Goldstein et al., 1968), except where they occur as specific antibodies to them. The production of antibodies to protein hormones such as insulin and gonadotrophins is well known as a cause of lessening therapeutic efficiency in cases where these drugs have been used for a long time. With growing recognition that many chemically simpler drugs are capable of behaving as haptens or antigens, the possibility that some patients can develop circulating antibodies capable of binding a drug avidly and extensively must be considered.

The overwhelming mass of plasma lipids are transported bound to certain proteins known collectively as lipoproteins. Free fatty acids, at low concentrations, are transported preferentially by serum albumin, but at higher fatty acid levels binding to α and β lipoproteins may also occur (Mora et al., 1955; Polonovski, 1966). The formation of a bilirubin-lipoprotein complex has also been reported to occur at high plasma bilirubin levels (Cooke and Roberts, 1969). However, the preferential association of bilirubin is with albumin (Lathe et al., 1966) as is the binding of
endogenous prostaglandins (Raz, 1972a). The drug tetrahydrocannabinol and its metabolites are reported to bind to lipoproteins (Wahlqvist et al., 1970; Widman et al., 1973). The interaction of both exogenous and endogenous compounds with erythrocytes has been reported and include promazine and chlorpromazine (Jänchen et al., 1971), steroid (Brinkman and van der Molen, 1972), bilirubin (Bratlid, 1972), salicylate and phenobarbital (McArthur et al., 1971), although usually the amount bound is small, the significance of erythrocyte binding should not be overlooked. For the majority of drugs and exogenous compounds, albumin would appear to be the primary binding plasma protein, although interaction with globulins, usually at high drug concentrations has been shown for some drugs e.g. penicillins, tetracyclines and chlorpromazine, methadone (Olsen, 1973) sulphonylureas (Judis, 1972).

3. **Nature of Drug-Albumin Interactions**

Serum albumins can potentially interact with anions, cations and neutral compounds, although the binding of acidic compounds to albumin has been extensively studied (Goldstein, 1949; Meyer and Guttman, 1968), the binding of neutral (Steinhardt and Reynolds, 1969), and basic compounds (Borga, et al., 1969; Franksson and Anggard, 1970), has not. At one time binding to plasma protein was attributed to simple electrostatic attraction between the ionic form of the drug and a charged group on albumin. However, additional forces must be invoked to explain the observed relationship between degree of binding and chemical structure. In a series of barbiturates, for example, all of which have the same $pK_a$ (7.6), barbitone is hardly bound, but as the chain length is extended the binding increases to 55% with pentobarbitone (Brodie, 1966). It seems likely that ionic bonds, hydrogen bonds, London-van der Waal forces or hydrophobic bonds are also concerned in these interactions. (Goldstein, et al., 1968;
The concept of hydrophobic bonding has arisen largely from the early work of Frank and Wen (1957). However, despite a number of other fundamental studies on the thermodynamic properties of hydrocarbons in aqueous solutions (Nemethy and Scheraga, 1962 a, b, c, & 1963; Crothers and Ratner, 1968; Hermann, 1971; Tanford, 1972) differences of opinion exist as to the nature of these interactions (Klotz, 1958; Pauling, 1961; Hildebrand, 1968; Nemethy, et al., 1968; Holtzer and Emerson, 1969). Hydrophobic bonding has been broadly defined as the tendency of non-polar groupings, particularly hydrocarbons, to aggregate together in aqueous solution. The structure of water in close proximity to hydrophobic groups is believed to play a significant role in this bonding since such adherence processes are accompanied by entropy effects (Nagwekar and Kostenbauder, 1970). The resulting entropy gain has been suggested to be the driving force for the formation of hydrophobic bonding and has been ascribed to the disruption of the quasi-crystalline water structure ('ice-berg'), around the non-polar groups in aqueous solution (Kauzmann, 1959; Schachman, 1963; Molyneux and Frank, 1969). However, it should be emphasised (Frank and Evans, 1945) that the structure of the icebergs need not be the same as that in ordinary ice nor the same for all icebergs.

Hydrophobic bonding could explain the increasing positive entropies observed for the binding of the anions; octyl, decyl, and dodecyl sulphates (Karush and Sonenberg, 1949). More recently a number of studies have further implemented the important role of hydrophobic bonding in drug-protein interactions. Hansch has studied the binding of a wide variety of compounds with bovine serum albumin (Hansch, et al., 1965; Helmer, et al., 1968; Hansch, 1969, 1971). An excellent correlation was found between log P, π (Hansch-Fujita constant, 1964, see Chapter Two), and the molar
concentration of a compound necessary for a one to one complex of the compound with the protein. In an extensive study by Scholtan (1968), using a variety of drugs, including sulphonamides, penicillins, cardenolides and steroid hormones, it was shown that the affinity of binding increased if the hydrophobic character of the drug was increased by the introduction of non-polar substituents such as; alkyl groups, halogen atoms and aromatic rings. Correlations between binding and lipophilic character have also been obtained for a number of other compounds e.g. penicillins (Bird and Marshall, 1967), p-hydroxybenzoic acid esters (Patel et al., 1968), a homologous series of fatty acids (Teresi and Luck, 1952; Goodman, 1958); sulphates, sulphonates and alcohols (Ray et al., 1966; Reynolds et al., 1967), hydrocarbons (Wildnauer and Canady, 1966; Mohammadzadeh, 1969 a, b), aromatic compounds and alkanes (Wetlaufer and Lovrien, 1964; Sachyun, 1966). Wishnia and Pinder (1964), in a detailed account of hydrophobic interactions of the alkanes with BSA have suggested that possible models for the binding of hydrocarbons to proteins could be:

a) penetration into the interior of the protein  
b) penetration into surface clusters  
c) binding on the protein surface with the formation of a more favourable ice-cage  
   or  d) binding with completion of a partially formed clathrate cage

Correlations have also been found between partition coefficient and the interaction of basic compounds with BSA, for phenothiazines (Kriegstein et al., 1972 a, b; Nambu and Nagai, 1972), but not for the binding of eight tricyclic antidepressants (Jorgensen, et al., 1973).

Hansch (1971), has concluded from calculations of the free energy of binding for a wide variety of drugs e.g. sulphonamides, tetracyclines,
penicillins, cardenolides, steroid bisquanylhydrazones, acridines, steroid hormones, that steric effects and specific hydrogen bonding (not accounted for in the partitioning reference model) are not generally important in the binding of drugs to albumin.

Attempts to use the water solubility (Vandenbelt, 1954; Sahyun, 1966), of the small molecule as an extrathermodynamic reference do not generally appear to give as good a correlation as partition coefficients. This is perhaps surprising in view of the finding of Saracco and Marchetti (1958) and later Hansch (1968) that a linear correlation between \( \log P \) (partition coefficient) and \( \frac{1}{S} \) (aqueous solubility) existed for a number of compounds.

The studies with neutral compounds are limited, and therefore it has generally not proved possible to confidently single out hydrophobic bonding as the prime determinant of drug-albumin interactions, thus calculation of the free energy of hydrophobic bond formation is complicated by the fact that other interactions may be simultaneously operative. Studies of the solubility of hydrocarbons in aqueous solution have suggested \( \Delta G \) values of 0.7 (Nemethy and Scheraga, 1962c) or 0.85 K cal/mole (Davis et al., 1972) for one H–C–CH\(_2\) interaction. Whether these values are in any way appropriate to drug-protein interactions for the assessment of the thermodynamic contribution for hydrophobic bonding is unknown. The determination of the thermodynamic contribution from various chemical groupings would appear to be a necessary prerequisite to a fuller understanding of the importance of hydrophobic forces in small molecule-protein interactions.

The sulphonamide drugs have frequently been used as a model to evaluate the contribution of these various binding possibilities to serum albumin, however, clear differences of opinion exist in the interpretation of the results. Thus Fujita (1972), has recently confirmed his support for
the importance of hydrophobic bonding, rather than electrostatic interactions, for the binding of sulphonamides to serum proteins. Jardetzky and Wade-Jardetzky (1965), using NMR methods, have concluded that a hydrophobic part of the sulphonamide structure, probably the p-aminobenzene moiety, does participate in the binding. Agren, et al., (1971), however, while agreeing that there is some correlation between partition coefficient and binding, found a much better correlation between the binding and electrostatic charge, as calculated using the extended Hückel method. These studies thus clearly illustrate the problem of interpretation of data when more than one binding force is involved. Dearden and Tomlinson (1970), in a study of the protein binding of a series of p-substituted acetanilides to BSA have shown good correlation between the thermodynamics of their binding and Hammett's substituent constant (\(\sigma\)), a reasonable agreement with \(\pi\) was also found. Such studies are capable of providing valuable information on the nature of the interaction and of the structure-activity relationships in certain series of drugs. Binding studies over changes in pH, ionic strength and temperature could be used to further clarify the nature of such interactions.

Clearly, it is unlikely that a common mechanism of interaction exists for all drugs since binding is obviously dependent upon the individual physico-chemical properties of the drugs. For ionic drugs, it may be that once distributive and diffusive processes have brought the drug molecules into the vicinity of a receptor surface then it is likely that the first forces to be involved are the comparatively strong ionic bonds. These could bring the drug molecule nearer to the receptor surface, the complex formed then, being stabilised by secondary binding forces such as hydrophobic bonds, hydrogen bonds or London-van der Waal forces.

Brodie's statement (1966), 'it is doubtful if the binding of a single drug to plasma protein is fully understood,' is still largely true, but
certain molecular features can be recognised as likely to enhance protein binding, namely substituents which:

a) cause an increase in hydrophobicity e.g. aromatic ring or methylene group substitution.

b) produce an increase in the degree of electrolytical dissociation of an ionizable drug at the pH of the plasma. Binding affinities have also been shown to increase in the order $\text{SO}_4^{2-} > \text{SO}_3^{2-} > \text{COO}^- = \text{OH}$ (Reynolds, et al., 1968).

Other factors likely to effect the degree of binding include, protein concentration, drug concentration, temperature, pH, ionic strength and physiological parameters such as: disease, age, etc.

4. Nature of Binding Sites in Albumin

It is generally assumed that some binding sites on the protein contain complementary hydrophobic areas, whereas others may consist of only hydrophobic areas. Table I,1 lists the charged amino acids of bovine serum albumin which are the potential binding sites of charged drug molecules.

Table I,1

<table>
<thead>
<tr>
<th>Residue</th>
<th>Group</th>
<th>Number of groups per molecule</th>
<th>pKa</th>
<th>Number ionized at pH 7.4</th>
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<tr>
<td>C-terminal</td>
<td>$\alpha$-carboxyl</td>
<td>1</td>
<td>3.75</td>
<td>1</td>
</tr>
<tr>
<td>Aspartic</td>
<td>$\beta$-carboxyl</td>
<td>99</td>
<td>3.97</td>
<td>99</td>
</tr>
<tr>
<td>Glutamic</td>
<td>$\gamma$-carboxyl</td>
<td>19</td>
<td>10.35</td>
<td>0</td>
</tr>
<tr>
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<td>Phenolic</td>
<td>19</td>
<td>10.35</td>
<td>0</td>
</tr>
<tr>
<td>Histidine</td>
<td>Imidazole</td>
<td>16</td>
<td>6.9</td>
<td>6</td>
</tr>
<tr>
<td>N-terminal</td>
<td>$\alpha$-amino</td>
<td>1</td>
<td>7.75</td>
<td>0.8</td>
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<tr>
<td>Lysine</td>
<td>$\varepsilon$-amino</td>
<td>57</td>
<td>9.8</td>
<td>57</td>
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<tr>
<td>Arginine</td>
<td>Guanidine</td>
<td>22</td>
<td>&gt;12</td>
<td>22</td>
</tr>
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</table>

a After Tanford et al., 1955
In view of the large number of charged amino acids concerned, it is perhaps rather surprising that for a large number of drugs the highest affinity (primary) binding sites number less than ten (Jonas and Weber, 1971), and for many no more than two (Thorp, 1964). Many compounds also bind to lower affinity (secondary) binding sites. Thorp (1972), has suggested that there are potentially three separate primary binding sites for organic acids on human albumin. One or more of these sites may become uncovered through local changes in the conformation of the albumin molecule, specifically by the structural features of the compound. Such a site may become essentially 'unaccessible' to related compounds with even minor structural changes.

Thorp (1964) considered that the N-terminal amino acid group (aspartic acid for bovine and human albumin) acts as a binding site for fatty acids, coenzymes such as pyridoxal phosphate and hormones such as: thyroxine and steroid sulphates or glucuronides, while Whitehouse et al., (1967) have suggested that the ε-amino group of lysine is a binding site for a number of triterpenoid acids. Swaney and Klotz, (1970) have investigated the properties of the tryptophan residue of human serum albumin (HSA), which earlier workers (Reynolds et al., 1967; Herskovits and Sorenson, 1968) had suggested might be a potential binding site. Following chymotrypsin hydrolysis and tryptic digestion they were able to determine the primary structure adjoining the lone tryptophan in HSA as:

Lys   Ala   Trp   Ala   Val   Ala   Arg

They suggested that the highly apolar character of the region and the presence of the two basic residues, arginine and lysine make it especially suitable for the binding of anions, particularly those which contain both apolar and electronegative regions. The same reasoning would also apply to the neighbourhood of the 'reactive' tyrosine (Sanger, 1960):-
Arg Tyr Thr Lys in HSA and Arg Tyr Thr Arg in BSA.

However, the finding that the interaction of anions, such as dodecylsulphate, with HSA perturbs the absorption of tryptophan (Herskovits and Laskowski, 1962; Steinhardt et al. 1971), may support the hypothesis that tryptophan is in close proximity to the binding site. Furthermore the findings of Ryan (1968) and Ryan and Gibbs (1970), that the binding of testosterone, progesterone and cortisol, led to the perturbation of the lone tryptophan (while androstelanone did not), is compatible with binding at the tryptophan site, insofar as either lysine or arginine would be a good proton donor for hydrogen bonding. It is suggested that in the case of BSA, which has two tryptophans, only one is embedded in the protein structure (Ivkova, et al., 1971). Initial studies (Sugae and Jirgensons, 1964), would suggest that both the tryptophans lack these positive charges, at least on the C-terminal side, although they may be found on the N-terminal side. Whether or not both tryptophan residues bind anions with equal avidity is difficult to ascertain, but it appears unlikely since the determined numbers of primary binding site for anions is often between one and two.

Little information is available on potential binding sites for basic compounds, in either BSA or HSA, although single primary binding sites on BSA for several phenothiazines has been reported (Krieglstein, et al., 1972a). Promazine can apparently be displaced by acidic as well as basic drugs (Franz, et al., 1969), paradoxically however, highly bound drugs were either not effective or less effective as displacing agents, than lesser bound drugs. Furthermore, several drugs appeared to cause an increase in the binding of phenothiazines to albumin. Borga, et al. (1969), reported that the binding of tricyclic antidepressant desmethylimipramine (DMI) was not significantly affected by the presence of several highly bound anionic drugs, e.g. phenylbutazone, clofibrate, salicylate, or by several basic
compounds. A possible explanation for this may be that DMI binds to a large number of sites.

Unfortunately relatively little is known about the tertiary and quaternary structure of the albumin molecule. It has been established that at pH 4 albumin undergoes a conformational transition which results in an increased viscosity and greater electrophoretic mobility (normal to fast, hence N-F transition). A model to account for such conformational transitions was proposed by Foster (1960), based on an original model by Harrington, et al., (1956). Optical rotatory (Sogami and Foster, 1968) and viscosity studies (Tanford, et al., 1955) revealed that three albumin transition forms could be distinguished at low pH. Based on Foster's model (1960), of four compact fragments held together in pairs by hydrophobic bonds and linked by short randomly coiled polypeptide chains, these can be considered to arise through an increase of the distance between the units from pH 4.5 to 3.9, a more or less stable situation between 3.9 and 3.6 and a partial unfolding at lower pH's (acid-expanded form). Bradshaw and Peters (1967, 1969), have sequenced the first 24 amino acid residues of the N-terminal end of human, rat and bovine albumin, and shown a high degree of similarity between the albumins. Recently King and Spencer (1970), using cyanogen bromide cleavage of bovine plasma albumin in 75% formic acid, obtained two fragments which could be further reduced to fragments of 88, 98, 211, 148 and 34 amino acid residues. The single sulphhydroyl group of albumin was found to be located in the 88 amino acid residue peptide which occupies the N-terminal position of the molecule. These workers also found that defatted bovine albumin subjected to limited tryptic hydrolysis gave a fragment of molecular weight of about 40,000, the fragment being derived from the C-terminal two-thirds of the albumin molecule. Comparison of this tryptic fragment and defatted albumin showed that they both had the same primary binding site for octanoate and L-tryptophan. The results of their
study are summarised in the schematic structure of bovine plasma albumin (Fig. I, 3). More recently Meloun and Kusnir (1972), sequenced the 37 residues at the C-terminal end of human plasma albumin and Kusnir (1973) the 36 residues between the first and second methionine residues. Studies with such 'fragmented' albumins, despite their incompleteness, and also chemically 'modified' albumins (Means and Feeney, 1971) are likely to play an important role in characterising binding sites for drugs and other small molecules.

Fig. I, 3
Schematic Structure of Bovine Plasma Albumin.
(King and Spencer, 1970).
Figures in parenthesis are amino acid number.
It is possibly a misleading concept to consider a specific number and class of binding sites, because the act of binding to one site may either 'create' or 'destroy' other sites. This concept of conformational adaptability was first proposed by Karush (1950a). The idea that a bound molecule may induce adjacent or remote conformational changes in the protein structure which may facilitate further binding, is similar to that of induced fit and allostery transitions in enzyme-substrate interactions (Koshland, 1958; Monod, et al., 1963). The binding of some small molecules to albumin have been shown to cause changes in a number of properties of the protein e.g. stability to heat denaturation (Boyer, et al., 1946), increases in viscosity (Franz, et al., 1969). A number of ligands, especially long chain detergents have been shown to affect the ultraviolet difference spectrum of albumin, often such conformational changes are so marked as to cause unfolding of the protein structure (Reynolds, et al., 1967, Steinhardt and Reynolds, 1969). As a result of producing such conformational changes a second drug may interfere with the first in a 'non-competitive' manner, thus phenylbutazone interacts with bromsulphthalein (Rudman, et al., 1971), and ethacrynic acid with warfarin by non-competitive inhibition (Sellers and Koch-Weser, 1970a). Alternatively two drugs may compete directly for the same binding site on the albumin molecule, for example phenylbutazone competitively inhibits the binding of warfarin, (O'Reilly and Kowitz, 1967). Finally the binding of the first drug to albumin may also induce conformational changes which increase the capacity of albumin to bind a second class of drug molecule, thus the diuretic chlorthiazide increases the capacity of plasma albumin to bind the ganglionic blocking agent pempidine (Dollery, et al., 1961). Consideration of the clinical implications of such drug competition are presented later.

It is clear from the above considerations that suitable data can provide information on both the affinity of binding and the number of
binding sites. Such data analysis inevitably employs the theory of multiple equilibria (Klotz, 1953; Rosenberg and Klotz, 1960, Steinhardt and Reynolds, 1969) in which it is assumed that all drug molecules are bound to the protein with equal affinity (or can be grouped into distinct classes within each of which the association constants are equal). Furthermore, the internal degrees of freedom of the bound drugs are neglected and therefore it must be assumed that all drugs in a given class bind in an identical manner. One frequently used method of data plotting involving such considerations is that due to Scatchard (1949) (see also Chapter Two)

\[ r/D_f = nk - rk \]

where \( r \) = number of moles drug/bound/mole albumin

Deviations from linearity of such plots are usually taken as indicative of binding to more than one class of binding site. Interpretation of such curvature can be complicated if interaction between binding sites occur, interaction may result from steric or charge repulsion phenomena or from a conformational change in the protein decreasing the affinity for the remaining sites (negative cooperativity) (Gutfreund, 1972). Laiken and Nemethy (1970a) have suggested that while the assumptions of the theory of multiple equilibria may be reasonable for small ions and other compounds with a rigid molecular structure they may not be for a 'chain-like' molecule. They suggest that such compounds, (flexible ligands), are composed of several functional groups, not all of which have to be in contact with the protein simultaneously. The binding of such ligands is best considered as occurring through interaction with several sites, where each site is defined as an
area on the protein surface which serves as a point of attachment for a single functional group, or segment, of the ligand. Although the majority of drugs would be unlikely to qualify as 'flexible ligands', it is apparent that a number of detailed binding studies have been performed on compounds that would probably qualify (e.g. long chain anions, hydrocarbons), such a model may be of considerable value in interpreting such binding phenomena.
(i) Absorption

A high degree of plasma protein binding may enhance the absorption of a drug by rendering the concentration gradient favourable for absorption. Brodie (1966) has suggested that this is particularly important with a drug poorly soluble in water, since binding will affect the equilibrium between drug already absorbed and drug remaining in the gut, both dissolved and undissolved. It may be that drugs with poor solubility characteristics, such as dicoumarol, would not be well absorbed unless they were avidly bound to plasma proteins. However, few examples are at present apparent to substantiate the role of plasma protein binding in influencing intestinal absorption. Kakemi et al. (1969) have demonstrated a correlation between intestinal absorption rate and protein binding for a series of barbituric acid derivatives in the rat, but concluded that binding to the mucosal surface is of more significance in affecting absorption rate, for these compounds, than plasma protein binding. Recently, however, Dearden and Tomlinson (1971) have suggested that protein binding may be important in determining the rate of buccal absorption for a series of p-substituted acetanilides.

(ii) Distribution and pharmacological activity

Most drugs distribute through the body water and tissues by passive distribution down a concentration gradient. It is generally considered that only the unbound plasma concentration of the drug is available for transport to the extravascular receptor sites (Goldstein, 1949; Brodie, 1966). Experimental observations
lend support to this concept, particularly with respect to the reduced potency of drugs such as sulphonamides (Davis, 1943; Anton, 1960; Krüger-Thiemer et al., 1965), penicillins (Rolinson and Sutherland, 1965; Kunin, 1967), barbiturates (Goldbaum and Smith, 1954) and prostaglandin E₂ (Raz, 1972b), when bound to albumin.

Providing they do not bind preferentially or irreversibly to tissue sites, highly protein-bound drugs are likely to be located mainly in the plasma compartment, at least at doses where the high affinity binding sites are unsaturated (Brodie and Hogben, 1957; Martin, 1965a,b). Under these circumstances the protein bound drug can serve as a reservoir which replenishes (by dissociation) some of the drug that is lost by metabolism and excretion. In this way the concentration of unbound drug can be maintained at a therapeutically useful concentration for a period of time, over a range of total plasma concentration. Furthermore, plasma protein binding can reduce the unbound concentration of toxic drugs below that required to elicit a toxic response, thus rendering the drug safe for therapeutic use. In the steady-state the concentration of unbound drug in the tissues, and tissue compartments of the extracellular fluid, such as the synovial fluid, equals that in the plasma (Howell et al., 1972).

Martin (1965a), has discussed a theoretical model describing the binding of four hypothetical drugs ranging from weakly bound to highly bound from which it can be calculated that only for drugs with association constants (k) greater than \(10^4\) M\(^{-1}\) will binding have any appreciable effect on distribution. At low plasma
levels a drug with a high association constant \( k \geq 10^5 \text{ M}^{-1} \) will be almost completely bound to plasma proteins. When the concentration of the drug increases however, the available plasma protein binding sites become fewer, as saturation approaches, with the result that more of the drug diffuses into the tissues and the fraction of drug remaining within the vasculature is reduced. For such drugs there is a fairly narrow range over which small changes in the plasma level can exert a profound change on the distribution of the drug within the body. Martin's model is highly simplified, but illustrates that the therapeutic dose range may be critically narrow for highly bound drugs which undergo large dose-dependent changes in distribution. Levy and Nagashima (1969) have demonstrated that the distribution and elimination of the highly bound drug dicoumarol is affected by its plasma protein binding, but they emphasise that such effects are likely to be less pronounced for drugs not so extensively bound. Curry et al. (1970) has reported that the plasma concentration of chlorpromazine fluctuates after intravenous doses in dogs and in man. Since chlorpromazine is a highly protein bound drug (Curry, 1970a), the suggestion has been made that movement of the drug between tissue and plasma stores could result in small changes in protein binding by plasma and tissues (Curry, 1970b). Although tissue binding has not yet been intensively investigated, it seems likely that some highly bound drugs have even higher affinities for certain tissue proteins which may or may not be receptor proteins. The cardiac glycosides, for example, have been found \textit{in vitro} to have a higher affinity for cardiac actin and myosin than for bovine serum albumin (Genazzani and Santamaria, 1969), such that \textit{in vivo} their concentration in the heart is about twenty to thirty times that in plasma.
Competition between endogenous and exogenous compounds for plasma protein binding sites can be summarised as:-
(1) mutual competition between endogenous substances,
(2) displacement of endogenous substances by drugs,
(3) interaction between two drugs

Although competition may occur between endogenous compounds such as steroid hormones (Keller et al., 1966), the importance of this phenomenon remains to be ascertained. However, a number of examples of displacement of endogenous compounds by drugs are known. Odell (1959) has shown the toxic effects that can be produced by displacement of bilirubin by sulphonamides. This effect is of particular significance in premature infants with low plasma albumin concentration, or in individuals with an impaired capacity to metabolise bilirubin. The view that the antirheumatic activity of certain drugs, e.g. salicylate, phenylbutazone, indomethacin, prednisolone, chloroquine, may be mediated through their ability to displace endogenously plasma protein bound tryptophan and other small peptides has been proposed (McArthur et al., 1971; Smith et al., 1971). It has also been suggested (Brodie, 1965), that some anti-inflammatory drugs may act by displacing steroids from plasma binding sites.

Numerous drugs have been shown in vitro and in animal experiments to compete for plasma protein binding sites (Meyer and Guttman, 1968), the many potential drug-drug interactions have been extensively reviewed (Hussar, 1969; Hartshorn, 1970; Koch-Weser and Sellers, 1970a,b; Sher, 1971; Swidler, 1971; Baker and Neuhaus, 1972). The data relating to human subjects is limited and largely anecdotal. Important examples where displacement may manifest
itself in a clinically undesirable side effect include, displacement of the anticoagulant drug warfarin by highly bound drugs such as phenylbutazone, chlorophenoxyisobutyric acid, indomethacin, salicylate, fatty acids and various sulphonamides, and these in turn may compete with one another for binding to plasma proteins (Solomon and Schrogie, 1967; Solomon et al., 1968).

The displacement of warfarin by phenylbutazone results in an increased anticoagulant effect which may give rise to spontaneous haemorrhage, yet also results in a more rapid excretion of the drug. A possible explanation is that phenylbutazone may displace warfarin not only in the plasma, but also from non-specific tissue binding sites, particularly in the liver. The oral hypoglycaemic drug, tolbutamide, which is normally quite extensively bound to plasma proteins may be competitively displaced by the administration of bishydroxycoumarin, with the consequent result of a sudden hypoglycaemic crisis. The converse situation also applies, for if tolbutamide and bishydroxycoumarin are given simultaneously, the concentration of the unbound anticoagulant is also elevated. Displacement phenomena may be of particular practical importance in the case of those drugs which are frequently self-prescribed and not regarded as drugs by patients. Drug displacement may however be of potential clinical value (Mroszczak et al., 1969), thus highly albumin bound pharmacologically relatively inert excipients (such as sodium trichloroacetate) which lower the dose of a drug necessary to produce a given pharmacological response, could be included in a pharmaceutical preparation of a drug (e.g. warfarin plus sodium trichloroacetate). Such a preparation could give stable blood levels for warfarin even when other highly bound drugs (known to displace warfarin) were concurrently administered.
From a clinical standpoint it is important to distinguish between potential and actual drug interactions since competitive phenomena, which are readily demonstrable \textit{in vitro}, may not be of sufficient magnitude to have a clinical effect or may be obscured by other factors \textit{in vivo}. It is unfortunately difficult to predict the displacement of one drug by another merely from a knowledge of binding data and plasma concentrations. A drug with a higher association constant (k) does not necessarily displace a drug of lower affinity, unless both share common binding sites. It is apparent that until such mechanisms are better understood great caution must be exercised when administering novel combinations of drugs known to be protein bound. Reports that hospitalised patients in America (Seidl et al., 1965) received an average of 13 different drugs, emphasises this need for caution. Appropriate \textit{in vitro} screening tests must be sought since it is unrealistic to test all combinations in the far more complex \textit{in vivo} experiments.

\textit{iii) Metabolism}

It is generally believed that only unbound drug is available for metabolism and filtration at the glomerulus. The limited number of studies on the kinetics of binding have been discussed by Meyer and Guttman (1968), and there is general agreement that in most systems rates of association and dissociation are very rapid. From these considerations it would seem unlikely that protein binding is normally the limiting factor in determining the rate of metabolism and excretion of most drugs.

A theoretical consideration of the effect of protein binding on metabolism has recently been made by Gillette (1971). He suggests
that if the activity of the drug-metabolising enzyme in liver is so high that virtually all the drug is cleared from the blood as it passes through the liver (i.e. extraction ratio nearly one) then an increase in binding of the drug by plasma proteins could accelerate its metabolism, by increasing the rate at which it is carried to the liver. Alternatively, if very little of the drug is metabolised as it passes through the liver (i.e. extraction ratio nearly zero) an increase in the binding of the drug in the blood may either decrease its metabolism or have little effect. The effects of protein binding on half-lives of drugs which are entirely metabolised have not been elucidated, largely because variations in drug clearance (extraction ratio) tend to obscure the effects of drug binding. Investigation of the effect of protein binding on the metabolism of drugs is complicated because changes in chemical structure, which can affect the extent of protein binding, may also affect the nature and rate of metabolism.

Newbould and Kilpatrick (1960), found that addition of plasma to the fluid perfusing a rabbit liver preparation, reduced the rate of acetylation of two 'long-acting' sulphonamides, and that the rate of metabolism was dependent on the concentration of unbound drug. Later Anton and Boyle (1964), and Wiseman and Nelson (1964), also reported a correlation between rate of metabolism of a sulphonamide and the extent of protein binding. Raz (1972c) has shown that both the rate of metabolism of prostaglandins (F₂ and A₂) and the nature of the metabolites formed, are affected by plasma
protein binding. The higher affinity of digitoxin \((k = 10^5 \text{ M}^{-1})\) for serum albumin compared to digoxin \((k = 10^3 \text{ M}^{-1})\) has been held responsible for the higher plasma concentration, lower urinary excretion rate and longer plasma half-life of the former drug in man (Lukas and DeMartino, 1969). The presence of warfarin in the plasma and the absence of unchanged drug in urine has been suggested to result from its binding to albumin and its non-polar character (O’Reilly, 1969). On the other hand, hydroxylated metabolites of warfarin, which are more weakly protein bound are virtually absent from plasma, but present in urine. However, highly protein bound drugs such as sulphobromophthalein \((k = 10^7 \text{ M}^{-1})\) are rapidly metabolised (Brodie and Hogben, 1957).

iv) Elimination

Considering the importance of this subject remarkably few experimental studies have been made. Kakemi et al. (1962) found an approximate inverse correlation between the binding of a series of salicylates and their rate of excretion. However, such a correlation is likely to hold for only a few drugs, for which the renal excretion is predominantly determined by the rate of glomerular filtration. Several authors have considered the elimination of drugs possessing high binding affinities for plasma proteins from a theoretical point of view, but without complete agreement (Martin, 1965b; Kruger-Thiemer, 1968; Keen, 1971).

Rieder (1963), in a study of a number of sulphonamides, was unable to observe a correlation between binding and rate of disappearance from plasma or rate of renal excretion, although Arita et al. (1971), in a study involving two sulphonamides, reported that protein binding did effect their glomerular filtration rate. Penicillins have a
very high clearance value reflecting tubular secretion which would support the finding that their $t_{1/2}$ values are relatively insensitive to protein binding (Notari, 1973). In contrast, it has been stated that the renal clearance of tetracyclines decreases with increased protein binding (Fabre et al., 1971). Bluestone et al. (1969) have reported increased excretion of urate following its displacement from plasma binding sites by aspirin and phenylbutazone.

The effect of plasma protein binding on biliary excretion is largely a matter for conjecture (Keen, 1971; Smith, 1971). Several highly bound anions (e.g., bromosulphthalein, bilirubin) have been shown to be rapidly excreted in the bile (Bradley et al., 1952). and a number of highly protein bound radio-opaque compounds were shown to be excreted in the bile, whereas only relatively poorly bound compounds were excreted in the urine (Lasser, et al., 1962). This would tend to suggest that protein binding may assist biliary excretion. However, Czok et al. (1970), have recently shown that a number of poorly bound compounds (phenolphthalein glucuronide, succinysulphathiazole) were excreted in the bile in unchanged form. Furthermore, it has been suggested that binding to plasma proteins may affect the uptake of the drug by the liver cells (Priestley and O'Reilly, 1966; Osorio and Myant, 1965).

6. **Physiological Factors Affecting the Plasma Protein Binding of Drugs.**

i) **Species differences**

Goldstein (1949), mentioned a number of reported inter-species differences in serum binding capacity. However, no consistent pattern was apparent, although rabbit sera appeared to exhibit superior binding capabilities. Despite the large number of reported binding studies since then, few have been concerned with evaluating
either inter- or intra-species differences in the plasma protein binding of drugs and no basis for prediction has emerged.

Sturman and Smith (1967) found that monkey, rabbit and guinea pig, like man, bound salicylate to the extent of 60-80% at the same total plasma concentration. Witiak and Whitehouse (1969), noted species differences in the binding of several acidic drugs and suggested that the rat may be an unrepresentative species as far as drug binding to albumin is concerned. They also observed differences between rat serum and isolated rat albumin. Species differences have been demonstrated in both the number of phenylbutazone binding sites and their affinities for the drug, with human, rat, dog and rabbit albumins (Chignell and Starkweather, 1971). Differences in the binding to different preparations of albumins (e.g. crystalline and fraction V) have also been reported for some drugs (e.g. warfarin, salicylate), however, no consistent interpretation is possible from these studies (Kostenbauder et al., 1970; Meyer and Guttman, 1970b; O'Reilly and Motley, 1971). Species differences in the binding of basic drugs; desipramine (Borgå et al. 1968), and amphetamine (Baggot et al. 1972) have been observed, such differences may account for reported species differences in distribution.

Inter-individual differences in the plasma protein binding in man of the tricyclic antidepressant drug, nortriptyline were observed (Alexanderson and Borgå, 1972). It has been reported that the protein bound iodine in Negro pre-adolescent children is significantly greater than that of white children (Starr et al., 1967). Differences in the thyroxine binding globulin, total gamma globulin and albumin concentration, but not thyroxine binding prealbumin were noted.
In contrast to the lack of unanimity on the binding consistency among healthy individuals, it is generally agreed that binding is decreased in disease (Goldstein, 1949; Reynolds and Cluff, 1960; Anton, 1968; Anton and Corey, 1971; Andreason, 1973). An increase in circulating metabolites, e.g. bilirubin, fatty acids, drugs, decreased albumin concentration, and altered protein structure have been suggested as possible causes for the binding deficiency, but the exact mechanism(s) has not been identified. For drugs that are mainly bound to albumin, a decrease in albumin concentration in disease has been shown to account for the proportionate decrease in the binding found with some drugs (Reynolds and Cluff, 1960; Wishinsky et al., 1962; Rolinson and Sutherland, 1965). Anton and Corey (1971) have suggested that this may be the reason for known decreases in the sulphonamide binding in anephric patients. Andreason (1973), recently reported decreased binding of the drugs acetylsalicylic acid, salicylic acid phenylbutazone, diphenylhydantoin, sulphadiazine and thiopental in patients with acute renal failure. Such decreases in protein binding could not be completely explained by lower albumin concentrations, although the drugs have been reported not to bind significantly to other plasma proteins. Interestingly, Reidenberg et al. (1971), although demonstrating decreased plasma protein binding for diphenylhydantoin in uraemic patients, found no effect on the binding of desmethylimipramine.

The concentration of unbound tryptophan in the plasma is thought to influence the concentration of tryptophan in the brain and hence the turnover of the putative transmitter 5-hydroxytryptamine (5HT). Recently, Coppen et al. (1972) have reported a lower unbound tryptophan concentration and increased percentage binding in depressive
patients, and postulated that it may account for the reduced cerebro-spinal fluid tryptophan levels and brain 5HT levels. Rheumatic patients have also been reported to have a higher unbound level of tryptophan in the plasma and a decreased percentage binding (McArthur et al., 1971). It is of interest that administration of antirheumatic drugs have an antidepressant action in rheumatic patients, and that the decrease in depression is correlated with an increase in plasma free tryptophan, (Aylward and Maddock, 1973). The binding of tryptophan to patients with Down's syndrome has also been shown to be reduced (Airaksinen and Airaksinen, 1972) and a similar effect was noticed for salicylate binding (Ebadi and Kugel, 1970). However, Airaksinen and Airaksinen (1972) suggest that this may be due to reduced plasma albumin levels.

ii) Age

Ganshorn and Kurz (1968) reported that the plasma binding of several sulphonamides was greater in adult than cord plasma, and Chignell (1971) has shown that sulphaphenazole has a lower affinity for plasma albumin isolated from cord and neonatal blood than for adult human plasma albumin. They suggest that differences could be due to the presence of a tightly bound endogenous compound, such as bilirubin, on fetal and neonatal albumin. The work of Pruitt and Dayton (1971) suggests that other factors such as quantitative or qualitative differences in albumin or binding to other proteins may also be operative. The physiological significance of the decreased binding capacity of fetal and newborn albumin is difficult to assess. Reduced binding capacity of albumin in the neonate could play a role in drug distribution and may therefore contribute to the extreme sensitivity of the newborn infant to various drugs.
Conclusions - Plasma protein binding may affect a drug's pharmacology, pharmacokinetics and toxicology. It is apparent that the detailed characterisation of drug-protein interactions are fundamental to our understanding of drug action. Such information is a necessity if we are to interpret inter- and intra-species differences, and the effect of age and disease states on the binding process. Only by such detailed knowledge of the plasma protein-drug interaction can the consequences of drug competition in the pharmaceutical and pharmacological phases of drug activity be rationalised. The practical implications of binding may necessitate determination of the degree of binding as a prerequisite to the use of plasma levels as a guide to drug therapy in man and screening tests in animals.
7. Binding of Drugs to Tissues

i) General

It is usual to assume that the fraction of unbound drug in the tissues is the same as the unbound fraction in the plasma. However, the situation in vivo may be complicated by binding to tissue proteins, and a series of equilibria will have to be established between the tissues of various organs and the tissue fluid. Evidence to support the identity of tissue fluid concentration with the unbound plasma level in the case of protein bound drugs was produced by Verwey and Williams (1962 a,b) in dogs. These authors measured the concentration of protein bound penicillins in lymph and related these to plasma concentrations. Furthermore, Scholtan and Schmid (1962) provided experimental evidence in mice which indicated that the levels of unbound penicillin and propicillin in serum were very similar to those in tissue fluid, even though levels of total drug were quite different. Kunin (1965) has shown that the distribution of penicillins in the tissues of rabbits was inversely related to their known binding to rabbit serum. Further support came from McQueen (1968) who, using an in vivo dialysis technique, has reported that the concentration of sulphormethoxine in the peritoneal fluid of the rat corresponded well with the unbound fraction in serum. However, in the case of drugs which are highly bound to tissues, drug levels per unit weight of tissue may well be in excess of the concentration of free drug in the plasma. Brodie (1952) demonstrated that 3 hours after an intravenous injection of the ultra short acting barbiturate, thiopentone, levels in the fat were 10 times higher than in plasma and Burns et al. (1953) reported higher unbound phenylbutazone levels in lung, heart and muscle than in plasma. Lullman and Van Zweiten (1969) demonstrated
that the cardiac glycosides were both highly bound to plasma proteins and to isolated atrica and showed that tissue-binding was not directly proportional to biological activity, indicating that plasma-binding and tissue-binding may be similar processes each sequestering drug and thereby lowering free drug concentration.

Drug displacement from tissue binding sites can also occur, and has been demonstrated by the interaction between the antimalarial drugs, mepacrine and pamaquine in patients (Brodie, 1966). This interaction potentiated the toxic effects of pamaquine, which exhibits a small margin between toxic and therapeutic concentrations. It is apparent that consideration of redistributionsal drug interactions must take into account both displacement from plasma protein, and tissue binding sites. The possibility of displacement from both intravascular and extravascular albumin, which is approximately three times that of the intravascular pool (Sellers et al., 1966), should also be considered.

Binding may occur at the level of the cell membrane or within the cell on the nucleus, on cytoplasmic organelles or in the cytoplasmic fluid. This binding may be with proteins or to polypeptides, lipids, and polysaccharides (Woolley and Gommi, 1966). A number of binding proteins to which both endogenous and exogenous molecules are known to associate have been isolated from cells of various organs. Rat kidneys have been found to contain aldosterone-binding proteins (Guidollet and Louisot, 1969). Two hepatic cytoplasmic proteins (Y and Z) have been isolated, purified and characterised (Levi et al., 1969; Reyes et al., 1971) and shown to bind sulphobromophthalein, bilirubin, corticosteroids and carcinogens and various dyes. The amount of the protein
(ligandin) (Litwack et al., 1971) was increased significantly in rat liver by phenobarbitone pretreatment (Reyes et al., 1969). The cytoplasmic proteins, Y and Z are found largely in the liver in a great variety of animals although they may be present in other tissues (Reyes et al., 1971; Ockner et al., 1972). The function of these various binding proteins is not known, but they may be involved in the uptake of small molecules from the plasma, intracellular transport or in mediating biological action in the case of some hormones.

It is likely that the physico-chemical factors which govern the plasma protein binding of drugs also predispose towards tissue binding. Some drugs may be more highly bound to tissues than to plasma proteins (Borg§ et al., 1969), and this may be for such drugs, a more important determinant of the distribution and persistence of a drug within the body, than plasma protein binding. It is apparent that the full scope and importance of the tissue binding of drugs, and other biological substances, necessitates more extensive study. Because of the practical problems associated with such studies, e.g. isolation of binding proteins, it may be that studies with other readily obtainable, relatively well characterised proteins, may serve as a basis for understanding tissue-drug interactions. Thus the study of the binding of drugs to plasma proteins might lead to a better understanding of the features governing the interaction of drugs with the drug metabolising enzymes, especially cytochrome P-450. A brief review of cytochrome P-450 and the nature of its spectral interactions is presented.
Klingenbur (1958) and Garfinkel (1958) first, independently, demonstrated the presence of the carbon monoxide binding pigment in liver microsomes later called cytochrome P-450, which appears to be involved in mixed function oxidase hydroxylation (Mason, 1957), of both endogenous and exogenous compounds. (Cooper and Brodie, 1955; Gillette et al., 1957; Conney et al., 1957; Omura and Sato, 1964; Cooper et al., 1965; Gillette, 1966).

Cytochrome P-450 is a b-type haemoprotein intimately associated with the endoplasmic reticulum membrane, it is mainly comprised of a protohaem, phospholipid and protein. Tissue and species differences in cytochrome P-450 and substrate specificities are likely to exist, due to variations in the association of cytochrome P-450 with the microsomal membrane or in qualitative or quantitative differences, in other components of the enzyme system. It is also possible that cytochrome P-450 may exist in different forms.

The importance to cytochrome P-450 stability of hydrophobic interactions, has been demonstrated by the potency with which dialkyureas convert cytochrome P-450 to P-420 (Ichikawa and Yamano, 1967), also alcohols in high concentrations cause the conversion. The observation that cytochrome P-450 can be solubilized by detergents, but not by proteases which solubilise about half of the microsomal protein (Omura and Sato, 1964) also favours the concept of a strong interaction of lipid and protein.

Although administration of phenobarbital to animals increases the level of cytochrome P-450, the haemoprotein nevertheless resembles that found normally. However, administration of 3-methylcholanthrene (3MC) and certain other polycyclic hydrocarbons, causes
the induction of a P-450 haemoprotein which differs from that seen normally (Sladek and Mannering, 1966, 1969). This species of cytochrome P-450 was named cyt.P_{1450} (Alvares et al., 1967; Hildebrandt et al., 1968). This 3MC induced haemoprotein appears not to bind type I compounds (Sladek and Mannering, 1966). Schenkman et al. (1969) suggested that cyt. P_{450} was simply an irreversible complex of the polycyclic hydrocarbon, or its metabolites, with the type I binding site of native cytochrome P-450. However, a considerable amount of evidence has suggested that this is not the case and that it reflects the formation of a new type of haemoprotein (Alvares et al., 1967; Kuntzman et al., 1969; Mannering, 1971; Fujita et al., 1973).

a) Substrate-induced spectral changes

A large number of compounds have been shown to produce characteristic absorbance changes in the difference spectrum of liver microsomes, which are thought to reflect interaction with cytochrome P-450 and these changes have been used to assess both the quantitative and qualitative character of drug P-450 interactions. (Remmer et al., 1966; Imai and Sato, 1966). Such spectral changes have been categorised into three types (Schenkman et al., 1967, Fig. I, 4), type I, type II and Reverse type I (type RI, also termed modified type II or Inverse type I, Diehl et al., 1970). The type I spectral change which is characterised by an absorption peak at about 385 nm and a trough at about 420 nm is typical of spectra produced by hexobarbital, aminopyrine, chlorpromazine, carbon tetrachloride. The type II spectral change is indicated by an absorption peak in the region 425-435 nm, and a trough at about 390 nm, e.g. aniline, pyridine
Types of Difference Spectra Induced in Hepatic Microsomes

Fig. 1, 4.

Absorbance

Wavelength (nm)

I = Type I
RI = Reverse Type I
II = Type II

and nicotinamide. The type RI spectrum (peak at about 420 nm and trough about 390 nm) is normally a mirror image of a type I spectrum (Schenkman et al., 1972) examples of compounds eliciting this type of spectral change are: agroclavine, ethanol (Orrenius et al., 1972). The magnitude of the spectral change observed in each case is dependent upon the concentration of substrate added, and can therefore be analysed by means of a Lineweaver-Burk (type) plot, for determination of the spectrally apparent dissociation constant (Ks) (see Chapter Two).

Oxidised cytochrome P-450 is considered the locus of these actions which are rapid, reversible and proceed substrate oxygenation. Several lines of evidence support the assumption that these spectral changes reflect the interaction of the added substrate with cytochrome P-450; a relationship between the magnitude of spectral change and the concentration of cytochrome P-450 has been demonstrated (Schenkman et al., 1967), also close similarities for a number of substrates between maximal type I spectral change (Ks) and hydroxylation K_m values (Imai and Sato, 1967; Schenkman et al., 1967). Solubilised and partially purified P-450 (Coon and Lu, 1969) preparations also show the same drug-induced spectral changes as native microsomes. However, the situation is complex for some compounds have been found to elicit difference spectra in different tissues (Orrenius et al., 1970) or at different concentrations (von Bahr and Orrenius, 1971), and furthermore, substrates of the microsomal mono-oxygenase systems may cause various types of spectral change (types I, II or RI Schenkman et al., 1967). The significance of the drug-induced
spectral changes is thus often difficult to assess.

The type I spectral change, generally acknowledged to be due to the binding of substrate to the apoprotein of cytochrome P-450, is probably caused by an increase in the electronegativity of one ligand of the haem. It has been suggested (Schenkman and Sato, 1968) that two forms of cytochrome P-450 exist, one as the unreacted enzyme and the other bound to an endogenous type I substrate (substrate-bound form). Type I interactions possibly occur by replacement of this endogenous substrate by a more strongly bound exogenous compound (Temple, 1972). The type I interactions apparently involve P-450-associated phospholipid. This view is supported by the finding that the type I site is destroyed by microsomal iso-octane extraction or digestion with phospholipases C and D (Chaplin and Mannering, 1970; Leibman and Estabrook, 1971). DiAugustine et al. (1970) and Eling and DiAugustine (1971) in a study employing the fluorescent probe ANS, a type I compound, also suggested that the binding locus involved a phospholipid, possibly phosphatidylcholine. They postulated that binding may involve both hydrophobic interaction and an electrostatic interaction with the cationic group of phosphatidylcholine. The type I spectral change can also be interpreted as a promoted transition of oxidised cytochrome P-450, from a low to a high spin electronic state, the converse high to low spin transition results from type II interactions (Mitani and Horrie, 1969; Gunsalus et al., 1971; Jefcoate and Boyd, 1971).

Although a close correlation between $K_s$ and $K_m$ is often found for type I interactions a large number of cases have also been reported where $K_s$ and $K_m$ are not related for type I compounds.
Likewise aniline and other type II compounds generally have dissimilar $K_s$ and $K_m$ values (Guarino et al., 1969; Schenkman, 1967). Also non-substrates have been shown to give rise to type I spectral changes, e.g. 2-hydroxydesmethylimipramine (von Bahr and Orrenius, 1971). Since $K_m$ values for microsomal drug metabolism encompass the individual kinetics of several components of a complex system, the poor correlation that often exists between $K_m$ and $K_s$ is not surprising; especially if the optical changes defined by $K_s$ values result primarily from conformational changes in cytochrome P-450, merely one component of the monooxygenase system (Schenkman and Sato, 1968). Also divergence between the two parameters will be even more likely if only a proportion of the cytochrome P-450 is engaged in formation of a spectrally apparent substrate complex and in subsequent oxygenation (Guarino et al., 1969; Ullrich, 1969).

The type II spectral change has been suggested to be associated with ferrihaemochrome formation involving electron transfer between the sixth ligand of iron and the nitrogen of the added compound (Schenkman et al., 1967). The type II interaction therefore would appear to be competitive with carbon monoxide and in all probability oxygen as well. It is likely that true type II changes are only given by basic amines, some e.g. aniline type II compounds are also substrates of the monooxygenase system. Although aniline and ethyl iso-cyanide both compete for oxidised and reduced P-450, there is evidence that the aniline type II and ethyl iso-cyanide sites are different (Alvares et al., 1971; Imai and Sato 1966 and 1967).
Type II compounds have been shown to competitively inhibit the type I spectral change, they also inhibit other type II interactions, but apparently never competitively. Type I compounds are mutually inhibitory, but not always competitively. The type II interaction has been shown to be enhanced by type I compounds (Leibman et al., 1969; Orrenius et al., 1970). The enhancement effects of type I compounds on type II spectral changes has led to the view that the type II spectral change may contain a hidden type I component (Orrenius et al., 1970). Such a type I component has apparently been demonstrated for aniline (Schenkman, 1970). The possible presence of a type I interaction may well explain the correlation found between the $K_s$ values for the type II n-alkyl amines and chain length. Such a correlation indicating the hydrophobic nature of the interaction between the n-alkylamines and cytochrome P-450.

The reverse type I (type RI) formerly referred to as the modified type II spectral change (Schenkman et al., 1967), or the Inverse type I (Diehl et al., 1970), has been shown to be the reverse of the type I spectral change in both absolute and difference spectra. The type RI appears to be as specific, with respect to wavelength, as the type I change. Because of the spectral similarities of type RI with type II, type RI spectral spectral changes have often been incorrectly interpreted as type II, however, whereas type II compounds will displace carbon monoxide from reduced cytochrome P-450, type RI compounds will not. The type RI would therefore appear to be the spectral manifestation of a class of compounds, not necessarily substrates, which bind to the apo-enzyme of the mixed function oxidase system (Schenkman et al., 1972). The type RI group of compounds appear to be as
chemically heterogenous as those producing type I spectral changes, and includes various drugs, alkaloids, alcohols, steroids and the amino acid tryptophan (Wilson and Orrenius, 1972).

Several hypotheses to account for the type RI change have been proposed: it could result from the displacement of some endogenously bound substrate (Schenkman et al., 1969; Schenkman, 1970; Diehl et al., 1970). This work was supported by the finding of Schenkman et al., (1972) that the magnitude of the RI change can be markedly increased by the prior addition of a type I compound to the microsomal preparation since it provided more substrate-bound enzyme (394 nm form). Also the formation of the type RI decreased the rate of P-450 reduction, as would be expected since the formation of a type I change enhances the rate of P-450 reduction. Schenkman et al. (1972) have more recently suggested that the type RI site on substrate bound form of cytochrome P-450 is at a site other than the type I site. They suggest that this interaction shifts the equilibrium between the substrate bound form (394 nm) and the free (419 nm) form towards the free form (419nm). The type RI spectral change does not appear to be related to substrate metabolism, like most type I spectral changes (Jansson et al., 1972). However, that some type RI compounds are substrates has been observed (Orrenius et al., 1972). To account for the type RI spectral change observed for the monoxygenase substrates: phenacetin and agroclavine, Orrenius et al. (1972) proposed that a type I interaction, related to metabolism, was present in their type RI spectral changes. They suggested that the lack of a type I component in the type RI spectral change may distinguish non-substrates from substrates. Evidently much remains to be elucidated about the nature of the 'drug' microsomal binding sites.
8. Methods of Studying Protein Binding

In his review Goldstein (1949) outlined three basic principles upon which the binding of drugs to proteins could be studied:

1. The concentration of free drug and its thermodynamic activity are reduced, and its biological action may also be diminished.

2. The drug may show changed properties, which can be measured with greater or less precision. Some of the effects may also be attributed to reduction of thermodynamic activity.

3. The protein component may be measurably altered with respect to its properties.

Only those established techniques still in frequent use, and the newer ones, the true potential of which is still to be fully assessed, will be discussed here. For summary see Tables I, 3 and I, 4.

Several comprehensive discussions on the methods of studying protein binding have been published including Goldstein (1949), Edsall and Wyman (1958), Rosenberg and Klotz (1960), Steinhardt and Reynolds (1969) and Chignell (1971).

Non-spectroscopic approaches include the classical techniques of equilibrium dialysis, ultrafiltration and gel chromatography.

i) Equilibrium dialysis

Confining the protein within a semipermeable membrane through which only unbound drug molecules can freely diffuse is one of the oldest and most widely used methods of studying binding (Osborne, 1906). Drugs are routinely dissolved in either the protein or solvent (buffer) containing compartments and dialysed against the same solvent until analysis shows that equilibrium has been attained.
The approaches which have been used for equilibrium dialysis studies range from the use of simple cellophane or cellulose bags (Klotz, 1946; Rosenberg and Klotz, 1960) to specially designed apparatus employing 'dialysis cells' (Stein, 1965; Farrell et al., 1971; Weder and Bickel, 1970).

Equilibrium dialysis is well suited for quantitative measurements of binding, but does have a number of problems (Goldstein, 1949; Steinhardt and Reynolds, 1969). Thus the presence of the membrane introduces the problem of Donnan inequalities (Donnan, 1924; Karush and Sonnenburg, 1949), or if the latter are suppressed by salts, possible competition effects between salt and drug (Klotz, 1953). Many buffers (e.g. trismaleate, veronal, chloride, acetate) used in in vitro experiments compete with the binding of drugs and other small molecules to bovine serum albumin, although little competition is apparent with phosphate (Klotz and Urquhart, 1949; Klotz, 1953; Keen, 1966). Adsorption of the drug onto the membrane can also occur (Agren, 1968; Steinhardt and Reynolds, 1969) although the binding of protein is generally negligible (Bennet and Kirby, 1965; Meyer and Guttman, 1970a). The time taken to attain equilibrium is variable depending on stirring, pretreatment of membrane, protein concentration, temperature, nature of the buffer, and volumes on both sides of the membrane (Steinhardt and Reynolds, 1969; Meyer and Guttman, 1970a). Dilution of the protein containing solution during dialysis may cause a change in the equilibrium of the drug-protein complex.

Several rapid non-equilibrium dialysis techniques have been described which have the advantage of much shorter experimental times. The methods described by Stein (1965), and Agren and Elofsson, (1967) and Colowick and Womack (1969) all employ automated flow-
through systems, the rate at which unbound compound enters the buffer containing compartment being proportional to the concentration of unbound compound in the protein-containing compartment. Data comparable to that obtained with equilibrium dialysis has been achieved and providing sensitive drug assays exist, this method provides a rapid method for studying binding.

ii) Ultrafiltration and diafiltration

Ultrafiltration (Flexner, 1937; Grollman, 1926) is in principle identical with equilibrium dialysis, except that a single solution, containing drug and protein in equilibrium, is used. By filtration under pressure a small volume of a protein-free phase is separated (ultrafiltrate), the concentration of which closely corresponds to the unbound drug concentration in the original protein solution. No dilution of the protein containing phase, apparent in the equilibrium dialysis technique, occurs. However, the continually changing protein concentration may result in a possible uneven distribution of protein and drug (Grollman, 1926). Some workers consider that the volume of ultrafiltrate should not exceed 10-15% of the total sample volume (Tait and Burstein, 1964; Sandberg et al., 1966), otherwise the equilibrium position may be disturbed through an increased protein concentration. However, Pearson et al. (1967) found for cortisol binding, that the removal of up to 70% of original volume as ultrafiltrate did not affect the initial equilibrium and they considered this to be in agreement with the considerations of Toribara et al. (1957), based on mass action theory.

Ultrafiltration employs the same type of membrane as used in dialysis, and the results obtained by ultrafiltration are therefore subject to the same potential sources of error as dialysis. The
technique is basically simple and many type of apparatus (Lavietes, 1937; Malinow and Karzon, 1947; Toribara et al., 1957; Anton, 1960) and various methods of achieving the necessary ultrafiltration pressure, e.g. vacuum, gas pressure, mercury columns, centrifugal force and even spring tension (Ames and Sakamore, 1964), have been described. The apparatus of Toribara et al. (1957) is especially suited to maintaining constant pH for studies on plasma samples.

Bennet and Kirkby (1965) using a specially designed vacuum ultrafiltration cell, showed that whereas stirring rate, ionic strength and viscosity did not markedly affect the kinetics of filtration, temperature, pH, volume of solution and size of ultrafiltration cell did. In their study the compounds investigated did not bind to the membrane. Their findings conflict with the view of Davis (1946) that because of the build up of protein on the membrane during ultrafiltration, agitation is necessary.

Ultrafiltration requires considerably shorter experimentation times than dialysis yet similarities in binding data obtained by the two techniques have been reported (Steinhardt and Reynolds, 1969). Ultrafiltration probably approximates most closely to the physiological process of glomerular filtration since the hydrostatic pressures employed are usually of a magnitude comparable to the renal filtration pressure (Goldstein, 1949; Dixon et al., 1967). The technique has been widely used to study the binding of drugs to plasma proteins (Anton, 1960; Reider, 1963; Keen, 1965; Meyer and Guttman, 1968)

A recently introduce technique is that referred to as 'diafiltration' (Blatt et al., 1968). The introduction of this technique stems largely from the advent of a new type of semi-permeable membrane ('Diaflo', Amicon, Lexington, Mass., U.S.A.)
which exhibits considerably higher flow rates than the classical membranes and are available with a range of cut-off values. In this technique the rate at which the protein or serum sample is ultrafiltrated is exactly balanced by the rate at which drug is replaced, thus protein is ultrafiltrated at a constant volume. Providing the rate of ultrafiltration is slow enough for equilibrium to be maintained between unbound and the protein-bound drug, the analysis of the eluate from the ultrafiltration cell enables the determination of the unbound drug concentration for a given addition of drug. The possibility should therefore exist of determining binding over a wide range of drug concentration in a single experiment of short duration. This technique is reported to combine the advantages of ultrafiltration and dialysis without their concomitant disadvantages. However, the number of applications of this technique are as yet limited (Blatt et al., 1968; Danon and Sapira, 1972; Crawford et al., 1972; Ryan and Hanna, 1971; Thompson, 1973) and findings such as those of Ryan and Hanna (1971), that the percentage bound of testosterone to bovine serum albumin was approximately 25% higher than compared with conventional equilibrium dialysis, require explanation.

iii) Ultracentrifugation

In a strong gravitational field sedimentation of protein and protein small molecule complexes can be obtained, whereas the unbound concentration remains unchanged. This technique has the advantage that it dispenses with the use of membrane. However, protein concentration changes also occur when prolonged centrifugation periods are required (Steinberg and Schachman, 1966). Care must also be exercised in removal of the unbound gradient to avoid contamination by the protein containing fraction. However, results in
agreement with those obtained by equilibrium dialysis have been reported (Kerp and Steinhilber, 1962) although the number of reported studies using this technique to study plasma protein binding is limited (Steinhardt and Reynolds, 1969; Weder and Bickel, 1970).

iv) Gel chromatography

Since Lathe and Ruthven (1956) and later Porath and Flodin (1959) described the use of columns of cross-linked dextran's ('Sephadex') in the separation of different molecular weight compounds, many workers have applied this technique to study small molecule-protein interactions (Meyer and Guttman, 1968; Kriegstein, 1969; Wood and Cooper, 1970).

The application of this technique to study drug-protein interactions utilizes the molecular sieve properties of dextran gel, in which only the unbound drug is able to penetrate into the internal volume of the gel matrix. A variety of gels are now available for proteins of different molecular weight. The use of this method involves application of a drug-protein mixture to a column of sephadex followed by zonal elution with buffer. However, for reversible equilibrium the rate of elution is generally much slower than the rate of dissociation of the drug-protein complex (Wood and Cooper, 1970) and thus results obtained by this approach may therefore often be misleading due to possible dissociation effects (DeMoor et al., 1962). This method has been used to study the binding to plasma proteins of barbiturates (Barlow et al., 1962), penicillins (Acred et al., 1963), salicylates (Hardy and Mansford, 1962; Sturman and Smith, 1967; McArthur and Smith, 1968). The use of dextran gels in a batchwise procedure has also been reported
(Scholtan, 1964; Ahtee et al., 1967; Saris, 1963; Von Hattenberg and Klaus, 1966; Pearlman and Crepy, 1967). Recently Cooper and Wood (1968), Burke (1969) and Krieglstein and Kuschinsky (1968) have extended the use of frontal analysis chromatography on gel columns to study drug-protein interactions. The technique of frontal analysis has been suggested (Cooper and Wood, 1968) to circumvent the problem of drug adsorption onto the gel and dissociation of the protein-drug complex. Similarly this difficulty can be overcome (Hummel and Dreyer, 1962) by applying protein-drug mixtures to a gel column previously equilibrated with a solution containing the same ligand concentration as the mixture, elution being carried out with the same solution. Recently the binding to serum albumin of phenothiazines (Nambu and Nagai, 1972) and bromo-sulphathalain (Crawford et al., 1971) have been investigated with this method and good correlation (Nambu and Nagai, 1972) with equilibrium dialysis reported. However, although theoretically sound, this method is extravagant with drug (Clausen, 1966), also integration of the protein and drug areas is required which can be laborious and imprecise, especially in those instances in which these areas are either diffuse or ill-defined. Careful selection of experimental conditions are also required to re-establish the equilibrium base-line.

v) **Electrophoresis**

Electrophoresis has been used to study the plasma binding of a large number of drugs, hormones and vitamins (Goldstein, 1949; Desgrez and DeTraverse, 1966; Meyer and Guttman, 1968; Cohen, 1971).

The technique is relatively simple and may provide valuable qualitative information on which proteins in the plasma are
responsible for binding. While the relative percentages of a small molecule or drug bound to individual proteins can be determined, molar binding ratios are not easily ascertained (Goldstein, 1949). It has an obvious advantage in that only very small samples are required. However, this also necessitates a sensitive assay for the compound and thus it is common-place for radioactively labelled compounds to be used.

Bickel and Bovet (1962) studied the interaction between proteins and a number of drugs by 'crossing electrophoresis'. In this a band of the free drug is laid perpendicularly to the protein strip, as the protein moves that portion which is continually exposed to drug binds the compound, its net charge may be altered and a change in mobility is exhibited as an acceleration or deceleration of that particular area. This approach is useful for rapid qualitative evaluation of the relative binding potential of a series of drugs. The interaction with drugs may or may not modify the electrophoretic mobility of the proteins (Murari, 1966). One serious drawback of paper electrophoresis is the difficulty of accurate assessment of radioactivity in the respective protein bands. To overcome this problem Murray (1956) has suggested a technique which involves producing an autoradiogram of the electrophoresis strip and then measuring the optical density of dark spots on the radiographic film. This method has been extensively used by Cohen et al. (1965).

Immunoelectrophoresis combined with autoradiography has also been used by Clausen (1966) to study the serum protein binding of 35S-sulphamido-6-methoxypyridazine. Two dimensional immunoelectrophoresis has been employed to investigate the binding of drugs (Freeman and Pearson, 1969; Kramer and Richens, 1973), and using a continuous flow electrophoresis method the binding of warfarin
(O'Reilly and Kowitz, 1967) and digitoxin (Lukas and DeMartino, 1969) has been studied. Disc gel electrophoresis (Ornstein, 1964) does not, as yet, appear to have found much application to the study of drug-protein interaction.

Electrophoresis has the disadvantage that with present methods the best separation is achieved at the non-physiological pH of 8.6. At this pH the binding characteristics of the protein may be modified and cause redistribution of the drug to occur, resulting in different binding patterns to that at in vivo pH 7.4. Dilution by the buffer, possible competition effects between the drug and the electrophoresis buffer and binding to the electrophoresis paper may also cause problems. Digitoxin, for example, was found to bind to starch gel (Lukas and DeMartino, 1969) with the result that it failed to migrate with the plasma proteins, but continuous flow electrophoresis on paper revealed that the glycoside migrated almost exclusively with albumin (Lukas and DeMartino, 1969).

The application of spectroscopic techniques in the study of drug-protein interactions has been discussed by Steinhardt and Reynolds (1969) and Chignell (1970b, 1971, 1972).

vi) Ultraviolet and visible spectrophotometry

The methods employed to study small-molecule protein interactions by absorption spectrophotometry can be broadly categorised into: those where binding alters the absorption characteristics of the drug, and those in which the absorption of the protein is affected.

A number of small molecules show changes in their visible spectrum on binding. Klotz (1946) used the reduction in the light
absorbance of azosulphathiazole at 500 nm, and the concurrent slight red shift, on binding to BSA to measure binding parameters and to indicate the nature of the interaction. It has been common practice to compare dye spectral changes in the presence of the protein with that when the dye was dissolved in organic solvents or detergents (Green, 1965). Marked species differences in the absorption spectrum of the anionic dye 2-(4'-hydroxyphenylazo)-benzoic acid (HABA) when bound to albumin has been observed (Baxter, 1964) which may imply that the dye-binding sites had different hydrophobic character.

Moriguchi et al. (1970) suggested that HABA spectral alterations on binding (metachromasy) are due to interaction with a lower polarity environment in the interior of the protein in which HABA is converted to the hydrazone form having a lower absorption maximum wavelength. This metachromasy of a number of dyes, including HABA, has been utilized for the quantitative determination of serum albumin (Rustein et al., 1954; Doumas et al., 1971). Binding of a range of sulphonamides to BSA has been determined by monitoring the displacement of the dye HABA (Moriguchi et al., 1968) assuming, perhaps without justification, that competition for the same binding sites are involved.

For a drug exhibiting significant ultraviolet absorption the spectral changes in this wavelength region which accompany binding are often small and difficult to isolate from the absorption of the protein. Such interactions are best observed by means of ultraviolet difference spectrophotometry which employs 'tandem' or 'split' cells (Herskovits and Laskowski, 1962; Herskovits, 1967; see also Chapter Two). Difference spectral studies can provide
information on both the environment of the binding site and also possible conformational changes consequent on binding. This latter phenomenon can be detected as a result of absorbance changes (perturbation) of the amino acid chromophore (Herskovits and Laskowski, 1962). Conformational changes in albumin induced by the binding of a variety of compounds have also been studied (e.g. sodium dodecyl sulphate) (Steinhardt and Reynolds, 1969; Steinhardt and Stocker, 1973) and the spectral perturbations of the amino acid tryptophan produced by the binding of various steroids (Ryan, 1968; Ryan and Gibbs, 1970). The binding of phenylbutazone and flufenamic acid to albumin has also been studied by this method. The use of laser Raman spectrophotometry has recently been used to study the binding of methyl orange to bovine serum albumin (Carey et al., 1972). However, while a potentially promising technique, insufficient studies have as yet been performed to allow meaningful evaluation of its application.

vii) Fluorescence Spectrophotometry

Fluorescence stands out amongst the optical techniques used to investigate macromolecules in that the life-time of the excited state is sufficiently long for a variety of chemical and physical interactions to occur. These include: rotational motion, solvent reorientation, complex formation, proton transfer and transfer of the excited state energy to another chromophore. Fluorescence excitation and emission spectra, fluorescence polarization, quantum yields and decay times can thus provide information about these processes and therefore about the microenvironment of the chromophore (Brand and Gohlke, 1972).

Weber (1960) divided proteins into two classes according to their
native fluorescence:- Class A proteins containing phenylalanine and tyrosine but no tryptophan. In these proteins, phenylalanine fluorescence is absent and the fluorescence spectrum is characteristic of tyrosine with an emission maximum at 303 nm. Class B proteins containing tryptophan, as well as tyrosine and alanine. In all these proteins the fluorescence of tryptophan dominates the emission spectrum. Plasma albumin is known to be a Class B protein (Teale, 1960; Longworth, 1971).

When a given population of molecules is irradiated, not all will re-emit their absorbed energy as fluorescence. The quantum yield (Q) of fluorescence is therefore defined as the fraction of light absorbed that is re-emitted, i.e.

\[
Q = \frac{\text{number of quanta emitted}}{\text{number of quanta absorbed}}
\]

The binding of a compound to a protein may cause changes in one or more of the following parameters:

i) The fluorescence intensity (Q) of the protein, usually quenched.

ii) The fluorescence intensity (Q) of a probe molecule, which may or may not be a drug, attached (covalently or otherwise) to a protein.

iii) The fluorescence polarization of the drug.

When any or all of these changes accompanies drug binding to the protein, then fluorescence spectroscopy can be used to study the interaction.
**a) Fluorescence quenching**

Quenching is defined simply as reduction of the quantum yield and may be due to either 'static' or 'dynamic' mechanisms. A static quencher acts upon the fluorophores in the ground state, while a dynamic quencher interacts in the excited state. In both cases their effect is to reduce the light re-emitted. Life-time measurements can distinguish between static and dynamic quenching, the former is not accompanied by a change in $\tau$ (fluorescence life-time), while in dynamic quenching the decrease in quantum yield is proportional to a decrease in $\tau$. Several discussions of quenching mechanisms have appeared (Förster, 1951; Teale, 1960; Williams and Bridges, 1964; Honikel and Madsen, 1973), see also Chapters Four and Five. Some examples of compounds which have been reported to quench protein fluorescence on binding to albumin are: steroids (Attalah and Lata, 1968); fatty acids (Spector and John, 1968); warfarin and dicoumarol (Chignell, 1970a); anilinonaphthalene sulphonates (Daniel and Weber, 1966); digitoxin (Lukas and DeMartino, 1966); carbenoxolone (Lindup, 1971).

**b) Fluorescent probes**

Fluorescent probes may be defined as small molecules which undergo changes in one or more of their fluorescent properties as a result of interaction with a protein or other macromolecule (Edelman and McClure, 1968). Fluorescent probes such as 1-anilino-8-naphthalenesulphonate (ANS) and 2-p-toluidino-6-naphthalenesulphonate, along with others, have been used by many workers, with a variety of macromolecules (Stryer, 1970; Brand and Gohlke, 1972; Long and Hsia, 1972; Flanagan and Hesketh, 1973; Ma et al., 1973; Sudlow et al., 1973,
see also Chapters Five and Six). Studies with fluorescent probes can be used to investigate both the structure and function of macromolecules, and in particular provide information on their binding sites and interaction. Radda (1971) in an excellent review, discussed the rationale behind the design and use of probes for membrane studies. He also summarised the information available from such studies in terms of the polarity and fluidity of the binding sites, the molecular proximity of specific groups and the availability of the sites. Insight into the rotational and translational mobility, distance and orientation between pairs of chromophores and quantitative information on the affinity of binding can also be obtained. Stryer (1970), Chance et al. (1971) and Brand and Gohlke (1972) have further considered the application of fluorescent probes to the study of macromolecules.

Fluorescent polarization can also be used to study drug interactions. However, this method is only applicable to drug molecules which are highly fluorescent both before and after binding to the macromolecule.

viii) Optical Rotatory Dispersion and Circular Dichroism

In addition to fluorescence spectrophotometry other techniques finding increasing application to the study of small molecule-protein interactions include optical rotatory dispersion (ORD) and circular dichroism (CD).

When plane polarised light, consisting of a left and right circularly polarized component enter a medium containing asymmetric molecules the left and right components are transmitted at different speeds (i.e. they have different relative indices) and
the plane of the polarised light is rotated. Such molecules are said to be optically active. When optical rotation is measured as a function of wavelength, the resultant ORD curve is generally plain (N.B. the curve may be plain positive or plain negative depending on whether it rises or falls with decreasing wavelength.) However, in the wavelength region where the optically active compound absorbs light, the ORD curve becomes anomalous, often changing sign. This anomalous behaviour is termed a Cotton effect (Fig. I, 5a). In this wavelength region there is also an unequal absorption of the right and left circularly polarised components of plane polarised light, i.e. \( \varepsilon_L \neq \varepsilon_R \) where \( \varepsilon_L \) and \( \varepsilon_R \) are the extinction coefficients for the left and right circularly polarized components. This phenomenon gives rise to circular dichroism (CD) which is a second facet of the Cotton effect. When the differential dichroic absorption of a simple Cotton effect is plotted as a function of wavelength, it will have the shape shown in Fig. I, 5b, with the circular dichroism maximum and the absorption at the same wavelength. Detailed discussions of ORD and CD have been presented by Crabbe (1955), Velluz et al. (1965), Chignell and Chignell (1972).

Both CD and ORD have been extensively used to study the interaction of detergents with proteins (Jirgensons, 1952, 1962; Foster, 1960; Perrin and Hart, 1970). However, the interpretation of CD and ORD spectra from the detergent-protein complexes is often difficult, since detergents may increase \( \alpha \)-helical content, convert \( \alpha \)-helical to \( \beta \)-structure, abolish \( \beta \)- but not \( \alpha \)-helical or simply act as denaturing agents (Chignell and Chignell, 1972). Several workers have used the techniques to study dye-protein interactions (Markus and Karush, 1958; Winkler and Markus, 1959; Blout and Stryer, 1959; Helmer et al., 1968; Daniel and Yang, 1973).
Fig. I, 5

ORD and CD Curves Associated with an Optically Active Chromophore

a) ORD

\[ [\alpha] \]

Absorption

Positive Cotton effect (----), negative Cotton effect (-----)

The lower curves represent the absorption spectra of the optically active chromophore

\[ \alpha = \text{optical rotation}; \ \theta = \text{molar ellipticity (deg.cm}^2 \ \text{decimeter}^{-1}) \]

\[ \Delta \varepsilon = \text{differential dichroic absorption (} \varepsilon_L - \varepsilon_R \) \]

(N.B. \( \theta = 3,300 \Delta \varepsilon \))

From Chignell (1971)
The application of this technique to the study of drug-protein interactions was demonstrated by Chignell (1968) who showed that extrinsic (induced) Cotton effects were observed on the binding of phenylbutazone and several anions to BSA, and that introduction of hydrophilic groups, e.g. OH; NO$_2$; CH$_3$SO$_2$ into the phenyl groups of phenylbutazone reduced the extrinsic Cotton effects on binding to albumin. In contrast, the introduction of hydrophobic substituents, e.g. Cl; F into the phenyl rings or modification of the n-butyl side chain had a much smaller effect on optical activity. Rosen (1970) showed how CD data could be used to derive association constants ($k$) and number of binding sites ($n$) for the binding of phenylbutazone and oxyphenbutazone to human serum albumin and interestingly found an extra binding site for oxyphenbutazone compared with phenylbutazone. A number of other drugs have also been studied, e.g. flufenamic acid; meclofenamic acid; mefanamic acid (Chignell, 1968, 1969 a,b, 1971); fenoprofen (Perrin, 1973); bilirubin (Wooley and Hunter, 1970); tryptophan (Sjöholm and Grahn, 1972); sulphonamides (Kostenbauder et al., 1971; Wood and Stewart, 1971); indomethacin, 4-hydroxycoumarin, warfarin, salicylic acid (Perrin and Nelson, 1972); benzodiazepines (Müller and Wollert, 1973).

The main advantage of CD techniques in the study of drug-protein interactions is the rapidity with which measurements can be made, and that both qualitative and quantitative information on the binding sites and interaction can be obtained. A disadvantage is that only those binding sites causing a change in optical activity (ellipticity) are revealed, and data interpretation is sometimes difficult. Since a large variety of biological molecules as well as some drugs, are optically active, ORD and especially CD are likely to play an
important role in future drug-macromolecule interaction studies.

ix) **Nuclear Magnetic Resonance**

When a small molecule is bound to a protein, the relaxation characteristics of one or more of either its hydrogen atoms or those of the protein may be altered. This alteration may show up as a 'chemical shift', a frequency change, or as the broadening of a line, in the proton magnetic resonance spectrum. Selective broadening of some of the proton resonances of a drug is always observed when it is bound to a protein and this is considered to indicate an intimate contact between such protons, or the groups to which they belong, and the macromolecule. The chemical shifts are small and difficult to measure; they arise from rapid reversible changes of the state of the small molecule from bound to unbound, and reflect not only the shifts in the nuclei which are caused by binding, but also the time average of the fraction of small molecule bound (Steinhardt and Reynolds, 1969).

The application of nuclear magnetic resonance to pharmacological problems was exemplified by the pioneering studies of Fischer and Jardetzky (1965) on the binding of penicillin G, and Jardetzky and Wade-Jardetzky (1965) on the binding of sulphonamides, to BSA. They found that the phenyl ring of penicillin and the p-aminobenzene sulphonamide moiety of sulphonamides were the functional groups participating in the binding. The binding of diphenylhydramine and some methyl analogues to BSA (van der Vlies, 1970), and of acetylsalicylic acid to HSA (Sykes, 1970) has been studied.

Chemical shifts apparent on binding can also be used to monitor
drug-protein interactions. At present, however, relatively few interactions between drugs and proteins have been studied by NMR techniques. The major difficulty which has prevented the use of NMR in the study of other drug interactions is the relative insensitivity of the technique, and this has often necessitated the use of high concentrations of material. However, the development of computer techniques such as signal averaging and Fourier transform spectroscopy have helped to improve the sensitivity. Thus there is little doubt that NMR spectroscopy will become an important tool for studying drug protein interactions.

x) Summary of techniques

In Table I, 2 is summarised the well established and newer techniques for studying the interaction between small molecules and proteins, with comments on the relative theoretical advantages and disadvantages of each technique. Reference should be made to Chapters Three, Five, Six and Seven, where further discussion of the practical application of equilibrium dialysis, ultrafiltration, gel chromatography and fluorescence is presented. Table I, 3 provides a list of miscellaneous methods which have been used to study protein binding. Although some of these may have considerable application in future studies, their validity remains to be demonstrated. For others the limitation of the method is readily apparent and they are therefore unlikely to find widespread application.
<table>
<thead>
<tr>
<th>Technique</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Selected References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equilibrium Dialysis</td>
<td>Thermodynamically sound, accurate reproducible quantitative data. Most used technique, therefore comparison easy</td>
<td>Quantitative information only, which is subject to errors from binding to membrane and Donnan inequalities. Non physiological and prolonged equilibrium time may cause bacterial contamination and protein denaturation. Equilibrium disturbed by dilution.</td>
<td>Klotz (1953) Rosenburg and Klotz (1960)</td>
</tr>
<tr>
<td>Dynamic Dialysis</td>
<td>Rapid</td>
<td>Essentially same disadvantages as equilibrium dialysis. How method, therefore validity not fully established.</td>
<td>Stein (1965)</td>
</tr>
<tr>
<td>In Vivo Dialysis</td>
<td>Probably the most physiological method available.</td>
<td>Assumes unbound drug in serum = peritoneal fluid = concn in peritoneal dialysis sac New method largely untested. Binding to membrane and quantitative data only.</td>
<td>McQueen 1968, 1969</td>
</tr>
<tr>
<td>Dialfiltration</td>
<td>No protein dilution or concentration effects.</td>
<td>Membrane binding and Donnan inequalities. Calculations lengthy and involved.</td>
<td>Blatt et al. 1968, Crawford et al. 1972</td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>Thermodynamically sound and rapid. Suggested to closely approximate in vivo situation. Specialised apparatus ideally suited for plasma samples</td>
<td>Quantitative data only. Binding to membrane and Donnan inequalities. Equilibrium disturbed by protein concentration</td>
<td>Toribara et al. (1962), Bennet and Kirby (1965)</td>
</tr>
<tr>
<td>Ultra-centrifugation</td>
<td>No membrane binding or Donnan inequalities</td>
<td>Theoretical basis not so well established as for dialysis or ultrafiltration. Prolonged experimental time Quantitation more difficult Cannot use protein mixtures</td>
<td>Steinberg and Schachman (1966) Steinhardt and Reynolds (1969)</td>
</tr>
<tr>
<td>Gel Chromatography</td>
<td>Separation of several binding proteins possible Thermodynamically sound</td>
<td>Adsorption to gel may disturb equilibrium can largely be circumvented by Frontal Analysis and Hummel-Dreyer methods. Although these methods require large samples. Dilution occurs</td>
<td>Scholtan 1964 Cooper and Wood, (1966) Wood and Cooper, (1970) Hummel and Dreyer (1962)</td>
</tr>
<tr>
<td>Electrophoresis</td>
<td>Qualitative data. Can separate multi-protein mixtures and only small samples required. Can be combined with autoradiography and immunoelectrophoresis.</td>
<td>Quantitation difficult Non-physiological Interpretation can be difficult and may be complicated by binding to support medium.</td>
<td>Bickel and Bovet (1962) Clausen (1966) O'Reilly and Kowitz (1967)</td>
</tr>
<tr>
<td>Ultraviolet and Visible Spectroscopy</td>
<td>Sensitive technique can monitor absorption changes in either drug or protein consequent upon binding. Can provide both qualitative and quantitative information, although quantitation can involve lengthy calculation. Homogenous system, only microquantities of material required.</td>
<td>Cannot be used if interaction causes no spectral change. Non-physiological May need to compensate for compound's absorbance.</td>
<td>Klotz (1964) Herskovits (1967) Chignell (1971) Steinhardt and Reynolds (1969)</td>
</tr>
<tr>
<td>Fluorescence Spectroscopy</td>
<td>Sensitive technique. Can monitor fluorescence changes in both drug and/or protein consequent upon binding. Can provide both qualitative and quantitative information, although quantitation can be done by binding to support medium.</td>
<td>Cannot be used if interaction causes no spectral change. Non-physiological 'Inner-filter' effects may occur and make interpretation difficult</td>
<td>Teale (1960) Atteah and Lata (1968) Radda (1971) Chignell (1972)</td>
</tr>
</tbody>
</table>
Table I, 3.

Miscellaneous Methods for Studying Small Molecule - Protein Interactions

<table>
<thead>
<tr>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Biological action</td>
<td>Goldstein (1949)</td>
</tr>
<tr>
<td>2. Diffusion</td>
<td>Goldstein (1949)</td>
</tr>
<tr>
<td>3. Physical properties, e.g. osmotic pressure, vapour pressure, surface tension, viscosity, electrophoretic mobility, sedimentation of protein.</td>
<td>Goldstein (1949)</td>
</tr>
<tr>
<td>4. Solubility of small molecule</td>
<td>Goldstein (1949)</td>
</tr>
<tr>
<td>5. Adsorption of small molecule</td>
<td>Goldstein (1949)</td>
</tr>
<tr>
<td>6. Precipitation</td>
<td>Goldstein (1949)</td>
</tr>
<tr>
<td></td>
<td>Priestly and O'Reilly (1966)</td>
</tr>
<tr>
<td>7. Stabilization</td>
<td>Goldstein (1949)</td>
</tr>
<tr>
<td></td>
<td>a) Goldstein (1949)</td>
</tr>
<tr>
<td></td>
<td>b) Colombo et al. (1968)</td>
</tr>
<tr>
<td>8. Equilibrium partition of small molecule</td>
<td>Goodman (1958)</td>
</tr>
<tr>
<td></td>
<td>Spector et al. (1969)</td>
</tr>
<tr>
<td>9. Autoradiography</td>
<td>Meyer and Guttman (1968)</td>
</tr>
<tr>
<td>11. Conductivity and E.M.F. methods</td>
<td>Goldstein (1949)</td>
</tr>
<tr>
<td></td>
<td>Steinhardt and Reynolds (1969)</td>
</tr>
<tr>
<td>13. ΔpH method</td>
<td>Scatchard and Black (1949)</td>
</tr>
<tr>
<td></td>
<td>Steinhardt and Reynolds (1969)</td>
</tr>
<tr>
<td>14. Heatburst microcalorimetry</td>
<td>O'Reilly et al. (1969)</td>
</tr>
<tr>
<td></td>
<td>Reynolds et al. (1973)</td>
</tr>
<tr>
<td>15. Refractive index</td>
<td>Steinhardt and Reynolds (1969)</td>
</tr>
<tr>
<td>18. Saturation Analysis</td>
<td>Zettner (1973)</td>
</tr>
<tr>
<td>19. Isoelectric focussing</td>
<td>Van Baelen and De Moor (1972)</td>
</tr>
</tbody>
</table>
9. Introduction to Present Work

It is apparent from the above discussion that although a large number of binding studies have been reported we are still a long way from being able to predict how a novel compound will interact with plasma proteins, and how this will affect its pharmacological action.

The aim of this study has therefore been directed at providing information on the answer to some of the fundamental questions posed by Scatchard et al. (1954) concerning small molecule-protein interactions, namely: 'How many? How tightly? Where? Why? and What of it?' No consistent guide-lines are apparent from the literature to assist in the selection of the most appropriate technique, we have therefore considered the question of 'How best to measure it?' Clearly the nature of the information required (i.e. mainly qualitative or quantitative) will largely determine the type of technique to be used. The three classical techniques of equilibrium dialysis, ultrafiltration and gel filtration were comparatively evaluated by studying the binding to albumin of a number of drugs differing markedly in their degree of binding and physico-chemical properties. The validity of these \textit{in vitro} methods to the \textit{in vivo} situation was evaluated in a comparative study of the binding of salicylate to rat serum by \textit{in vivo} dialysis and \textit{in vitro} ultrafiltration.

The possible importance of lipophilicity as a major determinant in the binding of a number of small molecules and drugs to albumin has over the last few years received increasing prominence. However, the possible involvement of other binding forces has made interpretation difficult and prompted disagreement about the relative importance of the contribution from lipophilicity to the free energy of binding. We have therefore investigated the binding of a series of carbamates which
possess a wide range of physico-chemical properties, which make them suitable as model compounds for the study of protein binding. This homologous series, R-O-CONH$_2$ was stable and non-ionised in aqueous solution and showed a regular increase in partition coefficient as the chain length was incrementally extended by addition of a methylene group. It would seem likely therefore, that their interaction with plasma proteins would be largely hydrophobic in nature. Furthermore, the regular increase in chain length for the series should allow an assessment of the contribution of a single methylene group to the thermodynamic free energy of binding. The increase in partition coefficient between the methyl and n-hexyl group was calculated and considered to be sufficient to provide a sound basis for assessing the effect of more subtle molecular modifications, such as branching of side chain or introduction of aromatic ring, on the binding parameters (percentage bound, number of binding sites and apparent association constant). The possibility that binding of the carbamates to albumin might induce a conformational change in the protein was investigated by ultraviolet and fluorescence spectrophotometry. The role of molecular dimensional parameters in hydrophobic bonding was studied by comparison of the binding to albumin of butyl and pentyl acetates with the respective carbamate. The acetates were calculated to have significantly higher partition coefficients than the carbamates, but little difference in their molecular dimensions.

Hydrophobic bonding has also been suggested to play a role in the binding of the drug warfarin to albumin. However, other forces may be involved (O'Reilly and Kowitz, 1967). Although the effect of various substituents in the warfarin molecule, on its binding, has been investigated, little is known about the structure of its binding site on albumin. Warfarin is a particularly appropriate drug to study in view of the large numbers of drugs known to displace warfarin from its
binding site and the importance of the clinical implications of this displacement. Information on the nature of the binding to albumin and the structural properties of displacing agents should offer a more reliable basis for predicting drug interactions. In this study use was made of the finding that warfarin's fluorescence characteristics alter with change of solvent polarity. Such fluorescence behaviour can be used to obtain both qualitative and quantitative information on the binding process and therefore offers distinct advantages over conventional 'non-spectroscopic' techniques for such a study. The possible inter-species differences in the binding of warfarin, its binding to chemically modified albumins, and a study of its displacement by other drugs, were studied in an attempt to characterise the warfarin binding site on albumin.

The possible use of drug-albumin interactions as a model system for the tissue binding of drugs was investigated. A fluorimetric study in which the binding of R(+) and S(-) warfarin, 1-anilino-8-napthalene-sulphonate, and benzidine, to albumin was compared with their interaction with hepatic microsomal preparation was carried out. The nature of their difference spectral interaction with the microsomal preparation was also determined to further characterise the tissue binding sites.
CHAPTER TWO

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1. Materials

i) Chemicals

Crystalline (batch no. 79B-8080), Cohn fraction V (batch no. 12C-2920) and Essentially Fatty Acid Free Cohn fraction V (batch no. 31C-7470) preparations of bovine serum albumin were purchased from Sigma Chemical Co. Ltd., St. Louis, Mo., USA, as was rat serum albumin (Cohn fraction V, batch no. 42C-2980). Crystalline human plasma albumin (batch no. TV49641) was obtained from AB Kabi, Stockholm, Sweden. All crystalline albumins contained not less than 99% of total protein as albumin, a fatty acid content 0.1 - 0.2% and moisture content of not more than 2%. Fraction V albumins contained at least 97% albumin, the remainder being globulins. The essentially acid free albumin had a fatty acid content of less than 0.005%. All albumins were stored in a desiccator at 4°C.

The sulphonamides:-- sulphormethoxine (Fanasil), and sulphamethoxazole (Gantanol) were kindly supplied by Roche Products Ltd., Welwyn Garden City, Herts., England; sulphaphenazole (Orisul) was kindly donated by Ciba Ltd., Basle, Switzerland; and sulphanilamide was of Analar grade from BDH Ltd., Poole, Dorset, England; as was sodium salicylate. Clofibrate (Atromid-S) was kindly donated by ICI, Alderley Park, Macclesfield, Cheshire, England; and tryptophan (batch no. 90C-3310) was purchased from Sigma Chemical Co. Ltd. The carbamates:-- Methyl (Koch-Light Labs. Ltd., Colnbrook, Bucks., England), ethyl (BDH Ltd.), n-propyl and n-butyl (Kodak Ltd., Kirby, Liverpool, England) and benzyl carbamate (Aldrich Chemical Co., Milwaukee, Wisconsin, USA) were obtained commercially and used without further purification. Iso-Butyl (2-methylpropyl; m.pt. 61°C), tert-butyl (1,1-dimethylethyl; m.p. 104-6°C), tert-pentyl (2,2-dimethylpropyl; m.pt. 80°C), n-pentyl (m.pt. 53-4°C), tert-hexyl (3,3-dimethylbutyl; m.pt. 55-6°C), n-hexyl (m.pt. 59°C), n-heptyl (m.pt. 62°C) and n-octyl (m.pt. 67°C) were synthesised by the Chemistry Division of the Chemical Defence Establishment.
Porton Down, Salisbury, Wiltshire, England The purity of all the carbamates used was checked by infra-red spectroscopy and GLC, and was found in all cases to be >99%.

The acetates n-butyl and n-pentyl, were obtained from BDH Ltd., and redistilled before use. Racemic (±) warfarin, was purchased from Sigma Chemicals Ltd. (batch no. 5061 600). The isomers R (+) and S(−) warfarins were kindly donated by Dr. A. Breckenridge, Royal Postgraduate Medical School Hammersmith, London, England. 1-anilino-8-naphthalenesulphonate (ANS; ammonium salt) and benzidine (batch no. B-3503) were obtained from Sigma Chemical Co. Ltd.

The compounds used in the interaction studies with warfarin (Chapter Five), were obtained from the following sources:- phenylbutazone (B.P.) and imipramine were gifts from Biorex Laboratories Ltd., London; chlordiazepoxide (Librium) and diazepam (Valium) were kindly donated by Roche Products Ltd.; pentobarbitone (B.P) was a gift from May & Baker Limited, Dagenham, England; iprindole (Prondol) was donated by AB Kabi Ltd. (batch no. 4713). Biphenyl (m.pt 69-70°C) and the metabolites 2-hydroxy (m.pt. 56-57°C) and 4-hydroxy (m.pt. 166-170°C) were obtained from BDH Ltd., and were recrystallised from 96% ethanol, 100-120° light petroleum and methanol respectively. Carbon monoxide (CP grade) and the gas mixture CO₂:O₂ (5.95%) were obtained from the British Oxygen Company Ltd., Crawley, Sussex, England. Buffers (phosphate and tris-HCl, pH 7.4) were prepared as described in 'Data for Biochemical Research'.

All other chemicals used were of either general or analytical grade and were used as supplied.

Sephadex G-25 (fine, Lot no. 233) particle size 20-80u , and Biogel P-2 (batch no. 101482) particle size 50-100u, were obtained from Pharmacia Ltd., London, England, and Bio-Rad Laboratories, Richmond, California, USA respectively. All gels were pre-swollen overnight in 0.1M phosphate
buffer, pH 7.4 and the 'fines' removed by decantation before use. Visking dialysis membrane used with both equilibrium dialysis and ultrafiltration techniques, was obtained from Scientific Supplies Co. Ltd., London, England, and prepared by soaking overnight in several changes of deionized water to remove surface glycerol and other contaminants, followed by a further period (overnight) in changes of the appropriate molarity phosphate buffer.

ii) Animals

Male Wistar-albino rats (Porton strain) from a randomly bred colony, weighing either 350-400 g (16-17 weeks) were used in the in vivo dialysis studies, and rats from the same stock approx. 130g (5-6 weeks) were used for the microsomal binding studies. Breeding nucleus was obtained from Laboratory Animals Centre, Carshalton, Surrey. England. Syrian hamsters (approx. 50g, 12-14 weeks) pure line golden strain, category 2, were also used for microsomal binding studies.

Animals were randomly allotted, prior to experimental work (minimum 2 weeks) as litter mate cage groups (test and control) and bedded on Sterolit (Engelhard Corp., Edison, N.J., USA) with water and food (Spratts No. 1 autoclaved animal diet, Spratt's Patent Ltd., Barking, Essex, England), ad libitum. All animals were housed under conditions of constant temperature (20°C), humidity (relative 50%) and lighting on a 12 hour light/dark cycle (06.30-18.30 light phase). The weight of the animals was routinely recorded throughout the experiments.
2. Methods

Albumin solutions (0.5 to 29.0 x 10^{-5} M based on total protein concn) were prepared in pH 7.4 phosphate buffer (0.1 or 0.15 M) or 0.1 M tris HCl. pH 7.4. Solution was achieved in each case by allowing the albumin to dissolve overnight at 4°C. For fluorimetric studies this procedure was found to be sufficient for stabilisation of albumin fluorescence (Teale, 1960). Phosphate and tris-HCl buffers (pH 7.4) were prepared in either glass distilled water, or double glass distilled water for fluorimetric studies, as described in 'Data for Biochemical Research' (1969).

i) Gel Chromatography

All column chromatography studies employed glass columns which contained a glass sinter (porosity Grade 1) for gel support. Columns were made by the University glass-blowers and had minimal 'dead space'. Column performance was checked after packing using a 1% (w/v) blue dextran 2000 (Pharmacia Ltd.) solution in distilled water.

(a) Batch method

The long acting sulphonamide, sulphormethoxine, which is known to be relatively highly bound to bovine serum albumin (Engel and Melville, 1966; Elofsson et al., 1971), was selected as a suitable compound for a preliminary evaluation of the batch method. The use of pre-swollen gel was selected as it is more closely analogous to equilibrium dialysis than dry gel. (N.B. In practice there may be little difference between the results obtained using dry gel and those employing pre-swollen gel.)

To 200mg of Sephadex G25 fine (calculated internal volume, Vi, of 0.5 ml) in 10 ml glass stoppered tubes, was added 1.0 ml of 0.1 M phosphate buffer, pH 7.4, and the gel allowed to swell overnight at room temperature. 1.0 ml of a bovine serum albumin
solution (29.0 x 10^{-5}M) containing sulphormethoxine (concentration range 16.0 - 80 x 10^{-5}M) was added and mixed first on a rotary shaker for 30 minutes, then in a shaking water bath for a further 30 minutes to allow the gel to settle. At the end of each incubation 0.5 ml. of the supernatant (external phase) was removed and assayed. The sulphonamide concentration was determined by the Bratton and Marshall procedure (1939) and protein by the procedure of Lowry et al. (1951). An attempt to determine the concentration of sulphonamide imbibed in the gel (internal phase concentration) was made by using the separation procedure employed by Saris (1963). The sephadex gel was filtered through a Büchner funnel and the sulphonamide concentration assayed in the presence of the sephadex gel.

(b) **Zonal method**

The binding of the drugs sulphormethoxine, clofibrate and sulphamethoxazole to BSA was used to evaluate this method. As an alternative to sephadex, a cross-linked polyacrylamide gel Biogel P2, was also employed.

Columns (1.2 cm diameter) were packed with gel to various column heights. The sample volume was varied according to the bed volume of the column, but was typically either 2.0, 1.0 or 0.5 ml. of a drug-BSA (BSA concn. = 29.0 x 10^{-5}M) mixture. Sample was applied to the column in the standard manner and subsequently eluted with 0.1M phosphate buffer, pH 7.4 Column eluate was collected using a Central fraction collector (Central Ignition Co., London, England) employing a constant volume collecting syphon (5ml., 2ml., or 1ml.). All experiments were performed at room temperature 20° ± 1° C and at pH 7.4.
(c) **Frontal analysis**

The frontal analysis method used was essentially that described by Cooper and Wood (1968). In this method a large volume of protein-drug solution is applied to a sephadex column at a constant rate and eluted with a buffer. The steady-state elution profile reveals two plateaus, the first ($\beta$) corresponding to the total drug concentration in the solution applied to the column and the second ($\gamma$) to the unbound drug concentration. Unlike other gel filtration methods the most important advantage of this procedure is that it permits estimation of the degree of binding in the original solution.

Sephadex G-25 (fine) was packed into precision-bore glass columns (internal diameter 0.6 cm.) to column lengths of 15-20 cm. The glass columns were fitted with a polypropylene outlet valve, and contained a glass sinter at the end of the column. They had the advantage of minimal dead space, and showed acceptable elution profiles. The top of the column was connected to a LKB perpex pump 10200 (LKB Instruments Ltd., South Croydon, Surrey, England) which gave a non-pulsating flow at the rate of ~10ml/hr. The column outlet was connected to a LKB Ultrarac 7000 fraction collector, set to collect 7 drop fractions (~0.42 ml.). To evaluate the validity of the frontal method, the binding of sulphormethoxine to BSA was studied. Protein-drug solutions (12 ml, sulphormethoxine concn.range 7.5 -90 x 10^{-5}M; BSA 29.0 x 10^{-5} M) were applied to a Sephadex G-25 column previously equilibrated with 0.1M phosphate buffer and then eluted with the same buffer. Each fraction was assayed for both sulphonamide and protein; experiments were performed at 20° ± 1° C.
Elution of protein-containing solutions resulted in a variation in fraction size due to the effect of the protein on the surface tension of the solution. This was allowed for by weighing the collecting tubes, however, it was found by calculation of binding data that providing a steady-state plateau region had been obtained, this precaution was probably not necessary.

(ii) **Equilibrium Dialysis**

In our studies the commercially available Kontron Diapack equilibrium dialysis system was used, (the author is indebted to Anachem Ltd., Luton, Beds., England, for the loan of this equipment). This system is based on an original design by Weder (Weder and Bickel 1970). The equipment consisted of a series of individual dialysing cells located in four cell blocks, which fit into a rotational drive assembly, and allowed the cells to be rotated at selected speeds. This system allowed 20 separate dialyses to be performed simultaneously. Each dialysing cell consisted of two Teflon half-cells between which a dialysing membrane was inserted. Samples were inserted or removed through two injection ports located in each half-cell.

The chief advantage of this system over conventional dialysis is that small volumes can be used (100μl.-2ml.), and by employing a dialysis volume less than the half-cell volume a relatively large 'active' membrane area is obtained with rotation; this results in fairly short dialysing times (e.g. 5hrs.) thus reducing the likely risk of protein denaturation and bacterial contamination. The use of Teflon also tends to minimise drug adsorption onto cell components.

Visking dialysis tubing (18/32"), cut so that it opened to a single membrane surface, was pre-soaked as previously described. After
Fig. II, 1 Calibration of the Equilibrium Dialysis System:
Determination of Equilibrium Time for Dialysis of Sulphormethoxine (4.5 x10^-4 M) against Phosphate Buffer (0.1 M, pH 7.4)

Half cell volume = 1.0 ml
Temp. = 20°C, Rotation = 7 r.p.m.
Membrane: Visking dialysis (regenerate cellulose 0.025mm thickness)
soaking it was wiped with tissue paper, dried by blowing compressed air gently over its surface, and placed between the half-cells of the dialysis system. After assembly the cells were filled by the addition of 1ml. of solution (drug-protein) to one half-cell using a syringe followed immediately by the addition of 1ml. of 0.1M phosphate buffer, (pH 7.4) solution to the other side of the membrane. Solutions were dialysed at 20° C in a thermostatically controlled water-bath while being continually rotated at 7 r.p.m.

Initial experiments for the dialysis of sulphormethoxine \(45 \times 10^{-5}\text{M}\) in the presence of either 0.1M phosphate buffer pH 7.4 or BSA \(29.0 \times 10^{-5}\text{M}\) against the same buffer showed that equilibrium was reached by approx. 4hrs. whether protein was present or not (Fig. II, 1). A dialysing time of 5 hrs. was therefore selected for further experiments to ensure that equilibration had been attained.

(iii) Ultrafiltration

All ultrafiltration studies were carried out using a centrifugal ultrafiltration technique. Two different ultrafiltration units were employed depending on whether pH control was by buffer or a CO\(_2\):O\(_2\) (5:95) mixture (i.e. whether albumin solutions or serum samples were used).

(a) Of albumin solutions

The apparatus used for ultrafiltration of drug-BSA solutions consisted of a sintered glass disc (porosity grade 1), supported in a glass tube of suitable diameter to form a unit which fitted into a 50ml. polycarbonate centrifuge tube (M.S.E. Ltd., London, England) (Fig. II, 2a). The sintered disc-glass tube units were originally purchased from Scientific Supplies Ltd., but more robust units were subsequently manufactured by the University glass-blowers. These simple units were selected in preference to the more commonly used, relatively elaborate,
Fig. II. 2
Ultrafiltration Apparatus

a) Ultrafiltration Tube
(For albumin solutions)

b) Modified Toribara Tube
(For serum and plasma samples)

KEY
1. Glass tube
2. Polyethylene tube (2mm O.D.)
3. Glass sinter (Porosity Grade 0.5)
4. Adhesive and Parafilm seal
5. Rubber bung
6. Dialysis bag containing drug protein solution
7. Ultrafiltrate
8. Glass sinter incorporated into glass tube
9. Polycarbonate centrifuge tube (50 ml)
Toribara apparatus (Toribara, et al. 1957), for studies where buffered albumin solutions were used as they could be removed from the centrifuge tube which allowed thorough cleaning. For binding studies involving BSA solutions visking dialysis tubing (\(18/32''\)) was used and prepared as previously described. A knot was then carefully tied at one end of the tubing and the bag gently inflated. Drug-BSA (6ml) mixtures were transferred into the dialysis bags by means of a 10ml glass syringe fitted with a blunt luer-lock needle. As much air as possible was excluded from the bag and a second knot tied in the open end. After being folded into a 'U'-shape, with the two knotted ends uppermost, the bag was gently pushed down onto the sintered glass disc as shown in Fig. II, 2a. Care was exercised throughout to minimise handling and to avoid damage to the bag. The sintered glass tube contained in a 50 ml. polycarbonate centrifuge tube was centrifuged at 2,500\(g_{av}\) (2,700 r.p.m.) in a M.S.E. Mistral 6L centrifuge (employing a 6 x 1 L swing-out rotor with 24 x 50 ml. rubber adaptors) for 1 hour at thermostatically controlled temperature up to 45° C. The centrifugation procedure adopted was found by experimentation to produce slightly less than 10% of the original solution as ultrafiltrate, at such a low percentage removal, Donnan-equilibrium effects were shown to be small. Experiments also showed that slight variation in the centrifugation time did not significantly alter the results, nevertheless the procedure outlined above was routinely followed.

(b) Of serum samples
For ultrafiltration of serum samples, modified Toribara tubes were used (Toribara, et al., 1957; Fig. II, 2b). These were made by the University glass-blowers and had the advantage over conventional Toribara tubes in that they required smaller sample volumes (i.e. 1-2ml.)
and were also more robust and convenient to centrifuge. Serum samples containing added drug were transferred (using 2ml syringe) into dialysis bags, prepared from 8\"/32 dialysis membrane, and placed in the modified Toribara tubes. Individual Toribara tubes were then assembled in series and gassed with CO\textsubscript{2}:O\textsubscript{2} (5:95), it was found that approximately 15 minutes was sufficient to restore the serum pH to 7.4. The individual tubes were then sealed (as shown in Fig. II, 2b) and centrifuged in a Mistral 6L at 2,500 g for 20 minutes. This time was found sufficient to produce a suitable volume of ultrafiltrate, the pH at the end of ultrafiltration was invariably 7.4. No correction was found necessary for Donnan-equilibrium effects or, for the volume occupied by the protein and other solutes (see section 2.XIV this Chapter). Membrane binding was checked by control experiments (i.e. absence of protein) and recovery values. A large number of different techniques have been suggested for the preparation of dialysis membrane e.g. soaking in distilled water or buffer, boiling, etc. The procedure used in these studies was found to give reproducible results and no significant membrane adsorption could be detected for any of the compounds studied.

(iv) **Comparative Study of Equilibrium Dialysis, Ultrafiltration and Frontal Analysis**

The binding of the drugs: clofibrate; salicylate; sulphamethoxazole; sulphanilamide; sulphaphenazole and sulphormethoxine, to BSA was studied by the three techniques. Experimental procedures were as previously described, and the binding to BSA (29.0 x 10^{-5}M) was investigated over the drug concentration range (7.5 - 90.0 x 10^{-5}M) at 20° C, all drugs and BSA solutions were prepared in 0.1 M phosphate buffer, pH 7.4.
(v) **In Vivo Dialysis**

This method involves the implantation of a small dialysis sac into the peritoneal cavity of an animal. It is a tacit assumption that the concentration of unbound drug in the plasma reflects the peritoneal fluid drug concentration provided a steady plasma level of the drug is attained by suitable manipulation of the dosage regime. The measurement of the steady plasma level and the concentration inside the dialysis sac, should therefore reflect the degree of binding. In our study the binding of sodium salicylate to rat serum by *in vivo* dialysis was compared with its *in vitro* interaction as determined by the ultrafiltration technique described above (2 iii a). The method of *in vivo* dialysis employed in our study was essentially that described by McQueen (1968), the only modification being that we selected intragastric administration of the drug rather than mixing with the diet, in order to obtain a more reliable record of the dose intake.

Male Wistar albino rats (350-400g), housed in groups of four, were dosed with salicylate (in isotonic saline) at a dose level of either 10 or 20 mg/kg. body wt., once daily for eight successive days, the control group received saline (1ml/kg.). 4 hours after dosing, on the seventh day of treatment, dialysis sacs were implanted in the peritoneal cavity the sacs were removed 24 hours later (4 hrs after dosing on day 8). The sacs were made from sterilized Visking dialysis tubing (\(8'/32\)) and contained 1 ml. of 6% dextran (Pharmacia, T 700) in isotonic saline. Each dialysis sac was implanted into the peritoneal cavity under ether anaesthesia with sterile precautions, through a small abdominal incision, which was then carefully closed by stitching and suturing. After 24 hours the sacs were removed, again under ether anaesthesia, at the same time blood samples were obtained from the tail vein and by heart puncture, and serum obtained by centrifugation. In agreement with the
findings of McQueen (1968), very little irritation of the peritoneum was apparent and no excess of free fluid was observed. Salicylate concentration in the serum and the dialysis sac contents was determined by the fluorimetric method of Oie and Fristid (1970), serum albumin concentration was determined by the method of Doumas, et al. (1971). The presence of salicylate in the serum was found not to affect the assay of serum albumin. The variation of serum salicylate concentration with time in rats under the above experimental conditions was also determined. Blood samples (approx. 0.8 ml) were removed by tail puncture at intervals over a 24 hour period, and the serum obtained by centrifugation. After removal of approximately 3 ml of blood (after about 4 hrs.), subcutaneous isotonic saline was injected on further blood sampling to compensate for the reduction in blood volume. Serum was assayed for both salicylate and albumin. The binding of salicylate to rat serum was also determined by \textit{in vitro} ultrafiltration. Blood was removed from the aorta of normal, ether anaethetized, rats (male Wistar albino, 350-400 g) and the serum obtained. Salicylate was added to the serum to give a similar concentration range to that obtained \textit{in vivo} (i.e. 10-50 \textmu g/ml.) and ultrafiltration performed at 37\textdegree C.

(vi) \textbf{Ultraviolet Difference Spectrophotometry}

The approach used here to study carbamate-BSA (Cohn, fraction V) interaction (Chapter Four) was based on that described by Herskovits and Laskowski (1962). Although their procedure employed split absorption cells to compensate for any uv absorption of the drug, conventional stoppered single quartz cells were used in our study for the carbamates had no significant uv absorption. Albumin concentrations were either 0.58 or 5.8 \times 10^{-5}\text{M} in phosphate buffer, pH 7.4 and were titrated with
either 5 or 50 x 10^{-3} \text{M} \text{ carbamate depending on the carbamates solubility in phosphate buffer.}

2.5ml. volumes of albumin solutions (BSA V) were pipetted into both the test and reference cuvettes, fitted with Suba-seal stoppers, (Gallenkamp Ltd., London) and placed in the Pye-Unicam SP1800 spectrophotometer, (Pye-Unicam Ltd., Cambridge, England). The base-line was then recorded between 250-350nm, adjusted for a full scale deflection of 0.2 extinction units, with slit widths at 1.0nm. Small volume (\mu l) additions were then made from a 10\mu l Terumo syringe (Scientific Supplies Ltd., London, England) through the Suba-seal stoppers. Mixing was performed by inversion and the scan between 250-350nm obtained. Sequential microlitre additions up to 20 \mu l were continued until the carbamate-albumin ratios as used in the ultrafiltration studies (Chapter Four) had been reached. Studies were performed at 28 \pm 2^\circ \text{C}.

(vii) Fluorescence

All fluorimetric studies and estimations were performed in a Perkin-Elmer MPF 3 spectrophotofluorimeter coupled with a Perkin-Elmer model QPD-33 Recorder (10mv fullscale) (Perkin-Elmer Ltd., Beaconsfield, Buckinghamshire, England). The recorded spectra were not corrected for the spectral dispersion of excitation energy, nor for the spectral characteristics of the dispersion system. (Parker and Rees, 1960; Melhuish, 1961; Chen, 1967). The stability of the xenon lamp was regularly checked by use of a fluorescent standard (5\mu g/ml quinine sulphate in 0.1N sulphuric acid), although day to day variations were observed, during the course of an experiment the instrument remained relatively stable. Standard readings were therefore only checked at the beginning and end of an experiment.
In Fig. II, 3(a and b) are represented the common modes of measuring sample luminescence. For all studies on either albumin-drug binding or assays where optical densities were low, luminescence was measured at an angle of 90° to the exciting light (Fig. II, 3a). For microsomal studies (Chapter Six) because of the high turbidity, measurements were made using a cell in the front-surface reflectance mode (i.e. samples were measured at an angle of 45° to the exciting light (Fig. II, 3b) in a specially designed cell holder, constructed in the department).

For all investigations the following instrument settings were used; excitation and emission spectral band width of 5nm, and when either wavelength was being scanned, a scan speed of 180nm/min and a recorder chart speed of 30mm/min. These settings were found to give a suitably high resolution.

Fig. II, 3

a) b)

All cells (stoppered 10nm. cells, Spectrosil, ChemLab, London, England) and glassware were routinely cleaned (except for studies employing ANS, Rosen and Weber, 1969), in dilute nitric acid to remove any fluorescent contaminants.
(a) **Fluorescence quenching**

A 2ml. volume of albumin solution was pipetted into a round stoppered cuvette and capped with a Suba-seal stopper. Emission wavelength scans were recorded at the excitation maximum wavelength $\lambda_{ex}$ (determined from scan of excitation wavelength at an emission wavelength in the region of the fluorescence maxima). The fluorescence value at the emission wavelength maximum ($\lambda_{em}$) was arbitrarily assigned a value of 100%. The $\lambda_{ex}$ and $\lambda_{em}$ are dependent upon the species and nature of the albumin being studied, but are usually in the region of 290 and 340 nm respectively. Small volume ($\mu l$) additions were made from a 10$\mu l$ Terumo syringe through Subaseal stoppers. The solutions were mixed by shaking and the emission wavelength scanned at $\lambda_{ex}290nm$, changes in either the intensity of fluorescence or $\lambda_{em}$ were being recorded. Successive microlitre additions were performed to achieve suitable albumin-drug ratios.

The fluorescence after each addition was measured at room temperature 28 ± 2°C, and corrected for dilution from a control titration with the appropriate buffer (i.e. 0.1M tris-HCl, 0.1M phosphate buffer, pH 7.4). Measurements revealed no sample photodecomposition.

(b) **Fluorescent probe studies**

The titration procedure was similar to that described by Daniel and Weber (1966).

*Albumin studies* Fluorescence intensities of the probe-protein complex as a function of probe concentration (0.5 - 30 x 10$^{-6}$ M) in pH 7.4, 0.1M phosphate buffer, 2ml of each protein solution and
phosphate buffer was titrated through Suba-seal stoppers with successive additions of $1 \times 10^{-3} \text{M}$ (for low concentrations) or $5 \times 10^{-3} \text{M}$ (high concentrations) of probe in either 0.1M phosphate buffer or ethanol, depending on the probe used. After mixing fluorescence intensities were measured at the respective excitation and emission wavelengths for the probe in either protein or buffer solution. Measurement of the quenching of albumin fluorescence (previous section) on additions of the probe was also recorded during these titrations. Dilution effects were corrected for by control titrations of albumin solutions with buffer or ethanol. At the volumes added ($0.2\% \text{ V/v}$) ethanol was shown not to affect the protein fluorescence apart from the dilution effects.

For drug interaction studies either probe or competitor were added first and titrations of the albumin-drug complex with the other performed as described. All experiments were performed at room temperature $28 \pm 2^\circ\text{C}$.

**Microsomal experiments** Titrations of microsomal suspensions (2mg/ml) and buffer, by the probe were performed in an analogous manner to that with albumin solutions except that observations were recorded with the cell in the front surface reflectance mode. Possible quenching of microsomal fluorescence by the probe was not recorded.

Increase of quantum yield accompanied by a fluorescence emission maximum (and possibly excitation maximum) shift to a lower wavelength on decrease of solvent polarity, was used to select suitable fluorescent probes. To properly characterise the probe's properties equimolar concentrations of the compound were prepared in several solvents of different polarity (e.g. phosphate buffer ethanol, butanol,
hexane (spectroscopic grade, BDH Ltd.) and the emission and excitation maxima in each case, determined as previously described. Quantum yields were determined by the method of Parker and Rees (1960).

**Treatment of fluorescent probe data**

Enhancement of the fluorescence of the probe upon addition to albumin at two different concentrations, and the subsequent alteration of fluorescence in the presence of binding competitors, was used to calculate the binding constants of both probe and competitors. The fraction of probe bound $X$, was calculated using the following equation (Brand, et al., 1967):

$$X = \frac{(I_0 / I_f) - 1}{(I_b / I_f) - 1}$$

where $I_0$ and $I_f$ refer to the fluorescence intensities of a given concentration of probe in solutions of low protein concentration and in solutions without protein, respectively; and $I_b$ refers to the fluorescence intensity of same concentration of probe in solutions of high protein concentration. Thereby, $I_b$ gives the fluorescence intensity of the probe in the presence of excess binding sites. After the value $X$ was found for each point along the titration curve the Scatchard equation (Scatchard, 1949) was applied to calculate the association constant of the probe.

The type of inhibition apparent for fluorescence probe competitors was analysed by reciprocal (Klotz, et al., 1946) or Dixon (1953) plots.

**(viii) Animal Pretreatment and Microsomal Preparation**

Hamsters (approx. 50g) or rats (approx. 130g) were dosed with sodium
phenobarbitone 100mg of a 0.1% solution (in 0.9% NaCl aq. soln.) per Kg. body weight intraperitoneally, once daily for 3 days. Respective controls were injected with 0.9% NaCl (1ml. per Kg body weight). Injections were performed at approximately the same time of day - 10 a.m. ± 1 hour, and the last dose administered 24 hours before sacrifice. Gillette sterile needles (25G x ½) were used for injections. The microsomal fraction was prepared as described by Burke (1972).

(ix) Determination of Spectrally Apparent Cyt. P-450 Interactions

This method is a modification of that described by Schenkman, et al. (1967).

2.5 ml. of a 2mg. protein/ml. liver microsomal suspension was pipetted into a matched pair of glass round-stoppered spectrophotometer cuvettes and the cells placed in the sample position nearest the photomultiplier of a Unicam SP 1800 or Perkin-Elmer 356, dual beam spectrophotometer. A base line of equal light absorbance was then recorded between 350 and 500 nm. The cuvettes were then capped with Subaseal stoppers and addition of microlitre volumes of substrate solutions, in ethanol, performed by injection through the stoppers from a 10μl Terumo syringe. Sequential microlitre additions up to 20 μl of substrate solution were performed to give a substrate concentration range 0.04-1.0 x 10⁻³M. The addition of up to 20μl volumes produced a negligible volume change in the cuvette components. For each addition of substrate to the sample cuvette an equal volume of ethanol was added to the reference cell. After each addition the cuvettes were shaken to achieve mixing, and the difference spectra generated between 350 and 500nm recorded.

For substrates having an absorption spectrum which interfered with
the 'binding' difference spectrum, determinations were performed using a matched pair of split cells. These consisted of a pair of 2 x 1 cm. cells, each partitioned with a thin quartz wall into two 1 x 1 cm. individually stoppered compartments (Lightpath Optical Co. Ltd). For this procedure 2.5 ml of microsomal suspension was pipetted into one compartment of each cell and 2.5 ml of phosphate buffer into the other compartment. After recording a baseline equal microlitre volumes of substrate solution were injected into the reference cell. The same volume of ethanol was then injected into the microsomal suspension in the reference cell and the buffer in the sample cell. For estimating the effect of spectral modifiers, the competing substrate was injected into both sample and reference microsomal suspensions before recording the baseline. Additions of substrate were then performed in usual manner. Types I, II, and RI compounds were distinguished experimentally by the characteristic spectral wavelength changes they produced on interaction with microsomes. Types II and RI were further differentiated by the inability of the later type compounds to displace aniline in oxidised, or carbon monoxide in reduced, microsomes.

Analysis of spectral binding data

Spectral binding data was analysed by means of the Lineweaver-Burk plot (Fig. II, 4) with the following changes of notation: greatest spectral change $\Delta E$, instead of initial velocity; maximal spectral change $\Delta E_{max}$, instead of $V_{max}$, spectral dissociation constant $K_s$, instead of $K_m$. $\Delta E$ represents the extinction difference between the maximum and minimum of the difference spectrum.
(x) **Protein Determination**

(a) **Total protein**
Protein concentrations were normally determined by the method of Lowry, et al., (1951). For all studies, except frontal analysis, proteins were determined by the manual procedure. For frontal analysis, however, because of the large number of samples requiring analysis, determinations were performed automatically on an autoanalyzer (Technicon Instrument Co., New York, U.S.A.), the manifold used was essentially that of Gaunce and D'Iorio (1970). Manual and autoanalyser determinations were shown to give similar results.

(b) **Albumin**
The method used was that described by Doumas, et al., (1971).
Chemical Modification of Albumin and Determination of Modified Groups

Crystalline BSA and HSA preparations were chemically modified with acetic anhydride and diethylpyrocarbonate and used in the fluorescent probe studies of the binding of warfarin (Chapter Five). Controls (in the absence of modifying reagent) were carried through the same experimental procedure.

Acetic anhydride treatment

The method used to acetylate the albumins was essentially that of Bethune, et al. (1964).

2 ml. of acetic anhydride was slowly added to albumin (1g) in 200 ml. of 0.02M phosphate buffer (pH 7.4) and the pH was maintained at 7.4 for 0.5 hour at 0°C.

Diethylpyrocarbonate treatment

The method used was that of Mühlrad, et al., (1967). Solutions of albumin (2%) in 0.1M acetate buffer pH 6.0, were reacted with different concentrations of a 10% alcohol solution of diethylpyrocarbonate: 10 and 100 fold excess per mole of albumin. The reaction time was 12 hours at 20°C.

All albumin solutions after chemical modification were dialysed against several changes of distilled water at 0°C. for 2-3 days and then freeze dried (ChemLab Manifold Freeze Drier SB3, ChemLab, Ilford, Essex, England) and stored in a desiccator at 4°C.

The following assays were used to detect protein chemical group modification:— All albumin solutions were prepared in 0.1M phosphate buffer pH 7.4.
(a) **Amino** The degree of amino group modification was determined by the ninhydrin method (Kabat and Mayer, 1961).

To 0.1 ml. of albumin solutions (modified and control) in 0.1M phosphate buffer (1 to 20μgN) and blanks (buffer) was added 0.5ml. of freshly prepared ninhydrin reagent. The solutions were mixed and test tubes stoppered (10ml. screw-top Sovirel test tubes, V.A. Howe and Co. Ltd., London, England) and heated in boiling water bath for 20 minutes. 2.0ml. of H₂O-propanol solution (equal volumes of H₂O and n-propanol) was added and the solutions mixed and centrifuged. Absorbance was then read at 570nm.

The ninhydrin reagent was prepared as follows:-

40mg SnCl₂.2H₂O were dissolved in 25ml. citrate buffer (0.2M. pH 5.) and added to 0.4g ninhydrin in 12.5ml. of methyl cellosolve. Free lysine residues in diethylpyrocarbonate treated albumins were also determined by the trinitrobenzenesulphonic acid procedure of Habeeb (1966). To 1ml. of albumin solutions (0.6-1mg/min) and blanks was added 1ml. of 4% sodium bicarbonate, pH8.5 and 1ml. of a 0.1% aqueous solution of TNBS (2,4,6-trinitrobenzenesulphonic acid). The solutions were incubated at 40°C for 2 hours and then 1ml. of 10% sodium lauryl sulphate was added, followed by 0.5ml. of N HCl. Absorbance was determined at 335nm. and the molar extinction coefficient of one free amino group by the TBNS method was assumed to be 0.995 x 10⁴ (Habeeb, 1966).

(b) **Phenol** Acylation of tyrosine residues was determined by the decrease in absorbance at 278nm (Simpson, _et al._, 1963).
(c) **Imidazole** The degree of histidine substitution was determined from the absorbance at 240nm (Ovadi, 1967).

(d) **Guanidino** These were determined by a modified Sakaguchi reaction (Gilboe and Williams, 1956).

All reagents were cooled to 0-2°C before commencement of determination.

To 5ml of albumin solutions or arginine standards (5-30μg arginine/ml) was added 1ml of 0.02% 8-hydroxyquinoline and 1ml. of 10% sodium hydroxide. The solutions were whirli-mixed and replaced in an ice-bath for 2 mins., 0.2ml. of 1% sodium hypobromite was then rapidly added to develop the colour. After mixing and within 15 seconds, 1ml of 40% urea was added to destroy the excess hypobromite and prevent fading of the developed colour. One minute after addition of the hypobromite, 5ml. of cold distilled water was added, the solutions mixed and absorbance read at 506nm within 5 minutes of colour development.

(xii) **Drug Assays**

(a) **Sulphonamide**

The method used was based on that described by Bratton and Marshall (1939).

(b) **Clofibrate**

Suitable aliquots of the clofibrate containing solution were taken (e.g. 0.1 ml. containing 0.01-0.02 μ moles) and 0.5 ml. of 3N HCl was added followed by 3.0 ml of 5% absolute alcohol in
trimethylpentane (B.D.H. Spectroscopic grade). The solutions were then rotary shaken for 10 minutes and the absorbance of the organic layer measured at 226 nm. Blanks were 0.1 M phosphate buffer, pH 7.4 and clofibrate standards (0.1-2.0 μmole/ml) in phosphate buffer.

(c) **Salicylate**

The method used was that of Oie and Fristid (1971) which employed a fluorimetric estimation of total salicylate.

A standard range of salicylate (Na) concentration (5-500 nmol/ml) was prepared both in aqueous solutions and rat serum (obtained by the same procedure as test serum, as the fluorescence was observed to be lower in haemolysed serum).

(d) **Tryptophan**

The procedure used was essentially that described by Denckla and Dewey (1967), with the modification that the use of trichloroacetic acid (TCA) in their procedure was replaced with perchloric acid (PCA). This modification was found to eliminate sample 'cloudiness' after incubation apparent when using TCA, due to the formation of an insoluble condensation product, and therefore dispensed with the need to centrifuge samples before fluorescence estimation. The reduction in carbon dioxide formation by the use of PCA also reduced the need to readjust sample volume after incubation. All glassware, centrifuge tubes and spectrofluorimeter cuvettes were routinely soaked in dilute nitric acid, to remove interfering fluorescence contaminants, and then thoroughly rinsed in double-glass distilled water before use.

To duplicate aliquots (0.1 ml) of serum, dialysates and tryptophan
standards (0.2 - 20 μg/ml. in 0.1 N ammonium hydroxide) was added
9 ml. of PCA/FeCl₃ solution (3 x 10⁻⁴ M FeCl₃ in 10% W/v PCA) and
then mixed. The solutions containing serum were then centrifuged in
10 ml. polyethylene centrifuge tubes in a M.S.E. Super Speed 50
centrifuge using a 10 x 10 ml. angle rotor at 20,000 g av (21,000 rpm),
for 10 minutes at 20°C. Aliquots (1.8 ml) were then removed in
duplicate from each of the samples and transferred into 10 ml.
Sovirel screw-top test-tubes, (calibrated at a volume of 2 ml.).
Formaldehyde (0.2 ml of a 1.8% v/v aq. soln) was then added to each and
the tubes firmly stoppered, the solutions were then incubated for
2 hr. in an oven at 110°C. After cooling, the solutions were
readjusted to the 2 ml. volume mark with 10% PCA, and the fluorescence
measured (excitation wavelength, 373 nm; emission wavelength,
452 nm).
Norharman yields by the above procedure were routinely 70-80%.

Gas-Liquid Chromatography
A glass column, with J-type configuration (53 cm. long by 0.4 cm. i.d.)
packed with Porapac P (mesh-size 100-120, batch no. B-941, Phase
Separations Ltd., Flintshire, Wales) was used for both carbamate
and acetate determination. After packing the column was conditioned
by heating at 230°C for 24 hrs. with "oxygen free" nitrogen (20 ml./min
as the carrier gas. Several injections of a standard solution
(carbamate or acetate) were made and the column reconditioned (this
continued until reasonable efficiency was obtained).
(e) Carbamate determination

<table>
<thead>
<tr>
<th>Carbamate</th>
<th>Column temp. °C</th>
<th>Retention time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl</td>
<td>160</td>
<td>4 min.</td>
</tr>
<tr>
<td>Ethyl</td>
<td>160</td>
<td>6 min.</td>
</tr>
<tr>
<td>n-Propyl</td>
<td>180</td>
<td>4.5 min.</td>
</tr>
<tr>
<td>n-Butyl</td>
<td>180</td>
<td>7 min.</td>
</tr>
<tr>
<td>i-Butyl</td>
<td>180</td>
<td>6 min.</td>
</tr>
<tr>
<td>t-Butyl</td>
<td>200</td>
<td>6 min.</td>
</tr>
<tr>
<td>n-Pentyl</td>
<td>200</td>
<td>6 min.</td>
</tr>
<tr>
<td>t-Pentyl</td>
<td>200</td>
<td>6 min.</td>
</tr>
<tr>
<td>n-Hexyl</td>
<td>200</td>
<td>9 min.</td>
</tr>
<tr>
<td>t-Hexyl</td>
<td>200</td>
<td>9 min.</td>
</tr>
<tr>
<td>n-Heptyl</td>
<td>220</td>
<td>7.5 min.</td>
</tr>
<tr>
<td>Benzyl</td>
<td>220</td>
<td>15 min.</td>
</tr>
</tbody>
</table>

The detector temperature was approximately 50 °C above column temperature. Samples (2μl) were injected onto the column, with a 5μl. Hamilton microsyringe. Appropriate standards in 0.15M phosphate buffer, pH 7.4 were run in a similar manner. Peak areas were calculated by triangulation.
(f) **Acetate determination**

The same column and packing material as used for carbamate determination, was used for the assay of butyl and pentyl acetate, the only difference being in the operating conditions used.

<table>
<thead>
<tr>
<th>Column conditions</th>
<th>Gas flow rate (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>'Oxygen-free' Nitrogen:</td>
<td>200</td>
</tr>
<tr>
<td>Air flow:</td>
<td>120</td>
</tr>
<tr>
<td>Hydrogen flow:</td>
<td>45</td>
</tr>
<tr>
<td>Column temperature:</td>
<td>150°C</td>
</tr>
<tr>
<td>Detector temperature:</td>
<td>200°C</td>
</tr>
<tr>
<td>Retention times:</td>
<td></td>
</tr>
<tr>
<td>Butyl acetate 1.75 min.</td>
<td></td>
</tr>
<tr>
<td>Pentyl acetate 3.5 min.</td>
<td></td>
</tr>
</tbody>
</table>

No injector temperature was used, and the attenuation was varied according to concentration.

Because of the volatility of the acetates, 0.2 ml. of ultrafiltrate and standards (0.5-4mM) were rapidly transferred into small stoppered glass vials (1ml.) kept at 0°C. After addition of 40μl of internal standard (corresponding acetate, in 0.15M phosphate buffer) the solutions were mixed and the vials stoppered and kept at 0°C until injection onto the column.

The alternate analogue was routinely used as internal standard in the manner previously described. Because of the two acetates butyl and pentyl showed good peaks (i.e. low 'w' values, peak width at half height) (Scott, 1960) and sufficient difference in their retention times they could be used as the corresponding internal standard. They also had the advantage of similar volatility.
Determination of Apparent Partition Coefficient

The apparent partition coefficients for the carbamates series $R\text{-}0\text{-}\text{CO}\text{-}\text{NH}_2$ were determined in duplicate at 37°C using n-octanol and 0.15M phosphate buffer, pH 7.4. Both the octanol and buffer were saturated with the relevant aqueous or organic phase before use. Several carbamate concentrations (5-15mM) were then prepared in either the buffer ($R=G-C_5$) or the octanol phase ($R = C_6$). Equal volumes (25 ml.) of both phases were then mixed and shaken in stoppered conical flasks for 6 hours to achieve equilibrium (Leo, et al., 1971). The concentration of carbamate in both phases at equilibrium was then determined and good agreement (> 99%) found between duplicates.

The use of octanol for the determination of partition coefficients and its use as a reference system for a living biophase have recently been discussed (Hansch and Dunn, 1972). In support of the use of octanol Hansch has published numerous examples of excellent correlation between biological activity and octanol water partition coefficients (Hansch, 1971; Hansch and Clayton, 1973; Hansch and Dunn, 1972). The determined partition coefficients for the homologous series of carbamates ($R=0-CO-NH_2$) are presented in Table II, 1; The addition of a methylene group to the R side chain resulted in a regular increase in partition coefficient as determined by $\log P$ (logarithm of apparent partition coefficient) or the Hansch II constant (Hansch and Fujita, 1964), i.e. $\log P$ or $\Pi = 0.5$ for addition of CH$_2$ group in the straight chain homologous series where $\Pi$ is defined as:

$$\Pi = \log P_R = C_2 - C_6 - \log P_R = C_1$$

In Fig II, 5 is shown the linear correlation obtained between $\log P$ and the number of carbon atoms (x) in the R side chain for straight-
### Table 11.1. Partition Coefficients for Straight Chain Aliphatic Carbamates (R-0-CO-NH₂)

<table>
<thead>
<tr>
<th>R Group</th>
<th>Partition Coefficient a</th>
<th>log P</th>
<th>b</th>
<th>c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl</td>
<td>0.22</td>
<td>-0.66</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ethyl</td>
<td>0.70</td>
<td>-0.16</td>
<td>0.5</td>
<td>0.50</td>
</tr>
<tr>
<td>n-Propyl</td>
<td>2.30</td>
<td>0.36</td>
<td>1.02</td>
<td>0.52</td>
</tr>
<tr>
<td>n-Butyl</td>
<td>7.10</td>
<td>0.85</td>
<td>1.51</td>
<td>0.49</td>
</tr>
<tr>
<td>n-Pentyl</td>
<td>22.5</td>
<td>1.35</td>
<td>2.01</td>
<td>0.50</td>
</tr>
<tr>
<td>n-Hexyl</td>
<td>70.8</td>
<td>1.85</td>
<td>2.51</td>
<td>0.50</td>
</tr>
</tbody>
</table>

**a** Apparent partition coefficients determined using n-octanol and 0.15M phosphate buffer (pH 7.4) at 37°C.

**b** \(\pi\) derived from \(\pi = \log P_{R=C_2-C_6} - \log P_{R=C_1}\)

**c** \(\pi \ast\) derived from \(\pi \ast = \log P_R - \log P_{R-1}\)
### Table 11.2. 
Partition Coefficients for Branched Chain and Aromatic Carbamates (R-O-CO-NH$_2$)

<table>
<thead>
<tr>
<th>R Group</th>
<th>Partition Coefficient $^a$</th>
<th>log.P</th>
<th>$\pi^b$</th>
<th>$\pi^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>i-Butyl</td>
<td>4.5</td>
<td>0.65</td>
<td>1.31</td>
<td>-0.20</td>
</tr>
<tr>
<td>t-Butyl</td>
<td>3.0</td>
<td>0.47</td>
<td>1.13</td>
<td>-0.38</td>
</tr>
<tr>
<td>t-Pentyl</td>
<td>8.7</td>
<td>0.94</td>
<td>1.60</td>
<td>-0.41</td>
</tr>
<tr>
<td>t-Hexyl</td>
<td>28.0</td>
<td>1.45</td>
<td>2.11</td>
<td>-0.40</td>
</tr>
<tr>
<td>Benzyl</td>
<td>17.0</td>
<td>1.23</td>
<td>1.89</td>
<td>-1.12</td>
</tr>
</tbody>
</table>

$^a$ Apparent partition coefficients determined using n-octanol and 0.15M phosphate buffer (pH 7.4) at 37°C.

$^b$ $\pi$ derived from ($\pi = \log P_{R=C_2-C_8} - \log P_{R=C_1}$)

$^c$ $\pi^*$ derived from $\pi^* = \log P_R - \log P_{R-1}$

where $R^1$ is the respective straight chain analogue.

For benzyl carbamate $R^1$ = heptyl carbamate (log P = 2.35, $\pi = 3.01$)
The Relationship between Apparent Partition Coefficient and Number of Carbon Atoms in R Group for the Aliphatic Carbamate Series R-O-CO-NH₂

Log Apparent Partition Coefficient (Octanol/Buffer)

N (Number of Carbon Atoms in Side Chain)
The line of best fit (calculated by the method of least squares) had the following regression equation:

\[
\log P = 0.50 (\pm 0.0025) x - 1.152 (\pm 0.0013)
\]

\[n = 6, \ r = 0.999, \ s = 0.0169\]

where \(n\) is the number of data points, \(r\) is the regression coefficient and \(s\) is the standard deviation of the line. Figures in parenthesis are the standard deviations of the constants. The slope of this equation corresponded to the \(\Pi\) value for addition of a \(\text{CH}_2\) group (Table II, 1). The partition coefficients of the branched chain, \textit{iso}-butyl; \textit{tert}-butyl, \textit{tert}-pentyl, \textit{tert}-hexyl, and aromatic benzyl carbamate were also determined and demonstrated the effect of steric factors in the partitioning process (Table II, 2). Although the partition coefficients for the branched chain carbamates were lower than their respective straight chain analogues they nevertheless displayed a regular increase in \(\log P\) and \(\Pi\). For a single branch \(\log P\) and \(\Pi\) were found to be lowered by 0.2 with respect to the straight chain analogue, whereas for a double branch values were lowered by 0.4, for benzyl carbamate values were lowered by 1.12.

(xiv) **Experimental Design of Albumin Binding Studies**

Phosphate buffer (0.1 or 0.15M, pH 7.4) was used for all binding studies involving gel filtration, equilibrium dialysis and ultrafiltration techniques, although unrepresentative of a physiological buffer, it has been shown to exhibit low competitive effects on the binding of small molecules to albumin (Klotz and Urquhart, 1949, Keen, 1966).

Experimental binding data from the three techniques; frontal analysis, equilibrium dialysis and ultrafiltration was calculated according to the methods of Cooper and Wood (1968), Rosenberg and Klotz.
(1960), and Toribara, et al. (1957), respectively.

Under certain conditions (Karush and Sonnenburg, 1949), the Donnan effect may not be negligible and corrections to allow for such effects must be made (Higuchi, et al., 1954, Scatchard et al., 1950). Possible Donnan equilibrium effects for the three systems used were studied by the sodium ion distribution procedure of Keen (1965). Sodium determination by flame photometry, within experimental error, revealed negligible Donnan effects. However, calculation of the Donnan ratio (r) from Tanford, et al's (1955) data on the net charge on the albumin molecule at different pH's revealed an r value of 1.03. Keen (1965 and 1966) has also suggested that correction for the volume occupied by the protein and other solutes should be made when determining small molecule unbound and protein-bound concentrations. To account for this he suggested the following correction factor (f) for the concentration in albumin containing compartment or zone;

\[ f = \frac{100}{199.6 - (g\text{ albumin/100ml.})^{0.75}} \]

this assumes that the specific gravity of albumin is 1.33 and that 0.4% of the solution volume is occupied by solutes other than protein (McLean and Hastings, 1935).

Similarly for diffusate concentration of small molecule multiplication by 100 = 1.004 to allow for the space occupied by the 99.6 buffer solution. Therefore to account for both protein solute volume and Donnan effects the following equation can be used:

\[ \text{bound concentration} = \text{total concentration} \times f - \text{diffusate concn.} \times 1.004 \]
For our experimental systems the equation was resolved to:-

\[ \text{bound concentration} = \text{total concn.} \times 1.02 - \text{diffusate concn.} \times 0.97 \]

Similar corrections were needed for all three techniques, in view of this and the theoretical nature of \( r \) it was considered that the use of the formula:

\[ \text{bound concn.} = \text{total concn.} - \text{unbound concn.} \]

was within experimental error and sufficiently accurate, and was therefore used for all data calculation.

Experimental data was routinely interpreted by the use of the Scatchard plot (Scatchard, 1949, see next section), however for some studies (competition experiments, Chapter Five, tryptophan binding, Chapter Three, microsomal substrate interactions, Chapter Six) other plots i.e. reciprocal (Klotz, 1946) or Lineweaver-Burk (1934), or Langmuir-isotherm plots (Langmuir, 1917) have also been employed.

(xv) Treatment of Data from Binding Studies

The reversible interaction of both endogenous and exogenous compounds with plasma proteins is a dynamic equilibrium and as such is considered to obey the law of mass action sufficiently to allow treatment of the data by methods derived from this law (Goldstein, 1949, Edsall and Wyman, 1958, Kruger-Thiemer, 1968, Meyer and Guttman, 1968, Klotz and Hunston, 1971).

From a consideration of the law of mass action, in which certain assumptions are made, for a single site or class of sites reversible binding can be described by the equation:

\[ r = \frac{nkD_f}{1 + kD_f} \]

(1)

where \( r \) = moles of small molecule bound to total moles of protein in
the system

\[ D_f = \text{molar concentration of unbound small molecule} \]

\[ n = \text{number of binding sites} \]

\[ k = \text{apparent association constant (also termed affinity constant) for the binding of the small molecule.} \]

It is apparent that the binding of a charged molecule alters the overall electrostatic environment of the macromolecules, which increases the difficulty of adding another similar ion. Values can be corrected for such electrostatic interaction factors by using a rather complex equation (Scatchard, et al., 1950). Most authors, however, report uncorrected data and the association constants are therefore only 'apparent'.

The assumptions made in the equation (1) are:-

a) All sites within a class are equivalent in binding affinity

b) Binding of a small molecule at one site does not affect binding at another site i.e. no interaction occurs between binding sites

c) Concentrations are assumed to be equivalent to thermodynamic activities.

The earliest method of plotting data was adsorption isotherm methods of Freundlich (1907), and Langmuir (1917), however for several reasons discussed in detail by Goldstein (1949), Kruger-Thiemer (1968), Edsall and Wyman (1958), they are no longer the methods of choice.

A method of plotting according to Karush (1950) has also been used however it makes the assumption that the binding sites fit into two and only two, distinct groups, and involves long extrapolations to obtain \( E_n \) and \( E_{nk} \). For evaluation of the parameters of equation (1), two linear transformations have been widely used, the reciprocal and
Scatchard plots. Equation 2 shows the equation for the so-called reciprocal plot, (Klotz, 1946), Fig. II, 6 which is analogous to the Lineweaver-Burk plot.

\[
\frac{1}{r} = \frac{1}{n} + \frac{1}{nk D_f} \quad \ldots \ldots \ (2)
\]

in which \( \frac{1}{r} \) is plotted as a function of \( \frac{1}{D_f} \). If the binding sites are equivalent and independent, then \( n \) and \( k \) can be estimated directly from the slope and intercept values. Deviation from linearity in this plot, may however, be apparent.

**Fig. II, 6**
Reciprocal Plot

**Fig. II, 7**
Scatchard Plot

(For single class of binding site)
Scatchard (1949) proposed a different form of equation (1):

\[ \frac{r}{D_f} = nk - rk \ldots . . . . . . . \ldots (3) \]

Where \( \frac{r}{D_f} \) is plotted as a function of \( r \).

For a single class of binding site, the plot will be linear, and extrapolation to the abcissa gives the number of binding sites in the class, and extrapolation to the ordinate allows estimation of \( nk \). (Fig. II, 7). Curvature of the Scatchard plot is frequently encountered and this produces difficulties of interpretation. Curvature may result from one or more of the following causes:-

a) Binding sites are not equivalent i.e. there exists more than one class of site each with characteristic values for \( n \) and \( k \) thus -

\[ r = n_1k_1D_f + n_2k_2D_f + n_3k_3D_f \ldots \ldots \ldots (4) \]

b) Binding sites are not independent i.e. interaction occurs between the sites e.g. from electrostatic forces.

c) Curvature may result from a small molecule-induced conformational change in the protein.

d) A small molecule having several groups involved in binding may bind in different ways to the protein thus producing curvature.

It is apparent from (a) that for non-linear Scatchard plots no simple relationship between the intercepts and slopes and \( n \) and \( k \) values for the different sites will exist. In this case the intercepts and slopes will be composites of contributions from the various sites, and their respective \( n \) and \( k \) values must be segregated out (Klotz and Hunston, 1971).

Such curves may also be fitted as the summation of two or more
straight lines each due to a different class of binding site. Several manual methods of curve fitting have been described. (Hard, 1965, Rosenthal, 1967, Weder and Bickel, 1970, Feldman, 1972, Halfman and Nishida, 1972). However the complexity and tediousness of this procedure has led to computerised curve fitting procedures based on non-linear regression analysis (Fletcher and Spector, 1968, Crothers, 1968, Raz and Goodman, 1969, Rodbard, et al., 1969, Feldman, 1972). The reciprocal plot heavily weights those experimental points at low $D_f$ values (Klotz, et al., 1946, Meyer and Guttman, 1968) often non-linearity is observed and can therefore lead to misinterpretations concerning binding at high $D_f$. The Scatchard plot does not suffer from this disadvantage, and although curvature of the plot makes resolution complex, it is the graphical method of choice, (Meyer and Guttman, 1968, Krieglstein, 1969).

The reciprocal and Scatchard plots cannot be applied to experimental data obtained from experiments in which the amount and molecular weight of the protein involved in the binding are unknown. However, Sandberg, et al. (1966) and Rosenthal (1967) have proposed a Scatchard-type plot based on equation (5):

$$\frac{D_b}{D_f} = nkP_t - kD_b \quad \ldots \ldots (5)$$

Where $D_b = \text{concentration of unbound drug}$

$P_t = \text{total molar concentration of protein}$

A plot of $\frac{D_b}{D_f}$ (ordinate) against $D_b$, is independent of protein concentration and allows estimation of $nkP_t$, $nP_t$ and $k$, from the ordinate intercept, abscissa intercept and slope respectively. Curvature of the plot results when more than one species of binding protein is present or when there is more than one class of binding site.
on the protein.


Statistics

Linear regression analysis, based on the method of least squares, was performed on an Olivetti 101 programmeable calculator. Binding data exhibiting curved Scatchard plots was analysed by non-linear regression analysis performed using a Fortran computer programme, very kindly supplied by Dr. J. Francis, Roche Products Ltd., Welwyn Garden City, Herts.

Where appropriate data has been expressed as either mean ± S.E.M., or in the case of regression equations as mean ± S.D. Tests for statistical significance were performed using either the regression correlation coefficient or student-t-test.
CHAPTER THREE

A COMPARATIVE EVALUATION
OF SEVERAL METHODS OF MEASURING
PROTEIN BINDING
1. Introduction

A wide variety of methods have been employed to study drug-protein interactions and their relative theoretical merits and disadvantages reviewed (Goldstein, 1949; Edsall and Wyman, 1958; Rosenberg and Klotz, 1960; Steinhardt and Reynolds, 1969; Chignell, 1971). Although authors have stated their preference for one approach in favour of another, few extensive practical studies have been performed comparing available techniques. There is therefore, little experimental data to support the selection of one technique in preference to another for studying the binding of a novel compound to a protein.

Equilibrium dialysis is at present the most widely used technique in studying protein binding, but the methods of ultrafiltration and gel filtration have also received considerable attention. There is a clear need for a detailed comparison of these techniques in order to ascertain their appropriateness for studying particular drug-protein interactions.

The criteria we selected for such an evaluation were that the results obtained with each method should be reproducible over given variations in degree of binding, and changing physical parameters of the drug, such as lipid solubility, functional groups and pKa. Factors such as experimental duration, sample volume requirement, temperature control and pH control were all implicit in these considerations. The three commonly used methods of gel filtration, namely the batch, zonal and frontal analysis methods were evaluated in order to select the most suitable for comparison with the techniques of equilibrium dialysis and ultrafiltration.

The drugs; clofibrate (Atromid-S); sodium salicylate; sulphamethoxazole (Gantanol); sulphanilamide (Prontosil Album); sulphaphenazole (Orisul); and
sulphormethoxine (Fanasil), were selected for this study not only because
their physical properties were well documented and their protein binding to
albumin known to show the desired large variation in degree of binding at
comparable concentrations, but also the fact that they were relatively reliable
and simply assayed. Bovine serum albumin (BSA) was selected as a suitable protein.

One valid criticism of the above technique is that binding is determined
under non-physiological in vitro conditions. Little data is available to
establish the pertinence of such determinations to the in vivo situation.
McQueen (1968), described an in vivo method of investigating the protein
binding of drugs which involved the implantation of a dialysis sac in the
peritoneal cavity of a rat. He reported good agreement for the binding of
sulphormethoxine to rat serum albumin between this method and conventional in
vitro equilibrium dialysis. Unfortunately only the binding of
sulphormethoxine appears to have been investigated by this method, it was
therefore included in our comparative study of in vitro binding techniques.
The determination of salicylate binding by in vivo dialysis was also
investigated and compared with in vitro derived results. Salicylate was
selected because its metabolism, pharmacokinetics and in vitro binding are well
documented (Moran and Walker, 1968; McArthur and Smith, 1969), and it has
different functional groups and physico-chemical properties to the sulphonamide
(sulphormethoxine) selected by McQueen (1969). In particular salicylate has a
plasma half-life of considerably shorter duration than sulphormethoxine
(Ritschel, 1970), and may therefore be of value in exposing any possible
limitations of the in vivo technique in determining the binding of drugs with
relatively short plasma half lives. The use of this method to study the
in vivo displacement from serum protein binding sites of the tryptophan by
salicylate was also investigated.
2. Experimental Approach

In the following experiments all drug and BSA solutions were prepared in 0.1 M phosphate buffer, pH 7.4, and all experiments performed at 20°C.

a) Comparative Study of the Batch, Zonal and Frontal Analysis Methods of Gel Chromatography

A comparative study of the binding of the drugs sulphormethoxine (24.0 X 10^-5 M) and clofibrate (31.0 X 10^-5 M) to BSA (29.0 X 10^-5 M) was performed with these three gel chromatography methods. Sephadex G-25 (fine) was used as the gel filtration medium, although the use of a polyacrylamide gel (Biogel P2) was also investigated in the zonal chromatography studies.

b) Comparative Evaluation of the techniques of Equilibrium Dialysis, Ultrafiltration and Gel Chromatography (Frontal Analysis)

The binding of the drugs; clofibrate; sodium salicylate; sulphamethoxazole; sulphanilamide; sulphaphenazole and sulphormethoxine to BSA (29 X 10^-5 M) was studied over a wide concentration range (7.5 to 90 X 10^-5 M)by the three techniques.

c) Comparison of Salicylate Binding to Rat Serum by In Vitro and In Vivo Techniques

The binding of salicylate to rat serum and its ability to displace bound tryptophan was determined by the in vivo dialysis technique.

Salicylate, in isotonic saline, was intragastrically administered to male Wistar albino rats (10 or 20 mg/Kg. body wt.), control rats received isotonic saline only, once daily for seven days before implantation of dialysis sacs. The dosage regime was continued until removal of the sacs 24 hours after implantation (i.e. day 8). Sacs were implanted and removed 4 hours after oral dosing, blood samples were obtained from the tail vein and
by heart puncture, at the same time as the sacs were removed, and serum obtained by centrifugation. The concentration of tryptophan in the serum and dialysis sac was also determined. Serum salicylate-time curves were also determined over an equivalent 24 hour period in a similar group of rats.
3. Results
Table III, 1

Use of the Bath Method of Gel Filtration to study the Binding of Sulphormethoxine to Bovine Serum Albumin:

**Distribution of Sulphormethoxine between Internal and External Phases**

<table>
<thead>
<tr>
<th>Initial Sulphonamide Conc. ($x 10^{-5}$M)</th>
<th>Internal Phase (Free Conc.$^n$.) $x 10^{-5}$M</th>
<th>External Phase $x 10^{-5}$M</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.1</td>
<td>10.4 ±0.8</td>
<td>7.9 ±0.4</td>
<td>56.9 ±2.3</td>
</tr>
<tr>
<td>24.2</td>
<td>12.3 ±0.5</td>
<td>11.6 ±0.3</td>
<td>49.4 ±1.4</td>
</tr>
<tr>
<td>32.2</td>
<td>14.7 ±1.4</td>
<td>15.0 ±0.5</td>
<td>46.1 ±2.1</td>
</tr>
<tr>
<td>48.3</td>
<td>25.8 ±0.9</td>
<td>19.9 ±2.6</td>
<td>47.3 ±4.3</td>
</tr>
<tr>
<td>64.5</td>
<td>37.4 ±0.7</td>
<td>28.6 ±0.9</td>
<td>51.2 ±3.3</td>
</tr>
<tr>
<td>80.6</td>
<td>42.1 ±4.7</td>
<td>35.6 ±1.3</td>
<td>48.2 ±2.7</td>
</tr>
<tr>
<td>Control in buffer</td>
<td>50.2 ±6.0</td>
<td>35.4 ±3.9</td>
<td>88.6 ±8.4</td>
</tr>
</tbody>
</table>

*a 29.0 x $10^{-5}$M BSA in 0.1M phosphate buffer (pH 7.4)
*b Concentration imbided in Sephadex gel after equilibration.
All values mean ± S.E.M. of 3 determinations.
Experiments performed at 20°C.
USE OF ZONAL GEL CHROMATOGRAPHY TO STUDY THE BINDING OF DRUGS TO
BOVINE SERUM ALBUMIN (Figs. III, 1-3)

Fig. III, 1 Elution Profiles of Sulphormethoxine in the presence of
Bovine Serum Albumin or Phosphate Buffer

Column: Sephadex G-25 (fine), 20 x 1.2 cm
Sample volume: 2 ml
(24.0 x 10^{-5}M Sulphormethoxine
29.0 x 10^{-5}M BSA)

% of total eluted

BSA

Sulphormethoxine + BSA (○—○)
Sulphormethoxine + Phosphate Buffer (pH 7.4, 0.1M)(○—○)

Elution volume (ml) (5ml fractions)
**Fig. III, 2** Effect of Reduction of Column Length on the Elution Profile of BSA-Sulphormethoxine Mixture.

**Fig. III, 3** Elution Profiles of Clofibrate in the presence of Bovine Serum Albumin or Phosphate Buffer.

Column: Sephadex G-25 (fine) 8 x 1.2 cm

Sample volume: 1 ml (24.0 x 10^{-5} M Sulphormethoxine; 29.0 x 10^{-5} M BSA)
Fig. III, 4

Frontal Analysis Chromatogram of Sulphormethoxine in the presence of Bovine Serum Albumin and Phosphate Buffer

Temp. = 20° C
All points mean of 2 determinations

- Sulphormethoxine (24.0 x 10^{-5} M)
- Sulphormethoxine (24.0 x 10^{-5} M) + BSA (29.0 x 10^{-5} M)
- BSA (29.0 x 10^{-5} M)

In all cases elution was carried out with 0.1 M phosphate buffer pH 7.4
### Table III, 2.

**Some Physico-Chemical Properties of the Drugs used in this Study**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Formula</th>
<th>M.Wt.</th>
<th>pKa</th>
<th>Log P (octanol:buffer)</th>
<th>% Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulphanilamide</td>
<td>$\text{H}_2\text{N} \text{SO}_2\text{NH}_2$</td>
<td>172.2</td>
<td>10.6</td>
<td>-0.72</td>
<td>13.9</td>
</tr>
<tr>
<td>Sulphamethoxazole</td>
<td>$\text{H}_2\text{N} \text{SO}_2\text{NH}$</td>
<td>253.3</td>
<td>5.6</td>
<td>0.88</td>
<td>62.6</td>
</tr>
<tr>
<td>Sulphormethoxine</td>
<td>$\text{H}_2\text{N} \text{SO}_2\text{NH}$</td>
<td>310.3</td>
<td>5.8</td>
<td>1.56</td>
<td>48.1</td>
</tr>
<tr>
<td>Sulphaphenazole</td>
<td>$\text{H}_2\text{N} \text{SO}_2\text{NH}$</td>
<td>314.4</td>
<td>5.9</td>
<td>1.57</td>
<td>76.9</td>
</tr>
<tr>
<td>Clofibrate</td>
<td>$\text{Cl} \text{O} \text{CH}_3 \text{COOC}_2\text{H}_5$</td>
<td>243</td>
<td>-</td>
<td>3.64</td>
<td>81.0</td>
</tr>
<tr>
<td>Salicylate (Na)</td>
<td>$\text{COONa}$</td>
<td>160.3</td>
<td>3.0</td>
<td>-0.85</td>
<td>83.9</td>
</tr>
</tbody>
</table>

a Data from Eloffson, et al. (1971); Knoefel (1972) and Reider (1963)
b Data from Leo et al. (1971)
c BSA concentration, 29.0 x 10^-5M; drug concentration, 45.0 x 10^-5M; pH 7.4 at 20°C.
Table III, 3.

Comparison of the Percentage Binding\(^a\) of Sulphormethoxine to Bovine Serum Albumin by Three Techniques

<table>
<thead>
<tr>
<th>Initial Sulphonamide Conc(^n) x10(^{-5}) M</th>
<th>Frontal Analysis</th>
<th>Ultrafiltration</th>
<th>Equilibrium Dialysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Bound</td>
<td>% Bound</td>
<td>% Bound</td>
<td>% Bound</td>
</tr>
<tr>
<td>7.5 ±1.2</td>
<td>-</td>
<td>74.6 ±4.8</td>
<td></td>
</tr>
<tr>
<td>15.0 ±0.8</td>
<td>57.5 ±1.2</td>
<td>55.4 ±1.2</td>
<td>54.1 ±5.1</td>
</tr>
<tr>
<td>22.5 ±1.7</td>
<td>49.9 ±0.4</td>
<td>52.5 ±0.5</td>
<td>44.8 ±0.5</td>
</tr>
<tr>
<td>30.0 ±1.1</td>
<td>48.3 ±1.3</td>
<td>51.1 ±1.3</td>
<td>43.1 ±3.3</td>
</tr>
<tr>
<td>45.0 ±2.3</td>
<td>46.1 ±1.2</td>
<td>48.1 ±1.2</td>
<td>41.6 ±3.0</td>
</tr>
<tr>
<td>60.0 ±1.6</td>
<td>42.1 ±1.3</td>
<td>45.6 ±1.3</td>
<td>40.5 ±2.8</td>
</tr>
<tr>
<td>75.0 ±1.7</td>
<td>40.6 ±1.5</td>
<td>45.0 ±1.5</td>
<td>41.3 ±1.9</td>
</tr>
<tr>
<td>90.0 ±1.1</td>
<td>38.3 ±0.7</td>
<td>42.6 ±0.7</td>
<td>36.2 ±1.2</td>
</tr>
</tbody>
</table>

\(^a\) Mean ± S.E.M. of at least two determinations using a BSA concn. of 29·0 x 10\(^{-5}\)M.

Experiments were performed at 20\(^\circ\)C.
Comparison of the Percentage Binding\textsuperscript{a} of Sodium Salicylate to Bovine Serum Albumin by Three Technique

<table>
<thead>
<tr>
<th>Initial Salicylate Conc\textsuperscript{n} $\times 10^{-5}$M</th>
<th>Frontal Analysis</th>
<th>Ultrafiltration</th>
<th>Equilibrium Dialysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Bound</td>
<td>% Bound</td>
<td>% Bound</td>
</tr>
<tr>
<td>7.5</td>
<td>-</td>
<td>91.8 ±0.5</td>
<td>97.2 ±1.3</td>
</tr>
<tr>
<td>15.0</td>
<td>93.6 ±0.5</td>
<td>91.1 ±0.4</td>
<td>94.5 ±1.4</td>
</tr>
<tr>
<td>22.5</td>
<td>91.2 ±0.4</td>
<td>90.9 ±0.7</td>
<td>88.9 ±0.7</td>
</tr>
<tr>
<td>30.0</td>
<td>-</td>
<td>88.9 ±0.1</td>
<td>85.4 ±0.3</td>
</tr>
<tr>
<td>45.0</td>
<td>87.6 ±0.9</td>
<td>83.9 ±0.1</td>
<td>80.4 ±0.5</td>
</tr>
<tr>
<td>60.0</td>
<td>-</td>
<td>78.9 ±0.4</td>
<td>77.2 ±0.3</td>
</tr>
<tr>
<td>75.0</td>
<td>77.8 ±0.2</td>
<td>75.4 ±0.3</td>
<td>75.1 ±0.2</td>
</tr>
<tr>
<td>90.0</td>
<td>-</td>
<td>75.2 ±0.2</td>
<td>65.2 ±0.7</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Mean ± S.E.M. of two determinations, using a BSA concn of 29.0 $\times 10^{-5}$M

Experiments were performed at 20°C.
Table III, 5

Comparison of the Percentage Binding\(^a\) of Sulphamethoxazole to Bovine Serum Albumin by Three Techniques

<table>
<thead>
<tr>
<th>Initial Sulphonamide Conc(^n) x10(^{-5})M</th>
<th>Frontal Analysis</th>
<th>Ultrafiltration</th>
<th>Equilibrium Dialysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Bound</td>
<td>% Bound</td>
<td>% Bound</td>
<td>% Bound</td>
</tr>
<tr>
<td>15.0</td>
<td>68.4 ±1.4</td>
<td>69.7 ±1.3</td>
<td>67.8 ±4.6</td>
</tr>
<tr>
<td>30.0</td>
<td>65.3 ±0.6</td>
<td>64.3 ±0.3</td>
<td>64.0 ±2.1</td>
</tr>
<tr>
<td>45.0</td>
<td>57.3 ±0.9</td>
<td>62.6 ±0.4</td>
<td>58.3 ±1.2</td>
</tr>
<tr>
<td>75.0</td>
<td>54.8 ±0.7</td>
<td>52.6 ±0.2</td>
<td>48.7 ±2.4</td>
</tr>
</tbody>
</table>

\(^a\) Mean ± S.E.M. of two determinations, using a BSA concn. of 29.0 x 10\(^{-5}\)M

Experiments were performed at 20°C.
Table III, 6

Comparison of the Percentage Binding\textsuperscript{a} of

Sulphamethoxazole to Bovine Serum Albumin by Three Techniques

<table>
<thead>
<tr>
<th>Initial Sulphonamide Conc. ( \times 10^{-5} \text{M} )</th>
<th>Frontal Analysis</th>
<th>Ultrafiltration</th>
<th>Equilibrium Dilaysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Bound</td>
<td>% Bound</td>
<td>% Bound</td>
</tr>
<tr>
<td>15.0</td>
<td>88.2 ±0.5</td>
<td>87.3 ±0.4</td>
<td>85.3 ±0.8</td>
</tr>
<tr>
<td>22.5</td>
<td></td>
<td>85.1 ±0.5</td>
<td>84.1 ±0.9</td>
</tr>
<tr>
<td>30.0</td>
<td>81.7 ±0.9</td>
<td>83.8 ±0.3</td>
<td>80.1 ±1.1</td>
</tr>
<tr>
<td>45.0</td>
<td>76.7 ±0.6</td>
<td>76.9 ±0.6</td>
<td>75.6 ±0.9</td>
</tr>
<tr>
<td>75.0</td>
<td>73.7 ±0.4</td>
<td>72.0 ±0.4</td>
<td>66.7 ±1.2</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Mean ± S.E.M. of two determinations, using a BSA concn. of 29.0 \( \times 10^{-5} \text{M} \)

Experiments performed at 20°C
Table III; 7

Comparison of the Percentage Binding$^a$ of Sulphanilamide to Bovine Serum Albumin by Three Techniques

<table>
<thead>
<tr>
<th>Initial Sulphonamide Conc. x10$^{-5}$M</th>
<th>Frontal Analysis</th>
<th>Ultrafiltration</th>
<th>Equilibrium Dialysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Bound</td>
<td>% Bound</td>
<td>% Bound</td>
</tr>
<tr>
<td>7.5</td>
<td>27.6 ± 0.9</td>
<td>28.3 ± 0.7</td>
<td>26.4 ± 2.1</td>
</tr>
<tr>
<td>15.0</td>
<td>23.5 ± 0.8</td>
<td>24.5 ± 0.5</td>
<td>22.3 ± 1.4</td>
</tr>
<tr>
<td>30.0</td>
<td>17.4 ± 1.2</td>
<td>15.8 ± 2.1</td>
<td>15.0 ± 0.4</td>
</tr>
<tr>
<td>45.0</td>
<td>7.9 ± 0.7</td>
<td>13.9 ± 1.4</td>
<td>11.5 ± 1.1</td>
</tr>
</tbody>
</table>

$^a$ Mean ± S.E.M. of two determinations, using a BSA concn. of 29.0 x 10$^{-5}$M.

Experiments were performed at 20°C
### Table III, 8

Comparison of the Percentage Binding\(^a\) of Clofibrate to Bovine Serum Albumin by Three Techniques

<table>
<thead>
<tr>
<th>Initial Clofibrate Concentration (x10(^{-5}) M)</th>
<th>Frontal Analysis</th>
<th>Ultrafiltration</th>
<th>Equilibrium Dialysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Bound</td>
<td>% Bound</td>
<td>% Bound</td>
</tr>
<tr>
<td>45.0</td>
<td>85.8 ±2.0</td>
<td>81.0 ±1.1</td>
<td>80.1 ±1.6</td>
</tr>
<tr>
<td>60.0</td>
<td>81.9 ±1.5</td>
<td>85.0 ±4.8</td>
<td>80.6 ±2.2</td>
</tr>
<tr>
<td>75.0</td>
<td>74.8 ±0.9</td>
<td>73.2 ±1.3</td>
<td>70.3 ±2.4</td>
</tr>
<tr>
<td>90.0</td>
<td>66.2 ±1.7</td>
<td>64.8 ±1.8</td>
<td>63.3 ±0.9</td>
</tr>
</tbody>
</table>

\(^a\) Mean ± S.E.M. of two determinations, using a BSA concn. of 29.0 x 10\(^{-5}\) M.

Experiments were performed at 20°C.
Fig. III, 5

Comparison of Scatchard Plots of Data for the Binding of Sulphormethoxine to Bovine Serum Albumin by Three Techniques

Temp. = 20°C, pH 7.4

- Frontal Analysis Data
- Ultrafiltration Data
- Equilibrium Dialysis Data

Each point is the mean of at least two determinations.
Fig. III, 6

Comparison of Scatchard Plots of Data for the Binding of Sodium Salicylate to Bovine Serum Albumin by Three Techniques

Temp. = 20°C, pH 7.4

- Frontal Analysis Data
- Ultrafiltration Data
- Equilibrium Dialysis Data
Table III, 9

Binding of Salicylate to Rat Serum Albumin as Determined by In Vivo Dialysis

<table>
<thead>
<tr>
<th>Oral Salicylate Pretreatment</th>
<th>Male Rat No.</th>
<th>Serum Salicylate Conc. x 10^-5 M</th>
<th>Rat Serum Albumin Conc. g/100 ml x 10^-5 M</th>
<th>r (moles salicylate bound/mole albumin)</th>
<th>% Salicylate Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mg/kg</td>
<td>1</td>
<td>11.27</td>
<td>3.97</td>
<td>0.16</td>
<td>81.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>10.48</td>
<td>3.78</td>
<td>0.17</td>
<td>86.6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>15.93</td>
<td>4.17</td>
<td>0.21</td>
<td>79.0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>9.82</td>
<td>3.75</td>
<td>0.15</td>
<td>82.6</td>
</tr>
<tr>
<td>Mean ± S.E.M. 1-4</td>
<td></td>
<td>11.88 ± 1.38</td>
<td>3.92 ± 0.10</td>
<td>0.17 ± 0.01</td>
<td>82.4 ± 1.6</td>
</tr>
<tr>
<td>20 mg/kg</td>
<td>5</td>
<td>23.60</td>
<td>3.88</td>
<td>0.36</td>
<td>85.7</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>23.71</td>
<td>3.81</td>
<td>0.34</td>
<td>82.0</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>21.34</td>
<td>3.96</td>
<td>0.31</td>
<td>84.4</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>27.71</td>
<td>4.25</td>
<td>0.37</td>
<td>82.6</td>
</tr>
<tr>
<td>Mean ± S.E.M. 5-8</td>
<td></td>
<td>24.09 ± 1.32</td>
<td>3.98 ± 0.10</td>
<td>0.35 ± 0.01</td>
<td>83.7 ± 0.85</td>
</tr>
</tbody>
</table>

a. Each value is the mean of 4 measurements.

b. Single daily oral dose for 8 consecutive days; peritoneal sacs implanted on day 7. All values determined 4 hours after last oral dose (day 8). c. Molecular weight assumed to be 69,000

Significance between groups: Total, P < 0.001; Df, P < 0.02; bound, P < 0.001; r, P < 0.001; non-significant differences in albumins concns. between groups and controls.
Table III, 10.

**Binding of Salicylate to Rat Serum Proteins as determined by In Vitro Ultrafiltration**

<table>
<thead>
<tr>
<th>Initial Salicylate Conc. x10^{-5}M</th>
<th>Unbound Salicylate Conc. x10^{-5}M</th>
<th>Bound Salicylate Conc. x10^{-5}M</th>
<th>% Bound</th>
<th>r (moles salicylate bound/mole albumin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.90 ±0.05</td>
<td>0.83 ±0.03</td>
<td>4.07 ±0.03</td>
<td>83.0 ±0.9</td>
<td>0.06 ±0.0005</td>
</tr>
<tr>
<td>10.40 ±0.01</td>
<td>1.82 ±0.03</td>
<td>8.58 ±0.03</td>
<td>82.5 ±0.15</td>
<td>0.13 ±0.0005</td>
</tr>
<tr>
<td>15.90 ±0.02</td>
<td>2.97 ±0.15</td>
<td>12.93 ±0.15</td>
<td>81.3 ±0.1</td>
<td>0.20 ±0.0002</td>
</tr>
<tr>
<td>21.20 ±0.13</td>
<td>4.32 ±0.20</td>
<td>16.88 ±0.7</td>
<td>79.6 ±0.7</td>
<td>0.26 ±0.0003</td>
</tr>
<tr>
<td>26.60 ±0.09</td>
<td>5.42 ±0.21</td>
<td>21.18 ±0.4</td>
<td>79.6 ±0.4</td>
<td>0.33 ±0.0003</td>
</tr>
</tbody>
</table>

All values ± S.E.M. of 3 determinations

a  At pH 7.4 and 37° C

b  Rat serum albumin concentration = 4.44 g/100 ml (64.35 x 10^{-5}M) ±0.05 ± 0.725
**Fig. III, 7a**  
Relationship between Unbound Salicylate and Rat Serum Salicylate Concentration

Unbound Salicylate Concentration, $D_f \times 10^{-5}$M vs. Total Serum Salicylate Concentration ($\times 10^{-5}$M)  
- **in vivo dialysis**  
  (individual values, albumin concn. = $3.92 \pm 0.10$ g/100 ml.)  
- **in vitro ultrafiltration**  
  (mean of 3 determinations, albumin concn. = $4.44 \pm 0.05$ g/100 ml.)

---

**Fig. III, 7b**  
Relationship between Percentage Salicylate Bound and Rat Serum Salicylate Concentration

% Bound vs. Total Serum Salicylate Concentration ($\times 10^{-5}$M)  
- **in vivo dialysis**  
  (individual values)  
- **in vitro ultrafiltration**  
  (mean of 3 determinations)
Fig. III, 8a  Relationship between \( r \) and Unbound Rat Serum Salicylate Concentration

\[
\text{moles salicylate bound/mole albumin}
\]

- in vivo dialysis (individual values)
- in vitro ultrafiltration (mean of 3 determinations)

![Graph for Fig. III, 8a](image)

Unbound Salicylate Concn., \( D_f \) (x 10^{-5}M)

Fig. III 8b  Relationship between \( r \) and Total Rat Serum Salicylate Concentration

\[
\text{moles salicylate bound/mole albumin}
\]

- in vivo dialysis (individual values)
- in vitro ultrafiltration (mean of 3 determinations)

![Graph for Fig. III, 8b](image)

Total Serum Salicylate Concn (x10^{-5}M)
Table III, 11. Serum Salicylate Concentrations and Half-Lives following the 8 day Oral Administration of Salicylate (either 10 mg or 20 mg/kg body wt.) to Rats.

<table>
<thead>
<tr>
<th>Time after oral dose on day 8 (hr)</th>
<th>Oral dose 10 mg/kg body wt.</th>
<th>Serum Salicylate Concentration (x 10^-5 M)</th>
<th>Mean + S.E.M. of Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oral dose 20 mg/kg body wt.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat No.:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>14</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2 ±0.5</td>
<td>1.3 ±0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.4</td>
<td>1.5 ±0.5</td>
</tr>
<tr>
<td>17-20</td>
<td></td>
<td>1.6 ±0.5</td>
<td>1.5 ±0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22 ±6</td>
<td>25.7 ±2.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16.6 ±2.6</td>
<td>17.7 ±2.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12.4</td>
<td>12.2 ±2.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15.0 ±2.4</td>
<td>27.2 ±2.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>28.3</td>
<td>23.5 ±2.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9.2</td>
<td>12.5 ±1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.4 ±1.8</td>
<td>20.1 ±1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.5</td>
<td>24.0 ±2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9.9</td>
<td>4.8 ±2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.4</td>
<td>4.3 ±0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.2</td>
<td>5.8 ±0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.8</td>
<td>9.2 ±0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.1</td>
<td>9.2 ±0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>4.9 ±0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>5.8 ±0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.1</td>
<td>2.0 ±1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each value is the mean of at least duplicate measurements (reproducibility > 97% between duplicates). Animal groups 13-16 and 17-20 have followed equivalent dosage regimes to groups 1-4 and 5-8 respectively employed in the in vivo binding study (Table III, 9).
Fig. III, 9  Serum Salicylate Concentration-Time Curves in Rats after 8 Day Oral Administration of Salicylate

a) Daily oral dose: 10 mg/kg body wt.

Curves are for individual rats, each point is the mean of duplicate measurements.

b) Daily oral dose: 20 mg/kg body wt.
Fig. III. Effect of 8 day Oral Administration of Salicylate on
Total, Bound, Unbound and Percentage Bound Rat Serum
Tryptophan In Vivo

a. Total Tryptophan

b. Bound Tryptophan

c. Unbound Tryptophan
d. % Tryptophan Bound

Control (Saline)  Salicylate 10 mg/kg body wt.
Salicylate 20 mg/kg body wt.

Tryptophan levels measured 4 hrs. after last oral salicylate dose.
Results are mean ± S.E.M. of 4 rats.

p>0.05 considered non significant (NS) with respect to control.
4. Discussion

Comparative Study of the Batch, Zonal and Frontal Analysis Methods of Gel Chromatography

The results for the study of the binding of the sulphonamide, sulphormethoxine, to bovine serum albumin by the batch method of gel filtration are presented in Table III, 1. For a compound which is significantly bound, the concentration in the external phase, in the presence of protein, should be considerably higher than its absence. This was not the case for sulphormethoxine, furthermore very low recovery values were obtained which could not be accounted for solely by experimental error. It is apparent that significant adsorption of the drug onto sephadex occurred. Estimation of the protein concentration in the external phase showed that there was no significant adsorption of the protein onto the gel. The low recovery values demonstrated that attempts to accurately separate the internal phase from the external phase by the method described by Saris (1963) were unsuccessful. Not only was it difficult to retain all of the sephadex beads, but it also proved impossible to completely remove the external phase. The results obtained thus reflect not only the problem of drug adsorption onto sephadex, but also the problem of external phase contamination of the interior phase samples. In similar experiments with the highly bound drug clofibrate a marked adsorption onto the sephadex was again apparent, (N.B. Reversible adsorption onto sephadex has also been demonstrated for other compounds, see Gelotte, 1960, Pearlman and Crepy, 1967). Although such adsorption effects can be allowed for, to some extent, the poor reproducibility makes any correction factor of questionable value. Although the batch method is potentially appealing because of its simplicity and faster equilibration time than equilibrium dialysis, because of the intrinsic problems this method is clearly not suitable for routine measurements of the protein binding of drugs and we therefore did not investigate it further.
The reversible adsorption of drug onto the sephadex, giving rise to a reduction in the degree of protein binding was also apparent in zonal chromatography studies using sulphormethoxine (Fig. III, 1). Attempts to minimise this by shortening of column length (bed volume <12 cc) (Fig. III, 2), or increasing elution rate, appeared to reduce the degree of dissociation, but introduced the concomitant problem of poor separation of the elution peaks of protein, free drug and bound drug, thereby making interpretation difficult. Studies with other drugs; clofibrate (Fig. III, 3), sulphamethoxazole and succinylsulphathiazole also showed similar dissociation effects. These results, which are in agreement with the findings of the batch method, demonstrate that sephadex adsorption and drug-protein dissociation on the column seriously restrict the use of this technique for the study of reversible drug-protein interactions. McArthur and Smith (1968), adopted a novel approach to the problem by studying the binding of salicylate to BSA, using sephadex columns of varying lengths, and then extrapolating the results back to a column of zero length. In this way they suggested that they could overcome the concomitant problems of drug adsorption and the dilution of the complex as it passed through the column. However, apart from being time consuming, this approach does have the added disadvantage that it is not always possible to accurately extrapolate back to zero column lengths. Great care is required to ensure that the elution profiles are not modified at shorter column lengths.

We also investigated the possibility that adsorption effects could be reduced by the use of different gel filtration medium. The binding of the drugs sulphormethoxine and sulphamethoxazole to BSA was studied using Biogel P2 which, unlike sephadex, is a polyacrylamide gel. Unfortunately while less dissociation of the drug-protein complex was apparent, there was still sufficient adsorption onto the polyacrylamide to make assessment of the degree of binding invalid. Certainly it is possible to correct for the degree of adsorption onto sephadex that occurs on column gel filtration as Brumbaugh and
Ackers (1971) have shown using a direct optical scanning method of columns, for binding studies. However the technique requires specific apparatus, is time consuming and the corrections are cumbersome. The zonal gel filtration method, because of the above problems was deemed unsuitable for further study.

The results for the study of the binding of sulphormethoxine to BSA obtained with the frontal analysis method are presented in Fig. III, 4. Elution of sulphormethoxine in the presence of BSA closely followed the elution of the protein. However, there was a slight lag in the leading boundary (α region) which indicated that, some degree of dissociation of the albumin-sulphonamide complex had still occurred. That this did not affect the final estimation of binding, however, was apparent from the presence of the two 'steady-state' plateaus (β and γ). The leading boundary (α region) for sulphormethoxine in the presence of BSA, represented the difference in elution profile between the BSA and free sulphormethoxine. The nature of this leading boundary would suggest that the equilibrium between sulphormethoxine was rapid and reversible. (Cooper and Wood, 1968). The β plateau corresponded (within experimental error) to the concentration of sulphormethoxine and BSA in the original solution. The rate of attainment of this plateau depended to some extent on the degree of dissociation, and accounted for some of the non-idealised plateaus obtained. The trailing boundary (γ) corresponded to unbound sulphormethoxine. The elution profile of sulphormethoxine would therefore appear to be in agreement with that expected from theoretical considerations (Nichol and Winzor, 1964; Cooper and Wood, 1968; Nichol, et al., 1971). To further establish the suitability of this method the binding of four different concentrations of sulphormethoxine to BSA were studied. The results showed that increasing the sulphonamide concentration produced the expected decrease in percentage bound. (Table III, 3). These experiments also confirmed that the position of the trailing boundary (γ region) which corresponded to unbound sulphonamide, was independent of sulphonamide concentration.
These findings substantiate the view that frontal analysis is a suitable method for studying drug-protein interactions. For routine measurements of binding it is only necessary to determine a statistically representative number of samples in the plateaus $\beta$ and $\gamma$ (corresponding to total and free drug respectively). It is important, however, when the binding of a drug not previously studied by this method, is investigated, that the complete elution profile is determined. So that the suitability of the plateau and the possibility of the dissociation of the drug-protein complex may be ascertained.

The above findings have shown that of the gel chromatography methods investigated, only frontal analysis is suitable for studying reversible drug-protein interactions.
Comparative Evaluation of the Techniques of Equilibrium Dialysis, Ultrafiltration and Gel Chromatography (Frontal Analysis).

The physico-chemical properties and percentage bound to BSA (Table III, 2) of the drugs selected for this study, confirmed that they satisfied the selection rationale. In Tables III, 3-8, are presented the results obtained by equilibrium dialysis, ultrafiltration and frontal analysis for the determination of the percentage binding to BSA of the drugs; sulphormethoxine; salicylate; sulphamethoxazole; sulphanilamide and clofibrate respectively. Of the three techniques studied, frontal analysis is to be theoretically preferred in that it determines the degree of binding pertaining in the original solution and is not subject to the concentration effects as in the case of ultrafiltration, or dilution effects as with equilibrium dialysis. Typical Scatchard plots (Scatchard, 1949) for the binding data of the drugs sulphormethoxine and salicylate with the three techniques are shown (Figs. III, 5 and 6). Analysis of the binding data suggested that the percentage bound value obtained by the three techniques were generally in good agreement. Although, slight differences were apparent in the values obtained by the three techniques, at some drug concentrations, no consistently significant differences were apparent for any of the drugs. Because only duplicate determinations were performed no detailed statistical treatment of the results has been presented. However, sufficient published data is available for each technique to verify its repeatability. Furthermore the nature of the duplicates and preliminary analysis of the data by 'two-way' analysis of variance would support the validity of these findings. The nature of the Scatchard plots obtained for sulphormethoxine and salicylate binding to BSA (Fig. III, 5 and 6) also emphasised the similarities of binding data obtained by the three techniques. It is apparent that the small differences observed in the percentage bound values did not detract from the common regression line that could be fitted to the Scatchard plots. Similarly good agreement was found for the other drugs
studied, when analysed by the Scatchard method. No attempt has been made to estimate either the number of binding sites (n) or the association constants (k) from these plots, as there were clearly insufficient data points to allow accurate regressional analysis determinations of the parameters.

The good correlation found using the three techniques for salicylate binding, disagreed with the observations of McArthur and Smith (1968). They reported higher percentage bound values with equilibrium dialysis than for ultrafiltration and frontal analysis, however, their binding studies were performed at only one salicylate concentration. Recently Keresztes-Nagy et al., (1972), confirmed our finding, showing good agreement for the binding of salicylate by equilibrium dialysis, and frontal analysis. They also suggested that although equilibrium dialysis produced reproducible results, larger experimental variations were apparent with this technique than for the frontal analysis. Some degree of support for this view might be concluded from our findings, but further studies would be needed to confirm this. Cooper and Wood (1969) in a limited comparative study of the binding of three sulphonamides by equilibrium dialysis and frontal analysis also reported good agreement. Generally the data obtained by ultrafiltration has not shown serious disagreement from that obtained by equilibrium dialysis (Steinhardt and Reynolds, 1969). Thus it can be concluded that even though the three techniques may affect the drug-protein equilibrium to different extents they would all appear to be appropriate for measuring the protein binding of drugs exhibiting similar physico-chemical properties to those studied here. Preference of one technique in favour of another must therefore be made on basis other than the binding data obtainable by these techniques.

A serious practical criticism of frontal analysis and equilibrium dialysis is the long experimentation times required by both techniques, Frontal analysis requires approximately 2 hours for each drug concentration, and
equilibrium dialysis, depending on the system used, from 5 to 24 hours. Non-equilibrium dialysis techniques (Stein, 1965; Meyer and Guttman, 1968) are generally faster, but can give cumbersome and sometimes questionable data analysis. Protein denaturation and bacterial contamination problems can clearly become manifest with such long experimental times, especially for experiments performed at 37° C. Frontal analysis also has the inherent problems of requiring large sample volumes, (although smaller volumes than used in this study could be employed) in order to attain a 'steady-state' plateau.

In conclusion ultrafiltration was selected as the routine technique for further studies on protein binding because it was shown to produce data comparable with that of the other methods and to require much shorter experimentation times. The ultrafiltration procedure used also has the important advantage of easier control of temperature, and much closer maintenance of pH for serum or plasma studies.

In this study it was perhaps fortunate that none of the drugs selected showed any significant adsorption onto the dialysis membrane. However, drugs and other compounds may bind to dialysis membrane (Klotz, 1953; Steinhardt and Reynolds, 1969; Meyer and Guttman, 1970,a). Such adsorption phenomena are not easily corrected for by 'blank' determinations made in the absence of protein, for the presence of protein can significantly affect the degree of binding to the dialysis membrane (Steinhardt and Reynolds, 1969). In such instances adsorption problems can generally be minimised by careful preparation of the membrane prior to use (e.g. boiling in water, pre-soaking in de-ionized water, etc.). Opinion seems varied as to which is the most suitable preparation. Adsorption effects may possibly be overcome by the use of different types of membrane (e.g. cellulose acetate, polyamide), although little guidance is available from the literature to aid the selection of an alternative membrane to the commonly used regenerated cellulose type. Where adsorption
phenomena cannot be reduced by pretreatment of the dialysis membrane, techniques not employing membranes should be investigated (e.g. frontal analysis, ultracentrifugation). It is regrettable that no universally applicable technique appears to be available, and the best available approach may well involve a combination of methods.
Comparison of Salicylate Binding to Rat Serum by In Vitro and In Vivo Techniques

The results presented (Table III, 9) were obtained after rats were orally dosed, daily for eight days with salicylate at either 10 or 20 mg/Kg body weight. Implantation, and removal of dialysis sacs was performed 4 hours after dosing. Despite the differences in total, unbound (dialysis sac concentration) and bound salicylate concentrations apparent at these two dosage levels, no difference in the percentage bound was detectable. Although the determined serum albumin concentrations showed individual variation, no significant effect of the salicylate on albumin level was apparent. The presence of salicylate did not appear to interfere with the estimation of albumin by the bromocresol green method.

Binding was also determined by in vitro ultrafiltration using normal rat serum to which salicylate had been added to give a similar range of concentrations to that attained in the in vivo experiment. (Table III, 10). A comparison of the binding of salicylate to rat serum by the in vivo and in vitro techniques showed good agreement (Tables III, 9 and 10, and Figs. III, 7a,b). Calculated \( r \) values (moles salicylate bound/mole albumin), which correct for the small individual differences in albumin concentration, were well correlated for both techniques when plotted against either total or unbound salicylate concentration (Figs. III, 8a,b). The results would therefore suggest that in vitro ultrafiltration gives a good reflection of the in vivo situation for salicylate. However under the dosage regime employed, only a narrow concentration range of total serum salicylate was achieved, such good correlation might not pertain at higher serum salicylate concentrations. In practice higher serum concentrations may be difficult to achieve without the concomitant problem of toxic side effects.

Serum salicylate concentrations for rats on the above dosage regime were
determined over a period of 24 hours (days 7-8, Table III, 11; Figs. III, 9a,b). Maximum serum concentrations were invariably reached within two hours of oral dosing, and although they slowly decayed over several hours, at the end of 24 hours concentrations were low. A wide inter-animal variation in both maximum concentrations and half-lives was apparent (Table III, 11). Half-lives were calculated assuming first-order kinetics (Bedford, et al., 1965, Wagner, 1967; Cummings and Martin, 1968). From the nature of the salicylate concentration-time curves in serum, it would appear that changes in the dosage regime had little effect in reducing the rate of elimination and it is unlikely that a 'steady-state' serum salicylate concentration was obtained. Although McQueen (1968) reported that sulphormethoxine attained a stable serum concentration, the majority of drugs appear to have shorter half-lives than that for sulphormethoxine (i.e. approximately 30 hours; in man Kruger-Theimer, 1962; Reider, 1963). If the attainment of a 'steady-state' plasma or serum drug concentration is a necessary prerequisite, then unless suitable dosage regimes can be employed, the application of this technique may well be limited. However, the good correlation obtained for the binding of salicylate by this technique with that of 'in vitro' studies, may suggest that a stable serum concentration, although desirable, is not essential. It may be that the main criteria required is one of a slowly changing serum concentration at the time of sac removal. This time should be sufficient to allow equilibration of serum and peritoneal fluid unbound drug concentrations, as well as attainment of equilibrium with the contents of the dialysis sac. (Hertzler, 1919; Putnam, 1922; Cunningham, 1926; Avey, 1963; Mattocks and El-Bassiouni, 1971). If this technique is to be widely used, information of the relative rates at which equilibration is achieved between blood and peritoneal fluid and the peritoneal fluid and dialysis sac contents are essential. In view of the widespread use of peritoneal dialysis for the treatment of kidney failure and for removal of dialysable exogenous and endogenous poisons, it is surprising that more
attention has not been paid to the use of peritoneal fluid in reflecting unbound drug concentrations in in vivo binding studies.

The serum and dialysis sac contents obtained from the in vivo binding study were also assayed for tryptophan (Fig. III, 10). At both salicylate dosage levels (i.e. 10 and 20 mg/Kg daily) reduction in total and bound serum tryptophan occurred, but only in the case of the unbound tryptophan concentration, was there any significant difference between the two salicylate dose levels. The nature of these changes was such that only the lower salicylate concentration showed a reduction in percentage tryptophan bound. The results have shown that the presence in vivo of salicylate has significantly reduced the binding capacity of albumin for tryptophan. The lack of a detectable increase in free tryptophan may be due to alterations in the rates of metabolism or excretion of tryptophan. The differences in the free tryptophan level between the two dosage levels of salicylate, may suggest the importance of salicylate concentration in affecting the metabolic fate of tryptophan. Although this remains to be verified, it may be of relevance in clinical conditions of rheumatoid arthritis, where prolonged salicylate treatment is required.

This study has therefore served as a further indication of the potential of in vivo dialysis in animal studies on protein binding and related displacement phenomena. Clearly this technique could have considerable potential in the study of the effects on protein binding and drug interactions on pharmacokinetics. Especially if, as suggested by McQueen (1968), the drug concentration level in the dialysis sac located in the peritoneal cavity reflects tissue fluid levels. It should be noted however, that Keen (1970) has argued this is not the case because the effective diameter of the pores in the dialysis tubing are very much less than that of capillary pores (Pappenheimer, 1953; Craig, et al., 1957). The potential of the in vivo dialysis method, would also be considerably extended if regular or
continual sampling of the contents of the dialysis sac during experimentation could be achieved. Theoretically there seems no reason why this should not be possible.

In summary, the techniques of equilibrium dialysis, ultrafiltration and frontal analysis have been shown to give comparable binding data for a number of drugs differing markedly in their physico-chemical properties. Comparison of the binding of salicylate to rat serum by in vivo dialysis and in vitro ultrafiltration would suggest that these in vitro techniques give a good reflection of the binding in vivo. The potential of the in vivo dialysis technique in studying the effect of protein binding on drug action is suggested.
CHAPTER FOUR

ALIPHATIC CARBAMATE-ALBUMIN INTERACTIONS: A POSSIBLE HYDROPHOBIC BONDING MODEL
1. Introduction

The determination of the protein binding of a series of structurally related compounds is a potentially valuable approach towards identifying functional groups and forces involved in the small molecule-protein binding process.

The binding of a homologous series of alkyl carbamates to bovine serum albumin was investigated in order to determine the significance of lipophilic character in small molecule-albumin interactions. Ultrafiltration experiments were performed at both 37°C and 10°C to allow calculation of the thermodynamic parameters for the carbamate-albumin interaction. The alkyl carbamates studied had the general structure: R-0-CO-NH₂, where R ranged from methyl to n-hexyl. The following were also included: t-butyl, t-pentyl, t-hexyl, i-butyl and benzyl, to enable the effect of branched chain and aromatic substituents on the binding to BSA to be evaluated. Carbamates were selected as appropriate model compounds because they are; stable, readily synthesised, purified and assayed, not ionized in aqueous solution and show a progressive increase in lipophilicity from methyl to n-hexyl. They are thus, very suitable model compounds, for a study of the role of the hydrophobic bonding in albumin-small molecule interactions. The protein binding of these short chain alkyl carbamates had not previously been reported, although Douglas, et al.,(1964) and van der Kleijn (1969), have investigated the albumin binding of some dicarbamates.

To extend the scope of this study, the interaction of the aliphatic acetates, butyl and pentyl to BSA was also examined. Their molecular dimensions are similar to butyl and pentyl carbamates, respectively, but the
lipid solubilities of the acetates, as determined by partition coefficient, are considerably higher. It was hoped that a comparison of their binding, with the corresponding carbamates, would assist in interpreting the respective roles of lipophilic character and molecular dimensional parameters, in the binding process.

The possibility that the carbamate-albumin interaction, might induce conformational changes in the albumin structure was also investigated by ultraviolet (UV) difference spectra (Herskovits, 1967; Polet and Steinhardt, 1968) and fluorescence studies.

2. Experimental Approach

All ultrafiltration studies of the carbamate-BSA (Cohn fraction V) binding were performed at both 37° and 10° C, while acetate binding studies were only determined at 10° C. Because of the problems associated with the low solubility it was not possible to use concentrations of carbamate in excess of 10 mM, or to use straight chain homologues higher than n-hexyl carbamate. The detection limit of the GLC assay dictated that starting concentrations of carbamate lower than 1 mM, could not be used for the binding studies. In addition to their low solubility, the acetates were too volatile to be satisfactorily studied at 37° C, therefore the binding of butyl and pentyl acetates was only measured at 10° C. The centrifuge tubes were routinely capped during the ultrafiltration to prevent losses by evaporation.

In the preliminary studies the concentration inside the bag after ultrafiltration, was also determined. This was discontinued as the presence of protein tended to deteriorate the performance of the GLC column used for carbamate assay. Controls in the presence of buffer (0.15 M phosphate), were routinely run, however, no significant binding to the dialysis membrane was
apparent for any of the compounds studied.

Ultraviolet difference spectra and fluorescence studies of the carbamate-albumin interaction were performed as described in Chapter Two. For these studies BSA solutions ($2 \times 10^{-5}$M in either 0.15M phosphate or tris-HCl buffer, pH 7.4) were titrated with successive volumes ($\mu$l.) of carbamate solution (10mM in appropriate buffer).
3. **Results**
Table IV,1.

Relationship between the Percentage Carbamate Bound to Bovine Serum Albumin\textsuperscript{a} and Apparent Partition Coefficient for the series R-O-CO-NH\textsubscript{2}

<table>
<thead>
<tr>
<th>R Group</th>
<th>Apparent Octanol/Buffer Partition Coefficient</th>
<th>% Bound\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl</td>
<td>0.22</td>
<td>5.47 ±0.15</td>
</tr>
<tr>
<td>Ethyl</td>
<td>0.70</td>
<td>10.47 ±0.15</td>
</tr>
<tr>
<td>n-Propyl</td>
<td>2.30</td>
<td>19.03 ±0.13</td>
</tr>
<tr>
<td>n-Butyl</td>
<td>7.10</td>
<td>33.2 ±1.70</td>
</tr>
<tr>
<td>n-Pentyl</td>
<td>22.50</td>
<td>41.80 ±1.33</td>
</tr>
<tr>
<td>n-Hexyl</td>
<td>70.80</td>
<td>64.33 ±0.33</td>
</tr>
<tr>
<td>t-Butyl</td>
<td>3.00</td>
<td>16.93 ±0.87</td>
</tr>
<tr>
<td>t-Pentyl</td>
<td>8.72</td>
<td>25.33 ±0.88</td>
</tr>
<tr>
<td>t-Hexyl</td>
<td>27.5</td>
<td>30.67 ±0.93</td>
</tr>
<tr>
<td>i-Butyl</td>
<td>4.36</td>
<td>29.17 ±0.60</td>
</tr>
<tr>
<td>Benzyl</td>
<td>17.60</td>
<td>45.53 ±0.37</td>
</tr>
</tbody>
</table>

\textsuperscript{a}0.29 \times 10^{-3} \text{ M BSA in 0.15 M phosphate buffer (pH 7.4)}

\textsuperscript{b} Mean ± S.E.M. of at least 3 determinations, using 1 \times 10^{-3} \text{ M carbamate solutions}

Ultrafiltration and partition coefficient determination performed at 37°C (pH 7.4).
The Relationship between Percentage Carbamate Bound to Bovine Serum Albumin and Apparent Partition Coefficient for the series R-O-CO-NH₂

Eqn. of regression line: \[ \log(\text{% bound}) = 0.40 \pm 0.12 \log P + 1.08 \pm 0.12; \]
\[ n = 11 \text{ (no. data points); } r = 0.96 \text{ (corr. coefficient); } s = 0.27 \text{ (S.D.)} \]
Figures in parentheses are S.D. of constants.
Scatchard Plots of Data for the Binding of Carbamates to Bovine Serum Albumin

Ultrafiltration at 37°C, each point represents mean of 3 determinations

Fig. IV.2
Methyl Carbamate
Regression Correlation Coefficient = 0.80

Fig. IV.3
Propyl Carbamate
Regression Correlation Coefficient = 0.84
Scatchard Plots of Data for the Binding of Carbamates to Bovine Serum Albumin

Ultrafiltration at 37°C, each point represents mean of 3 determinations

Fig. IV, 4.
t-Hexyl Carbamate

Correlation Coefficient = 0.76

Fig. IV, 5.
Hexyl Carbamate

Correlation Coefficient = 0.83
Fig. IV, 6  Scatchard Plot of Data for Binding of Benzyl Carbamate to Bovine Serum Albumin

Temp = 37°

Each point represents mean of 3 determinations

Regression Correlation Coefficient = 0.97
Table IV, 2.

Binding Data for the Interaction of Aliphatic and Aromatic Carbamates with Bovine Serum Albumin at 37°C

<table>
<thead>
<tr>
<th>Carbamate</th>
<th>$k \times 10^3 \text{M}^{-1}$</th>
<th>$n$</th>
<th>$nk \times 10^3 \text{M}^{-1}$</th>
<th>Regression Correlation Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl</td>
<td>0.18 ±0.03</td>
<td>1.28</td>
<td>0.23 ±0.01</td>
<td>0.80</td>
</tr>
<tr>
<td>Ethyl</td>
<td>0.31 ±0.05</td>
<td>1.74</td>
<td>0.53 ±0.03</td>
<td>0.93</td>
</tr>
<tr>
<td>n-Propyl</td>
<td>0.13 ±0.02</td>
<td>6.69</td>
<td>0.87 ±0.04</td>
<td>0.84</td>
</tr>
<tr>
<td>n-Butyl</td>
<td>0.47 ±0.16</td>
<td>5.60</td>
<td>2.63 ±0.82</td>
<td>0.55</td>
</tr>
<tr>
<td>n-Pentyl</td>
<td>0.85 ±0.13</td>
<td>4.40</td>
<td>3.74 ±0.17</td>
<td>0.85</td>
</tr>
<tr>
<td>n-Hexyl</td>
<td>5.80 ±0.89</td>
<td>3.30</td>
<td>19.14 ±0.88</td>
<td>0.83</td>
</tr>
<tr>
<td>t-Butyl</td>
<td>0.16 ±0.04</td>
<td>4.38</td>
<td>0.70 ±0.07</td>
<td>0.62</td>
</tr>
<tr>
<td>t-Pentyl</td>
<td>0.11 ±0.04</td>
<td>9.82</td>
<td>1.08 ±0.11</td>
<td>0.61</td>
</tr>
<tr>
<td>t-Hexyl</td>
<td>0.49 ±0.10</td>
<td>4.27</td>
<td>2.09 ±0.20</td>
<td>0.76</td>
</tr>
<tr>
<td>i-Butyl</td>
<td>0.56 ±0.11</td>
<td>3.66</td>
<td>2.05 ±0.23</td>
<td>0.75</td>
</tr>
<tr>
<td>Benzyl</td>
<td>0.78 ±0.12</td>
<td>5.20</td>
<td>4.06 ±0.51</td>
<td>0.97</td>
</tr>
</tbody>
</table>

a  Mean ± S.E.M., determined by regression analysis of a minimum of 21 data points

n  Number of binding sites

k  Apparent association constant

nk  Scatchard plot intercept at $r = 0$, termed here the 'apparent binding constant'
The Relationship between Carbamate Association Constants \( (k) \) for Bovine Serum Albumin and Apparent Partition Coefficient for the series \( R-O-CO-NH_2 \)

Regression line drawn for st. chain homologues \( R = C_3 - C_6 \)

Regression eqn:

\[
\log(k) = 1.05 (\pm 0.31) \log P + 1.71 (\pm 0.38)
\]

\( n = 4; \ r = 0.98; \ s = 0.27 \)

Figures in parentheses are S.D. of constants
Fig. IV, 8.

The Relationship between Carbamate Binding Constants (nk) for Bovine Serum Albumin and Apparent Partition Coefficient for the series R-O-CO-NH₂

Log (nk) = 0.65 (± 0.32) Log P + 2.73 (± 0.26)

n = 11; r = 0.915; s = 0.66

Figures in parentheses are S.D. of constants.
Table IV, 3.

Binding Data for the Interaction of Straight Chain Aliphatic Carbamate
with Bovine Serum Albumin at 10\(^{\circ}\) C

<table>
<thead>
<tr>
<th>Carbamate</th>
<th>% bound</th>
<th>k \times 10^3 M^{-1}</th>
<th>n</th>
<th>nk \times 10^3 M^{-1}</th>
<th>Regression Correlation Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl</td>
<td>14.0</td>
<td>0.34</td>
<td>2.26</td>
<td>0.77</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td>±0.30</td>
<td>±0.12</td>
<td>±0.59</td>
<td>±0.13</td>
<td></td>
</tr>
<tr>
<td>Butyl</td>
<td>49.2</td>
<td>0.70</td>
<td>6.42</td>
<td>4.49</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>±0.40</td>
<td>±0.05</td>
<td>±0.40</td>
<td>±0.23</td>
<td></td>
</tr>
<tr>
<td>n-Pentyl</td>
<td>68.3</td>
<td>1.28</td>
<td>6.74</td>
<td>8.63</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>±0.80</td>
<td>±0.16</td>
<td>±0.56</td>
<td>±0.59</td>
<td></td>
</tr>
<tr>
<td>n-Hexyl</td>
<td>86.8</td>
<td>6.68</td>
<td>6.72</td>
<td>39.67</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>±0.8</td>
<td>±0.52</td>
<td>±0.42</td>
<td>±1.85</td>
<td></td>
</tr>
</tbody>
</table>

- a 0.29 \times 10^{-3} M BSA in 0.15M phosphate buffer (pH 7.4)
- b Mean ± S.E.M. of 3 determinations, using 1 \times 10^{-3}M carbamate solutions
- c Mean ± S.E.M., determined by regression analysis of a minimum of 21 data points
### Table IV, 4.

**Thermodynamic Constants of the association of carbamates with Bovine Serum Albumin**

<table>
<thead>
<tr>
<th>Carbamate</th>
<th>Free Energy Change $\Delta G$ Kcal/mole</th>
<th>Enthalpy Change $\Delta H$ Kcal/mole</th>
<th>Entropy Change $\Delta S$ (e.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$37^\circ$</td>
<td>$10^\circ$</td>
<td>$37^\circ$</td>
</tr>
<tr>
<td>Methyl</td>
<td>-3.19</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ethyl</td>
<td>-3.52</td>
<td>-3.27</td>
<td>-0.59</td>
</tr>
<tr>
<td>n-Propyl</td>
<td>-3.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>n-Butyl</td>
<td>-3.78</td>
<td>-3.67</td>
<td>-2.56</td>
</tr>
<tr>
<td>n-Pentyl</td>
<td>-4.14</td>
<td>-4.01</td>
<td>-2.63</td>
</tr>
<tr>
<td>n-Hexyl</td>
<td>-5.32</td>
<td>-4.94</td>
<td>-0.91</td>
</tr>
<tr>
<td>t-Butyl</td>
<td>-3.12</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>t-Pentyl</td>
<td>-2.89</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>t-Hexyl</td>
<td>-3.80</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>i-Butyl</td>
<td>-3.90</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Benzyl</td>
<td>-4.09</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>R</td>
<td>Carboxylate R-O-CO-NH₂</td>
<td>Butyl</td>
<td>% Bound Bound b</td>
</tr>
<tr>
<td>-------</td>
<td>------------------------</td>
<td>-------</td>
<td>-----------------</td>
</tr>
<tr>
<td></td>
<td>Acetate R-O-CO-CH₃</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>39.4 ± 1.0</td>
<td>45.7</td>
<td>53.7 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>49.2 ± 1.2</td>
<td>62.3</td>
<td>170.0 ± 0.9</td>
</tr>
</tbody>
</table>

a 0.29 x 10⁻⁸ M BSA in 0.15M phosphate buffer (pH 7.4)
b Using 2.5 x 10⁻³ M carbamate and acetate solutions
c Mean ± S.E.M. % 4 determinations, ultrafiltration performed at 10°C
d Determined at 37°C
e Theoretical values (Hansch and Dunn, 1972)
Table IV, 6.

<table>
<thead>
<tr>
<th>Alkyl Group R</th>
<th>Carboxylic Acids a</th>
<th></th>
<th>Carbamates b</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R - CH₂ - COOH</td>
<td>k x10³ M⁻¹</td>
<td>n</td>
<td>ΔG Kcal/mole</td>
</tr>
<tr>
<td>Propyl</td>
<td></td>
<td>0.42</td>
<td>5.0</td>
<td>- 3.56</td>
</tr>
<tr>
<td>Butyl</td>
<td></td>
<td>0.56</td>
<td>4.5</td>
<td>- 3.73</td>
</tr>
<tr>
<td>Pentyl</td>
<td></td>
<td>1.55</td>
<td>4.7</td>
<td>- 4.32</td>
</tr>
<tr>
<td>Hexyl</td>
<td></td>
<td>6.45</td>
<td>4.2</td>
<td>- 5.16</td>
</tr>
</tbody>
</table>

a Data from Teresi and Luck (1952), equilibrium dialysis experiments performed at 23° C using crystalline BSA.

b Experiments performed at 37° C using BSA (Cohn Fraction V)

c = Apparent octanol-buffer partition coefficient at 37° C
Fig. IV, 9. Fluorescence Spectra of Bovine Serum Albumin in the Presence of the Aliphatic Carbamates

--- Emission maxn. 340nm obtained with BSA alone and BSA plus methyl → butyl, or benzyl carbamates

--- Emission maxn. 336nm obtained with BSA plus pentyl or hexyl carbamates

Excitation wavelength, 290nm
BSA concn $2 \times 10^{-5} \text{ M}$ in 0.1M Phosphate buffer, pH 7.4

Carbamate/Albumin ratio: 3.5 - 35
Table IV, 7

Interatomic Distances for the Carbamate Series

<table>
<thead>
<tr>
<th>R Group</th>
<th>Approx. Interatomic Distance (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl</td>
<td>5.25</td>
</tr>
<tr>
<td>Ethyl</td>
<td>6.57</td>
</tr>
<tr>
<td>Propyl</td>
<td>7.89</td>
</tr>
<tr>
<td>n-Butyl</td>
<td>9.21</td>
</tr>
<tr>
<td>n-Pentyl</td>
<td>10.53</td>
</tr>
<tr>
<td>n-Hexyl</td>
<td>13.74</td>
</tr>
<tr>
<td>Benzyl</td>
<td>9.55</td>
</tr>
</tbody>
</table>

a Interatomic distances calculated assuming:-

\[
\begin{align*}
\text{from data in Korolkovas (1970)}
\end{align*}
\]
4. Discussion

The series of carbamate homologues demonstrated a regular increase in lipophilicity, as measured by their octanol/buffer partition coefficients. The increase in log P (logarithm of partition coefficient) or Hansch II constant (Hansch and Fujita, 1964), for the addition of a methylene group was found to be 0.5, which was reduced to 0.4 and 0.2 respectively, when primary and secondary branching occurred. The aromatic ring of benzyl carbamate was found to possess approximately the same lipophilicity as the n-butyl group. These values are in good agreement with theoretical values (Hansch and Dunn, 1972). The regular increase in lipophilicity for the series demonstrated that either no significant inter- or intramolecular carbamate associations were involved or that they were constant for the series, since such effects would detract from their linear relationship (see Materials and Methods, Chapter Two).

For the binding of ImM carbamate solutions to BSA (0.29mM, Cohn Fraction V), an increase in the percentage bound was found to accompany an increase in chain length (Table IV, 1). When plotted as logarithmic functions the percentage bound correlated well with the apparent partition coefficient for the series (Fig. IV, 1, regression correlation coefficient, $r = 0.96$). It would appear, at least under the experimental conditions used, that this correlation is also valid for the branched chain and aromatic carbamates. It is important that even the ImM carbamate concentration (the lowest concentration which could be reliably detected) represents a relatively high carbamate/albumin molar ratio (3.45:1), a factor predisposed to lower percentage bound.

The binding of the carbamate series to BSA was investigated over the concentration range (1 - 7.5mM), and the data evaluated by the method of Scatchard (Scatchard, 1949). A typical Scatchard plot for the binding of
the carbamates: methyl, propyl, t-hexyl, hexyl and benzyl is shown (Fig IV, 2-6). For all the carbamate studies, except hexyl, a straight line plot was obtained, but for hexyl carbamate the plot was more difficult to interpret. Graphical presentation of the data (Fig. IV, 5) implied curvature possibly indicative of more than one class of binding site. On evaluation of this data by non-linear regression computer analysis, however, it could not be clearly resolved into more than one line. This difference probably resulted from the unequal distribution of the experimental data, that is the lack of experimental data at high r values (moles carbamate bound/mole albumin). The presence of more than one class of binding site could, therefore, not be verified. In view of this, the computer resolved, single regression line was used for the determination of binding parameters.

However, if the Scatchard plot is truly non-linear it could be attributable to either the presence of more than one distinct class of binding site or to negative cooperativity effects (Conway and Koshland, 1968; Laiken and Nemethy, 1970a). This negative cooperativity could be due to either protein conformational changes which may occur on binding, or the binding of the initial carbamate molecules interfering, possibly by steric repulsion phenomena, with the binding of subsequent molecules.

In Table IV, 2, are presented the mean values obtained for the apparent association constant (k), number of binding sites (n), nk (the intercept value, on the Scatchard plot at r = 0, termed here the apparent binding constant) and the regression correlation coefficients. It can be seen that for the straight chain alkyl carbamates an increase in the term nk accompanied an increase in chain length for the series. On closer analysis the data revealed important differences in the number of binding sites (n), and the apparent association constant (k), between methyl and ethyl
compared with the higher homologues propyl, butyl, pentyl and hexyl. For the homologues propyl to hexyl, an increase in $k$ and a corresponding decrease in $n$, accompanied incremental changes in chain length. However for methyl and ethyl carbamate, the $n$ values were found to be considerably lower than for the longer chain length homologues, while the $k$ values corresponded closely to propyl and butyl carbamate respectively. Such differences could not be explained by experimental error. For the branched chain tertiary carbamates (Table IV, 2) a progressive increase in $nk$ and $k$ values accompanied an increase in chain length.

A plot of log. $k$ (association constant) against log. $P$ (partition coefficient) for propyl n-butyl, n-pentyl and n-hexyl indicated a good correlation for these homologues. Benzyl and t-butyl carbamates also correlated well with this line, but methyl, ethyl, i-butyl, t-pentyl and t-hexyl carbamates did not (Fig. IV, 7). The anomalous position of methyl and ethyl carbamates is well demonstrated, both showed much higher association constants than was expected from their partition coefficients. A plot of the apparent binding constant ($nk$) against partition coefficient, however, produced a good correlation (Fig. IV, 8), even when methyl and ethyl carbamates were included. While a plot of association constant ($k$) against partition coefficient did not indicate such a relationship (cf. Figs. IV, 7 and 8). The importance of the term $n$ is clearly demonstrated. The poor correlation of the association constant with lipophilicity, and the variation in the number of binding sites would suggest that the nature of the carbamate-albumin interaction is non-specific. For the relationship between $nk$ and partition coefficient, the tertiary carbamates showed a small divergence from the regression line, whereas i-butyl carbamate was well correlated. Whether this divergence for the tertiary carbamates was significant could not be verified. The role of steric factors in the carbamate-albumin interaction although perhaps indicated, could not be
shown conclusively, but in support of this possibility Arvidsson et al. (1971), have demonstrated reduced association constant values for branched chain members of a series of long chain carboxylic acids.

It is important to note that whichever binding parameter was selected (i.e. percentage bound, k and nk) benzyl carbamate correlated well with that expected from its partition coefficient. The contribution of the aromatic ring would therefore appear to be largely one of an enhanced lipophilicity. The above good correlations of binding parameters with lipophilic character would strongly suggest that the carbamate-BSA interaction is hydrophobic in nature. In order to substantiate this, binding experiments at 10° C were performed for several carbamates; ethyl, pentyl, butyl and hexyl. An increase in the binding parameters (percentage bound, k, nk, and n) was observed at the lower temperature (Table IV,3). A similar increased value for the binding parameters (n and nk) has been reported for a series of p-hydroxybenzoic acid esters (Patel, et al., 1968). A slightly increased curvature of the Scatchard plots for both pentyl and hexyl was observed, however, the significance of this cannot be deduced.

The thermodynamic parameters, free energy $\Delta G$; enthalpy $\Delta H$; and entropy $\Delta S$, were calculated (Daniel and Alberty, 1966) (Table IV,4). The free energy ($\Delta G$) and enthalpy ($\Delta H$) changes accompanying the binding were negative in all cases, indicating a favourable spontaneous exothermic interaction. The positive entropy ($\Delta S$) changes associated with the binding are compatible with an interaction involving hydrophobic bonding. Such changes have been ascribed to the disruption of the 'ice-berg' structure of water, around the hydrocarbon groups in aqueous solution (Frank and Evans, 1945; Kauzmann, 1959; Schachman, 1963; Molyneux and Frank, 1969). Alternatively the binding could involve interaction of the carbamate with partial clathrate cages surrounding the apolar residues on the albumin surface (Klotz, 1958). This would not seem unlikely however, for binding by the clathrate mechanism would probably require a substantial negative enthalpy, whereas the enthalpy values found for the
carbamate binding, although negative, were relatively small.

As a result of the carbamate-albumin interaction increased rotational freedom of the carbamate and/or albumin, conformational changes in the albumin and/or carbamate, and increased translational freedom in the albumin might occur. Such affects would be consistent with the increased entropy (Aranow and Witten, 1960; Steinberg and Scheraga, 1963; O'Reilly, 1969).

Hydrogen bonding has been stressed as a major factor determining the values of the physical properties of the insecticidal carbamates (Hadaway, et al., 1970). However, it is probably not a major determinant for the carbamate-albumin interaction, for if this were the case the lower carbamates would be expected to bind disproportionately well in the series studied. If the binding of the aliphatic group to BSA is primarily determined by lipid solubility then more lipophilic compounds with similar molecular dimensions should show an enhanced binding. Replacement of the amino group in carbamates, by a methyl group as in acetates would be expected to increase lipid solubility without significantly altering molecular dimensions. Similar chain length acetates should therefore result in an increased binding compared with the corresponding carbamates because of their higher lipid solubility. A comparison of the binding of butyl and pentyl acetates with butyl and pentyl carbamates, showed this to be the case (Table IV,5), although the percentage bound values for the acetates were not as high as would have been predicted from their partition coefficients. These results further implicate the importance of lipophilic character in albumin binding. The possibility of a contributary role of molecular dimensional parameters in hydrophobic bonding must await further investigation.

The apparent association constants found for the carbamates are of similar magnitude (i.e. $x 10^3 M^{-1}$) to those reported for other short chain neutral compounds (Steinhardt and Reynolds, 1969).
The hydrophobic binding regions responsible for carbamate interactions may involve apolar groups or cluster of groups on the surface or in crevices of the protein (Steinhardt and Reynolds, 1969; Tanford, 1972). Hydrophobic interactions may also occur in the interior of the protein. Possible amino acids involved at these binding sites could be: alanine, leucine, phenylalanine, tryptophan, isoleucine, methionine, proline and cysteine.

The low n values (number of binding sites) for methyl and ethyl carbamate do not obey the relationship apparent for the higher straight chain homologues. It is possible that the very low percentage binding for methyl and ethyl might be a factor influencing their anomalously low n values. Binding studies using considerably higher albumin concentrations might clarify this. However, other hypotheses can also be proposed: it might be that the binding sites for methyl and ethyl are located in different regions of the protein from the binding sites for the higher homologues. Their hydrophilic-lipophilic balance could, for example, favour interaction with binding sites on the protein surface, in contact with the aqueous phase. Whereas the higher homologues might penetrate into the protein interior, or surface crevices. Alternatively, small hydrophobic regions which limit binding of chain lengths in excess of ethyl carbamate, could exist, according to Tanford (1972) this is, however, unlikely. Competitive studies should resolve whether methyl and ethyl and the higher analogues share common binding sites.

The observed decrease in the number of binding sites from propyl to hexyl carbamate may imply a common binding region of the protein for these homologues. An increase in the hydrocarbon chain length has been previously postulated, on theoretical grounds, to accompany a decrease in the number of binding sites for aliphatic compounds on albumin, (Steinhardt and Reynolds, 1969). It has been suggested that not all the carbon atoms in the hydrocarbon chain of a compound needs to be involved in its protein binding, and even contiguous methylene groups may not necessarily interact (Laiken and Nemethy,
1970a, b; 1971; Tanford, 1972). However, it remains to be demonstrated whether all or only part of the carbamate-hydrocarbon chain is involved in their interaction with BSA. It is possible that the binding interactions identified by the n values may not represent specific classes of binding site, alternatively they may reflect binding areas constituting several types of sites, (Laiken and Nemethy, 1970a, b; 1971).

The relationship between the number of binding sites (n) for the carbamates propyl and hexyl, and chain length differs in some respects from that reported for similar chain length alkyl carboxylic acids (Teresi and Luck, 1952, Table IV, 6). The number of binding sites for these short chain carboxylic acids showed insignificant variation with chain length. Although this contrasts with the relationship found for the carbamates, some similarities in n values for the two series are apparent. The reason for the different relationship between the number of binding sites and chain length, for the two series is unclear. However, it is of interest to note that for long chain anions e.g. dodecyl sulphate, the n value increased with increase of chain length (Ray et al., 1966; Steinhardt and Reynolds, 1969). It would also appear that the presence of an ionizable carboxyl group had little effect on either the association constant (k) or the free energy (ΔG) of interaction. (Table IV, 6). The binding of these short chain carboxylic acids would therefore appear to be largely governed by the lipophilic character of the side chain. It remains to be determined whether these short chain carboxylic acids and carbamates bind to the same site(s) on albumin.
The Effect of Carbamate Binding on Albumin Difference Spectra and Fluorescence

Wetlaufer and Lovrien, (1964) have suggested that a protein interacting with a hydrocarbon should show evidence of structural change (optical rotation, uv difference spectra etc.) if the binding takes place in the interior of the protein, but not if only the surface is involved. Ivkova, et al., (1971) have shown that while the binding of iso-butanol to the interior of BSA produced marked conformational changes as determined by fluorescence, binding to the surface of the protein did not.

The possibility that observed differences in n values for methyl and ethyl and the higher homologues, might represent differences in the location of the binding site (e.g. surface or interior of the protein) was studied. Possible induced conformational changes were studied by the techniques of uv difference spectrophotometry and fluorimetry.

No alterations in the uv difference spectra of the albumin was apparent when it was titrated with any of the carbamates, despite using a wider range of carbamate/albumin molar ratio than that used in the ultrafiltration study (3.5 - 35 molecules carbamate/albumin). The lack of observable perturbations of the albumin uv difference spectra on addition of the carbamates would imply that either conformational changes within the protein did not occur, or that they were so slight as not to be detected by this technique. It may be relevant that in most studies where perturbations have been demonstrated with this technique, high concentrations of long chain highly bound anionic compounds (r(moles bound/mole albumin)>5) have been necessary. Few studies with neutral compounds appear to have been performed (Ray, et al., 1966). Although Polet and Steinhardt (1968) have shown that octanol at high r values produced similar effects on the phenylalanine and histidine residues to those produced by the long chain anions.
No quenching of albumin fluorescence was observed with any of the carbamate homologues. However for homologues higher than butyl a 4nm shift to a lower emission maximum (blue shift) was observed (Fig. IV, 9). Blue spectral shifts in the emission wavelength have also been reported for the binding of other compounds to BSA (Lenz, 1969; Lenz and Steinhardt, 1969; Halfman and Nishida, 1971, Steinhardt et al., 1971). Spector and John (1968), showed that the binding of some carboxylic acids to BSA also produced blue shifts of the fluorescence emission. They found that for the homologous series up to caproic acid no blue shift could be detected, although the later compound did produce slight quenching. For higher analogues, however, blue shifts of approximately 4nm were obtained. These higher analogues also showed increased fluorescence quenching. After caproic acid further increase in chain length did not produce increased quenching. It is important to note that Spector and John (1968), used an excitation wavelength of 280 nm in their study. At this wavelength the emission spectra will contain a significant contribution from the amino acid tyrosine, as well as the major contribution from the amino acid tryptophan. In our carbamate studies, an excitation wavelength of 290nm, the maximum excitation wavelength for the protein, was used. A small contribution from tyrosine may occur under these conditions.

Halfman and Nishida (1971) who investigated the binding of dodecyl sulphate to BSA, suggested that the fluorescence alterations in BSA proceeded in two stages. The binding of the first four equivalents resulted in tryptophan quenching, whilst subsequent additions produced blue shifts in the tryptophan emission and increased fluorescence intensity. They suggested that quenching was probably due to the influence of binding on the state of ionization of ε-amino groups in the immediate vicinity of a tryptophan. Whilst the blue shift and enhanced fluorescence intensity was caused by an increase in the hydrophobic environment of the tryptophan residue on binding. Little influence on tyrosine occurred. Steinhardt et al., (1971) obtained similar findings and showed that
the degree of blue shift for these compounds increased with \( r \) (moles bound/mole albumin). They also showed that the binding of hexyl sulphate to HSA and octyl sulphate to BSA produced negligible effects on tryptophan and tyrosine fluorescence intensity, although blue shifts in emission wavelength did occur. The degree of blue wavelength shifts for these small chain anions was less than for the long chain. Few studies have been performed on the effects of neutral compounds on albumin fluorescence (Ivkova, et al., 1971).

The spectral shifts obtained for the carbamates could possibly occur by similar mechanisms to those produced by long chain alkyl anions and/or fatty acids. However anomalies such as lack of fluorescence quenching and enhancement effects have to be explained. For if the blue shift, apparent for the carbamates higher than butyl, is due to either similar environmental or conformational changes around the tryptophan or tyrosine residues, then effects on fluorescence intensity would have been expected.

**Carbamate-Albumin Interaction: a possible model to account for the fluorescence observations**

Steinhardt et al., (1971), have suggested a model for the observed spectral shifts in both \( uv \) difference and fluorescence spectra with long chain alkyl anions. Modification of this theory would account for most of the effects produced by the carbamates.

It is suggested that the blue shift is a result of the carbamates (pentyl and hexyl) binding to a hydrophobic region with one site sufficiently near to an exposed tryptophan for the hydrocarbon tail to cover the tryptophan. If this is the case then for the carbamate structure \( R-O-CO-NH_2 \) an \( R \) chain length of five methylene groups (approx. 5.28\( \AA \)) is required to cover the tryptophan. That benzyl carbamate does not produce the blue shift would tend to support this (approx. 4.30 A, Table IV, 7). An alternative explanation
might be that the lower homologues bind to different hydrophobic regions on the protein, from pentyl and hexyl carbamate. While the lower homologues may bind to surface sites on the protein, the binding of pentyl and hexyl carbamate possibly entails interaction with sites embedded in the protein structure. Surface binding would not be expected to effect tryptophan fluorescence (Ivkova, et al., 1971). This may explain the anomalously low n values (number of binding sites) for methyl and ethyl carbamate, but it would not appear to account for the correlation between the n and k values for propyl, butyl and benzyl, and those of pentyl and hexyl.

To account for the lack of fluorescence enhancement on binding, the model for the hydrocarbon tail covering tryptophan requires incorporation of the concepts of partial site occupancy and diffusion from site to site. With diffusion times less than the lifetime of the excited states (3-6 nano secs.). Only a few sites nearest the one which perturbs tryptophan need to be nearer than about 10\AA away from it, if the activation energy required is that of a diffusion-controlled process (Weber, 1970). It has been suggested (Steinhardt, et al., 1971) even for compounds with high association constants, diffusion from occupied to unoccupied sites may occur in times substantially lower than the $10^{-8}$ sec. required for fluorescence.

The lack of quenching of the tryptophan fluorescence in binding of the carbamates, would imply that either insignificant or undetectable protein conformational changes were produced on binding. It should be noted that in order for conformational quenching effects to occur changes in the spatial relationships of vicinal sulphydryl, $\varepsilon$-amino, $\alpha$-ammonium groups or disulphide links to the excited state of tryptophan would be required (Cowgill, 1967; Shinitzky and Fridkin, 1969; Longworth, 1970).

The model would therefore give at least a partial explanation to
observations for carbamate/albumin interactions. The results suggest that a
difference in degree of penetration of the protein structure may occur in the
series. Such difference could possibly be resolved by competitive studies and
the use of more sophisticated techniques (e.g. CD, NMR).

Summary

Our study of the carbamate/albumin interaction has demonstrated the value
of these relatively simple compounds as models for studying hydrophobic
bonding. The use of the homologous series of carbamates has permitted
determination of the contribution to the free energy of association, from both
aliphatic and aromatic groups, as well as the effect of branching of the
aliphatic side chain on the free energy of association. From these results
the $\Delta G$ values of the aliphatic or aromatic groups can be calculated. Such
values have been found to be in reasonable agreement with the values derived from
models on the aqueous solubility of the hydrocarbons (one $>\text{H}_2\text{C}...\text{CH}_2<$ interaction liberates approx. 0.62 Kcals/mole, Nemethy and Scharga (1963) reported
0.7 Kcals/mole). Such values should be applicable to drug-protein interactions
involving mixed forces, for theoretical assessments of the contribution from
hydrophobic forces to free energy of interaction. Application of this to a
comparison of the carbamates binding data with that published for the
carboxylic acids (Teresi and Luck, 1952), demonstrated that the presence of an
ionizable group in these short chain carboxylic acids appeared to have little
effect. A significant contribution to the free energy of association would
appear therefore to be from hydrophobic bonding. This study provides some
information on the mechanism of hydrophobic interactions, and reveals that for a
homologous series of short chain compounds, the number of binding sites ($n$) and
association constant ($k$) for interaction with BSA, may not obey the expected
trend for all members. Divergence from such a trend is possibly a function
of the compounds hydrophobic-hydrophilic balance. For such systems as the
carbamate-albumin, the term site as defined by multiple equilibrian theory may be inappropriate. Rather the concept of the site as a binding area which serves as a point of attachment for a single functional group or segment of the ligand, is to be preferred (Laiken and Nemethy, 1970a). Although interesting differences in the effect on albumin fluorescence are apparent for the series, it is unlikely that any of the carbamates studied produced significant conformational changes in the albumin molecule.
CHAPTER FIVE

INVESTIGATION OF THE PLASMA PROTEIN BINDING OF WARFARIN USING A FLUORESCENT PROBE TECHNIQUE
1. Introduction.

The previous chapters have demonstrated the suitability of the classical techniques: equilibrium dialysis, ultrafiltration and frontal analysis gel filtration in studying protein binding and revealed the nature of the information that can be obtained from these techniques, about small-molecule protein interactions. It is apparent from these studies that the information obtainable is largely of a quantitative nature and other techniques must be used if a qualitative insight into the binding process is required. However, techniques are available which can provide both quantitative and qualitative information on drug-protein interactions (e.g. circular dichroism, nuclear magnetic resonance and the fluorescent probe techniques).

The use of fluorescent probes stems from the early work of Weber and Laurence (1954) who demonstrated that several anilino-naphthalene sulphonates and aniline acridines, which were virtually non-fluorescent in aqueous solution, exhibited marked enhancement of fluorescence in non-aqueous solvents or when bound to protein. It was later shown that these fluorescent probes bind to hydrophobic sites on the protein molecule, through non-covalent bonds. These compounds have found considerable use as probes of the polarity of binding sites, as well as indicators of protein conformational changes occurring at the binding site (McClure and Edelman, 1966; Daniel and Weber, 1966; Weber and Daniel, 1966; Stryer, 1968; Radda, 1971; Brand and Gohlke, 1972). The most commonly used probe has been 1-anilino-8-naphthalene sulphonate (ANS), although recently the
fluorescent probe potential of biologically important tetracyclines (Popov et al, 1971; 1972), kynurenine (Churchich, 1972) and bilirubin (Krasner, 1973) has been demonstrated. There is, however, an obvious need for structurally different fluorescent probes of biological significance. Chignell (1970a, b), has reported that the binding of the anticoagulant drug warfarin to serum albumin resulted in a marked increase in the fluorescence yield of the drug, accompanied by a hypsochromic (blue) shift in its emission maximum. However the potential of warfarin as a fluorescent probe does not appear to have been further utilised.

The binding of warfarin to human serum albumin has been widely studied (O'Reilly and Kowitz, 1967; Solomon and Schrogie, 1967; Solomon et al, 1968; O'Reilly, 1969; O'Reilly et al., 1969; Perrin and Nelson, 1972) however, the interaction of warfarin with albumin from different species has not (Meyer and Guttman, 1970b; O'Reilly and Motley, 1971). In view of the established procedure for testing drugs in vivo in rodents, but examining their protein binding characteristics with bovine albumin preparations before clinically evaluating their efficacy in man, the need for more detailed information on the interspecies differences in the binding of drugs is apparent. In addition characterisation of the warfarin binding site on albumin should provide valuable information on the binding sites of drugs known to compete with warfarin for albumin binding sites.

This study has therefore investigated the potential of warfarin as a fluorescent probe and used this property to gain an insight into possible interspecies differences in the nature of the warfarin-albumin interaction. In order to further characterise these sites a study of the binding of warfarin to chemically modified albumin has been commenced. The potential of the fluorescent probe technique in studying drug competition has also been investigated.
2. Experimental Approach

For all fluorescence studies, albumin at concentrations of 0.5, 1.0 or 2.0 x 10^{-5}M in 0.1 M phosphate buffer, pH 7.4 was used. (N.B. Albumin concentrations in excess of 3 x 10^{-5}M exhibit concentration 'quenching' effects and therefore concentrations of 2 x 10^{-5}M were not exceeded.) Warfarin solutions (1 or 5 x 10^{-3}M) were routinely prepared in phosphate buffer (0.1 M, pH 7.4). Compounds used in the competition studies were prepared in either phosphate buffer or, where appropriate, ethanol. Fluorescence titrations were performed as described in Chapter Two, at 28 \pm 2^\circ C.
3. Results
Table V. 1.

Fluorescence Data\(^a\) of Warfarin in Different Solvents
and Human Plasma Albumin.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Solvent(^b) Dielectric constant (Debye units)</th>
<th>Relative Fluorescence</th>
<th>Quantum Yield</th>
<th>Emission Wavelength Max(^n.) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer (0.1 M, pH 7.4)</td>
<td>78.5</td>
<td>13.9</td>
<td>0.015</td>
<td>386</td>
</tr>
<tr>
<td>Ethanol</td>
<td>24.3</td>
<td>31.7</td>
<td>0.034</td>
<td>383</td>
</tr>
<tr>
<td>n-Butanol</td>
<td>17.1</td>
<td>37.0</td>
<td>0.04</td>
<td>383</td>
</tr>
<tr>
<td>n-Hexane</td>
<td>1.9</td>
<td>2.0</td>
<td>0.002</td>
<td>354</td>
</tr>
<tr>
<td>Human Plasma Albumin (1x10^-5 M)</td>
<td>-</td>
<td>100</td>
<td>0.09</td>
<td>374</td>
</tr>
</tbody>
</table>

a Warfarin concentration was 25 x 10^-6 M in all cases and solutions were excited at 320 nm.

Fig. V.1  Fluorescence Titration Curves of Human Plasma Albumin and Phosphate Buffer with Warfarin

Mean ± S.D. of 4 detn.

- HPA (1 x 10^{-5}M)
  - $\lambda_{ex} = 320$ nm, $\lambda_{em} = 374$ nm

- Phosphate buffer. 0.1M, pH 7.4
  - $\lambda_{ex} = 320$ nm, $\lambda_{ex} = 386$ nm
Fig. V, 2. Quenching of Tryptophan Fluorescence in Human Plasma Albumin and Phosphate Buffer ('inner filter' effect) by Warfarin

- HPA (1 x 10^{-5}M)
- 1 x 10^{-5}M tryptophan in 0.1M phosphate buffer, pH 7.4, ('inner filter' effect)

Fluorescence was monitored at \( \lambda_{ex} = 290 \text{ nm} \)

% Tryptophan Quenching

Warfarin Conc. (x 10^{-6}M)
The concentrations were: HPA, $2 \times 10^{-5}$ M; warfarin in HPA, $2 \times 10^{-5}$ M and alone in 0.1 M phosphate buffer, $29 \times 10^{-6}$ M; HPA solution was excited at 290 nm, while those solutions containing warfarin were excited at 320 nm. All solutions were in 0.1 M phosphate buffer, pH 7.4.
Fig V. 4. Titration of Tryptophan Solutions with Warfarin

Fluorescence monitored at $\lambda_{ex} = 320$ nm and $\lambda_{em} = 386$ nm

- ○ Phosphate buffer only (0.1M, pH 7.4)
- ▲ 1 x 10^{-5}M tryptophan (in phosphate buffer)
- □ 2 x 10^{-5}M tryptophan (in phosphate buffer)
- ● 4 x 10^{-5}M tryptophan (in phosphate buffer)
Fig. V, 5. Comparison of Fluorescence Titrations of Several Albumins with Warfarin

Albumin concns. 1 x 10^{-5}M in 0.1M Phosphate buffer
pH 7.4, 28°C.

\[ \lambda_{\text{ex}} = 320 \text{ nm}, \quad \lambda_{\text{em}} = 374 \text{ nm}, \text{ mean of 3 detn.} \]

- RSA (Fraction V)
- HPA (Crystalline)
- BSA (Crystalline)
- BSA (Fraction V) and BSA (Fraction V, Fatty Acid Free)

Fluorescence Intensity vs. Warfarin Concentration (x 10^{-6}M)
Table V, 2. Percentage Bound Values for the Binding of Warfarin to Different Albumins as Determined by the Fluorescent Probe Technique.

<table>
<thead>
<tr>
<th>Warfarin Conc. x 10^-6 M</th>
<th>% Bound&lt;sup&gt;a&lt;/sup&gt;</th>
<th>HPA (Crystalline)</th>
<th>BSA (Crystalline)</th>
<th>BSA (Fraction V)</th>
<th>RSA (Fraction V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.50</td>
<td>71.1 ± 2.0</td>
<td></td>
<td></td>
<td>97.8 ± 3.25</td>
<td></td>
</tr>
<tr>
<td>1.00</td>
<td>90.4 ± 4.7</td>
<td>74.5 ± 4.65</td>
<td></td>
<td>91.1 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>1.50</td>
<td>88.2 ± 1.85</td>
<td>70.4 ± 4.2</td>
<td>91.2 ± 2.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.00</td>
<td>84.5 ± 2.55</td>
<td>68.2 ± 3.75</td>
<td>97.4 ± 2.95</td>
<td>78.55 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>2.50</td>
<td>74.2 ± 3.4</td>
<td>66.2 ± 3.9</td>
<td></td>
<td>74.15 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>3.00</td>
<td>75.9 ± 3.3</td>
<td>63.7 ± 0.75</td>
<td>90.4 ± 2.3</td>
<td>70.05 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>3.98</td>
<td>69.9 ± 2.5</td>
<td>60.85 ± 2.45</td>
<td>85.8 ± 2.3</td>
<td>64.0 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>4.98</td>
<td>65.1 ± 2.6</td>
<td>56.2 ± 3.0</td>
<td>82.7 ± 3.8</td>
<td>55.5 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>5.96</td>
<td>61.8 ± 0.5</td>
<td>54.2 ± 2.6</td>
<td>79.1 ± 2.2</td>
<td>53.0 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>6.95</td>
<td>58.9 ± 0.2</td>
<td>51.4 ± 2.2</td>
<td>77.4 ± 1.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.94</td>
<td>56.0 ± 0.6</td>
<td>50.9 ± 2.45</td>
<td>74.3 ± 2.2</td>
<td>46.3 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>8.92</td>
<td>53.7 ± 0.7</td>
<td>47.7 ± 0.9</td>
<td>69.4 ± 1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.90</td>
<td>50.2 ± 0.4</td>
<td>46.0 ± 1.1</td>
<td>67.15 ± 0.9</td>
<td>41.5 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>12.35</td>
<td>45.3 ± 1.1</td>
<td>44.9 ± 0.45</td>
<td>62.1 ± 1.3</td>
<td>37.2 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>14.78</td>
<td>43.2 ± 0.5</td>
<td>42.9 ± 0.45</td>
<td>59.8 ± 0.9</td>
<td>34.1 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>17.20</td>
<td>41.2 ± 0.5</td>
<td>40.3 ± 0.1</td>
<td>56.7 ± 0.6</td>
<td>32.1 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>19.61</td>
<td>39.35 ± 0.6</td>
<td>38.7 ± 0.05</td>
<td>54.4 ± 1.1</td>
<td>33.7 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>24.39</td>
<td>37.3 ± 0.7</td>
<td>36.2 ± 0.35</td>
<td>49.4 ± 0.7</td>
<td>26.4 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>29.13</td>
<td>35.7 ± 0.3</td>
<td>33.7 ± 0.15</td>
<td>46.5 ± 0.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>33.82</td>
<td>34.8 ± 0.6</td>
<td>32.2 ± 0.45</td>
<td>44.0 ± 0.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± S.E.M. of two determinations, albumin concns. 25 x 10^-6 M, 0.1 M phosphate buffer, pH 7.4, 28°C.
Scatchard Plots of Fluorescence Probe Binding Data for the Interaction of Warfarin with Several Species of Albumin.
Table V.3  Binding Data for the Interaction of Warfarin with Different Species of Albumin - Comparison of the results obtained in this study with literature values.

<table>
<thead>
<tr>
<th>Albumin</th>
<th>$k_1 \times 10^6 M^{-1}$</th>
<th>$n_1$</th>
<th>$k_2 \times 10^4 M^{-1}$</th>
<th>$n_2$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPA (Crystalline)</td>
<td>6.46</td>
<td>0.9</td>
<td>4.20</td>
<td>8.1</td>
<td>This Study</td>
</tr>
<tr>
<td>BSA (Crystalline)</td>
<td>1.06</td>
<td>1.05</td>
<td>4.63</td>
<td>7.9</td>
<td></td>
</tr>
<tr>
<td>BSA (Fraction V)</td>
<td>3.60</td>
<td>1.6</td>
<td>11.75</td>
<td>7.4</td>
<td></td>
</tr>
<tr>
<td>RSA (Fraction V)</td>
<td>8.07</td>
<td>0.85</td>
<td>3.30</td>
<td>8.2</td>
<td></td>
</tr>
<tr>
<td>HPA (Crystalline)</td>
<td>0.23</td>
<td>2.6</td>
<td>-</td>
<td>-</td>
<td>O'Reilly and Motley (1971)</td>
</tr>
<tr>
<td>BSA (Crystalline)</td>
<td>0.05</td>
<td>2.9</td>
<td>-</td>
<td>-</td>
<td>Jun et al. (1972)</td>
</tr>
<tr>
<td>BSA (Fraction V)</td>
<td>6.24</td>
<td>1.0</td>
<td>2.61</td>
<td>6.0</td>
<td>Meyer and Guttman (1970b)</td>
</tr>
</tbody>
</table>

- Differences in experimental technique and slight differences in experimental conditions are apparent between studies.
- Mean values, determined from a minimum of 20 data points.
Table V, 4.

Comparison of the Amino Acid Composition of Albumins

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>HSA (Residues/mole of protein)</th>
<th>BSA (Residues/mole of protein)</th>
<th>RSA (Residues/mole of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>12</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>Alanine</td>
<td>62</td>
<td>46</td>
<td>58</td>
</tr>
<tr>
<td>Valine</td>
<td>41-42</td>
<td>36-37</td>
<td>33</td>
</tr>
<tr>
<td>Leucine</td>
<td>60</td>
<td>62</td>
<td>53</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>8</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td>Serine</td>
<td>19</td>
<td>26</td>
<td>23</td>
</tr>
<tr>
<td>Threonine</td>
<td>24-25</td>
<td>34</td>
<td>31</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>17-18</td>
<td>20</td>
<td>22</td>
</tr>
<tr>
<td>Aspartic</td>
<td>15</td>
<td>54</td>
<td>49</td>
</tr>
<tr>
<td>Glutamic</td>
<td>78-79</td>
<td>77-78</td>
<td>77</td>
</tr>
<tr>
<td>Histidine</td>
<td>16</td>
<td>17</td>
<td>14</td>
</tr>
<tr>
<td>Arginine</td>
<td>24</td>
<td>22-23</td>
<td>23</td>
</tr>
<tr>
<td>Lysine</td>
<td>62</td>
<td>62</td>
<td>50</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>30</td>
<td>27</td>
<td>23</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>37</td>
<td>36</td>
<td>31</td>
</tr>
<tr>
<td>Methionine</td>
<td>6</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Proline</td>
<td>26</td>
<td>30</td>
<td>29</td>
</tr>
<tr>
<td>Ammonia</td>
<td>34-35</td>
<td>32-33</td>
<td>38</td>
</tr>
</tbody>
</table>

Data from Kirschenbaum (1972).
Fig. V, 7. Comparison of the Tryptophan Fluorescence of Several Albumins when Titrated with Warfarin

Mean of 3 determinations.
Albumin concns. $1 \times 10^{-5}$M in 0.1M Phosphate buffer, pH 7.4, 28°C.
Fluorescence monitored at an excitation wavelength of 290 nm

- BSA (Fraction V), BSA (Fraction V, Fatty Acid Free) and BSA (Crystalline)
- HSA (Crystalline)
- RSA (Fraction V)
Fluorescence Titrations of Acetic Anhydride Treated and Control Human Plasma and Bovine-Serum Albumins with Warfarin.

a) Crystalline HPA (2 x 10^{-5} M)

\[ \lambda_{ex} = 320 \text{ nm} \quad \lambda_{em} = 374 \text{ nm} \]

\[ \Delta \text{ Control} \]

\[ \triangle \text{ Acetic Anhydride Treated} \]

b) Crystalline BSA (2 x 10^{-5} M)

\[ \lambda_{ex} = 320 \text{ nm} \quad \lambda_{em} = 374 \text{ nm} \]

\[ \Delta \text{ Control} \]

\[ \triangle \text{ Acetic Anhydride Treated} \]
Fluorescence Titration Curve of Control and Diethylpyrocarbonate Treated HPA with Warfarin.

$\lambda_{ex} = 320 \text{ nm} \quad \lambda_{em} = 374 \text{ nm}$

- Control
- 10 fold molar excess
- 100 fold molar excess

Diethylpyrocarbonate treated

HPA concn. $2 \times 10^{-5} \text{ M}$

Fluorescence Intensity

Warfarin Conc. $x 10^{-6} \text{ M}$
### Table V, 5.

**Intrinsic Fluorescence of Albumin Derivatives**

<table>
<thead>
<tr>
<th>Derivatives of Albumin</th>
<th>Emission Max(^a) (nm)</th>
<th>Tryptophan Quantum Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HPA (Crystalline)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>340</td>
<td>0.11</td>
</tr>
<tr>
<td>Acetic Anhydride</td>
<td>332</td>
<td>0.11</td>
</tr>
<tr>
<td>Diethylpyrocarbonate: (^c)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 fold excess</td>
<td>333</td>
<td>0.11</td>
</tr>
<tr>
<td>100 fold excess</td>
<td>334</td>
<td>0.09</td>
</tr>
<tr>
<td><strong>BSA (Crystalline)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>340</td>
<td>0.21</td>
</tr>
<tr>
<td>Acetic Anhydride</td>
<td>332</td>
<td>0.15</td>
</tr>
</tbody>
</table>

\(^a\) Excitation wavelength was 290 nm.

\(^b\) Quantum yields of native albumins from Longworth (1971).

\(^c\) '10 fold excess' denotes 10 fold excess of diethylpyrocarbonate per mole of albumin.
<table>
<thead>
<tr>
<th>Derivatives of Albumin</th>
<th>Percentage of Reacted Groups in Albumin Derivatives</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amino</td>
</tr>
<tr>
<td>HPA (Crystalline)</td>
<td></td>
</tr>
<tr>
<td>Acetic Anhydride</td>
<td>86.3 (57)</td>
</tr>
<tr>
<td>Diethylpyrocarbonate:</td>
<td></td>
</tr>
<tr>
<td>10 fold excess</td>
<td>26.1 (57)</td>
</tr>
<tr>
<td>100 fold excess</td>
<td>46.4 (57)</td>
</tr>
<tr>
<td>BSA (Crystalline)</td>
<td></td>
</tr>
<tr>
<td>Acetic Anhydride</td>
<td>90.0 (57)</td>
</tr>
</tbody>
</table>

a '10 fold excess' denotes 10 fold excess of diethylpyrocarbonate per mole of albumin.

b Results expressed as percentage reacted groups, with the respective native albumin taken as 0%.

Figures in parenthesis represent total number of groups present in native albumin.
Dixon Plot of Competition between Phenylbutazone and Warfarin with Human Plasma Albumin

Fig. V, 10

Albumin Conc. = 1 \times 10^{-5} \text{ M}

1
Relative Fluorescence

Phenylbutazone Conc. \times 10^{-5} \text{ M}

Warfarin Conc. (\times 10^{-6} \text{ M})

- 2.5
- 7.4
- 12.4
- 24.4
Table V, 7.

Effect of Various Compounds on the Fluorescence of Warfarin when Bound to Albumin.

<table>
<thead>
<tr>
<th>Competitor</th>
<th>Type of Displacement</th>
<th>Concn. Range of Competitor (x 10^-6 M)</th>
<th>Concn. Range of Warfarin (x 10^-6 M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylbutazone</td>
<td>Competitive</td>
<td>0.5 - 40</td>
<td>2.5 - 25</td>
</tr>
<tr>
<td>Sulphormethoxine</td>
<td>&quot;</td>
<td>1.5 - 100</td>
<td>2.5 - 25</td>
</tr>
<tr>
<td>ANS</td>
<td>&quot;</td>
<td>0.5 - 50</td>
<td>0.5 - 50</td>
</tr>
<tr>
<td>Clofibrate C</td>
<td>&quot;</td>
<td>1 - 80</td>
<td>0.5 - 20</td>
</tr>
<tr>
<td>Biphenyl 20 M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biphenyl 40 M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diazepam</td>
<td>None</td>
<td>1 - 100</td>
<td>2.5 - 10</td>
</tr>
<tr>
<td>Chlordiazepoxide</td>
<td>&quot;</td>
<td>1 - 100</td>
<td>2.5 - 10</td>
</tr>
<tr>
<td>Iprindole</td>
<td>&quot;</td>
<td>1 - 30</td>
<td>2.5 - 10</td>
</tr>
<tr>
<td>Pentobarbitone</td>
<td>&quot;</td>
<td>1 - 80</td>
<td>2.5 - 12.5</td>
</tr>
<tr>
<td>Carbamates (methyl + n-decyl)</td>
<td></td>
<td>5 - 50</td>
<td>2.5 - 12.5</td>
</tr>
</tbody>
</table>

a Similar effects were observed with both human plasma and bovine serum albumin (1 x 10^-5 M in 0.1 M phosphate buffer, pH 7.4).

b Drug/warfarin ratios included likely therapeutic ratios.

c Competition appeared to be non-competitive at low concentrations (up to 6 x 10^-6 M).
4. Discussion

A study of the fluorescence of warfarin in different solvents (Table V, 1) showed that warfarin exhibited a marked increase in fluorescence intensity or quantum yield, and also a hypsochromic (blue) shift in emission maximum on decrease of solvent polarity. One anomaly of this trend was the observed fluorescence in hexane, which although producing a 32 nm hypsochromic shift with respect to buffer, showed a lower fluorescence intensity than would have been expected from hexane's dielectric constant. Deviations between solvent dielectric constant and fluorescent intensity have also been observed for other fluorescent probes (McClure and Edelman, 1966; Edelman and McClure, 1968; Thomson, 1969; Kotaki et al., 1971). One possible explanation could be due to differences in solvent viscosity which are known to produce changes in quantum yield (McClure and Edelman, 1966; Turner and Brand, 1968).

The data presented in Table V, 1 for warfarin would tend to suggest that the solvent polarity was one of the major determinants affecting its fluorescence. This suggested that the electronic transition between ground state and the lowest excited singlet state was of $\pi \rightarrow \pi^*$ type (Kotaki et al., 1971) and that warfarin can therefore function as a fluorescent probe for hydrophobic environments. The hypsochromic shift produced in the emission wavelength in the presence of human plasma albumin would support this view (Table V, 1 and Fig. V, 1). The observed fluorescence of warfarin in albumin suggested that the dielectric nature of the environment of the binding sites was lower than that of water and that the intramolecular mobility of the drug was reduced (Kotaki et al., 1971).

The binding of warfarin to plasma human albumin (HPA) increased the warfarin fluorescence and also produced a concomitant quenching of
the native fluorescence of albumin (Fig. V, 2). The fluorescence of albumin when excited at 290 nm, chiefly reflects the fluorescence from the amino acid tryptophan, although a small contribution from tyrosine may occur. The quenching of tryptophan fluorescence on sequential addition of warfarin to HPA was accompanied by bathochromic (red) shift in the tryptophan emission. The degree of bathochromic shift of the emission wavelength increased with warfarin concentration., from 340 nm before warfarin addition to 364 nm at $8 \times 10^{-6}$M and finally to almost the emission maximum of the bound warfarin ($\lambda_{em} = 374$ nm) on further addition. This deactivation of a light excited tryptophan molecule ($T^*$) could occur by the following four processes:- fluorescence emission ($T^* \rightarrow T + h\nu$ where $h = $ Planck's constant, $\nu = $ frequency of emitted light), radiationless deactivation ($T^* \rightarrow T$), collisional quenching ($T^* + I$ (quencher)$ \rightarrow T + I^*$) and static quenching ($T^* + I \rightarrow [T - I]$), Honikel and Madsen, 1973).

The absorption band of warfarin was shown to overlap the emission band of HPA (Fig. V, 3). The quenching of tryptophan fluorescence promoted by addition of warfarin could therefore, at least partly, be explained in terms of radiationless energy transfer from the tryptophyl residues of HPA to the warfarin. Förster (1951) has suggested that for this energy transfer mechanism to be operative an extensive overlap between the emission band of the donor and the absorption band of the acceptor is required, this is clearly met in warfarin binding to HPA. Several authors have used this concept to determine the critical transfer distance of energy between the donor and acceptor (Chen and Kernohan, 1967; Chignell, 1970a; Wallach et al, 1970; Churchich, 1972).

Tryptophan quenching could also occur by 'inner filter' effects, such effects would be particularly noticeable in strongly absorbing solutions and result from the absorption of the excitation and/or fluorescence by the quencher. This was investigated by titrations
of tryptophan solutions comparable to that in the albumin preparations used (1.2 x 10^{-5} M, and also 4 x 10^{-5} M, in phosphate buffer, pH 7.4).

It was apparent that warfarin was able to quench tryptophan fluorescence by 'inner filter' effects, but comparison of the quenching of tryptophan in aqueous solutions with that of HPA (Fig. V, 2) suggested that 'inner filter' effects could only partially account for the quenching in HPA solutions. This could not be accurately quantitated as tryptophan's quantum yield is known to increase in non-polar environments (i.e. 0.12 in aqueous solution and 0.18 in bovine serum albumin, Chen, 1967; Flanagan and Ainsworth, 1968; Chignell, 1970a). Despite the effect of warfarin on the fluorescence of tryptophan, no effect of tryptophan on the fluorescence of warfarin could be detected (Fig. V, 4) which further established the suitability of warfarin as a fluorescent probe. Attallah and Lata (1968) have suggested that association constants can be derived from such tryptophan quench curves, however, in view of the assumptions made and difficulty of accurate correction of the 'inner filter' effects, the determined association constant values by this method are likely to be only approximate.

The fluorescence titration curves obtained with warfarin for the titration of:- human (crystalline); bovine (crystalline, fraction V, defatted fraction V), and rat (fraction V) albumins, are given in Fig. V, 5 which indicates interesting species differences. The fluorescence enhancement of warfarin was significantly greater on binding to human (crystalline) and rat (fraction V) albumins, than with bovine albumins (either crystalline, fraction V or defatted fraction V). Binding of warfarin to RSA exhibited the largest enhancement of fluorescence. The fluorescence increase in crystalline bovine serum albumin was greater than that with fraction V, and the removal of endogenously bound fatty acid had an insignificant effect. Such differences
between the crystalline and fraction V preparations may reflect the presence of the small amount of globulins in the fraction V preparation. (N.B. Albumin solutions were of equal total protein concentration, however the albumin concentration of fraction V preparations will be reduced by approximately 3% as compared with crystalline preparations.) This finding may well support the view (O'Reilly and Kowitz, 1967) that warfarin does not significantly bind to the serum globulins. Comparison of the warfarin fluorescence titration in human serum with that for human plasma albumin (HPA) showed insignificant difference, a similar finding was observed for rat serum and rat serum albumin (RSA). Little difference could be detected in the binding to albumin, of the R(+) and S(-) isomers of warfarin, this finding, which is in agreement with O'Reilly and Motley (1971), is important in view of the known differences in the plasma half-lives and pharmacological effects of the isomers, and the known species differences between rat and man (Eble et al., 1966; Breckenridge and Orme, 1972; Hewick, 1972; Hewick and McEwen, 1973). The observed interspecies differences in the titration curves for warfarin may be indicative of differences in the polarity of the binding sites and/or differences in the degree of binding.

Analysis of binding data revealed that for similar concentrations of warfarin, differences in the percentage bound to the different albumins were apparent (Table V, 2), which were reflected in the nature of the Scatchard plots (Fig. V, 6). For all species of albumin, marked curvature of the plots was apparent suggesting the presence of more than one distinct class of binding site. (N.B. Interpretation may be complicated by other factors such as negative cooperativity, see Chapter Four). One primary site (n₁) and approximately eight secondary
sites \( (n_2) \) were calculated for each species. (N.B. BSA (fraction V) indicated \( n_1 \) value of 1.6, slightly higher than that found for the other albumins.) (Table V, 3). The association constant \( (k_1) \) for the high affinity, primary binding site of crystalline, HPA and RSA (fraction V) was significantly higher than for crystalline bovine serum albumin (BSA), while little difference was detectable in the association constant \( (k_2) \) for the lower affinity secondary sites. Also, rather surprisingly, the BSA (fraction V) exhibited a higher \( k_1 \) value than did BSA crystalline, although it was lower than both HPA and RSA. The association constant \( (k_2) \) for the lower affinity secondary warfarin binding site on BSA (fraction V) was higher than for the other albumins. Comparison of these determined \( n \) and \( k \) values for the binding of warfarin with published data is complicated by the inconsistent nature of the literature values (Table V, 3). Our results would appear to exhibit reasonable agreement with those reported by Meyer and Guttman (1970b), although slight differences are apparent in the values for the secondary sites. These authors also reported insignificant differences between the binding of warfarin to HSA (fraction V) and BSA (fraction V). It is not known if our results would be consistent with the finding of O'Reilly and Motley (1971), that warfarin was more highly bound to HPA (fraction V) than to crystalline HPA. However, determination of the binding of warfarin to HPA (fraction V), by the fluorescent probe technique, should resolve this. The considerable differences in the reported values of the binding parameters \( n \) and \( k \) for warfarin may indicate that they have been calculated over a different, and for some studies, an insufficient range of \( r \) values (moles warfarin bound/mole albumin). Also it should be added that the use of different techniques and small differences in experimental conditions may also contribute to the differences in \( n \) and \( k \).
The differences in affinity of warfarin binding to different albumin preparations was disturbing in view of the considerable differences between manufacturers specifications for both crystalline and fraction V preparations. The need for standardised methods of isolation of the albumins is apparent if valid comparisons are to be made. This would support the need to purchase albumins from one manufacturer. At present, however, no single manufacturer produces a sufficiently large range of both crystalline and fraction V albumins from different species. These observed species differences in binding and fluorescence titration could result from differences in the amino composition of the albumins (Table V, 4).

The measurement of the decreased albumin fluorescence (tryptophan quenching) upon titration with warfarin revealed further interesting species differences. In general a much higher degree of tryptophan quenching was found for bovine albumin than for the other species of albumin. (N.B. Similar quenching was found for crystalline and fraction V, bovine serum albumins.) (Fig. V, 7). Rat serum albumin and HPA contrasted with the other albumins studied for although addition of low concentrations of warfarin (up to 2 x10^-6M) produced slight quenching of tryptophan fluorescence, at higher concentrations considerable enhancement of tryptophan fluorescence was observed. These changes were accompanied by a bathochromic shift (red) in the tryptophan emission maximum (λem = 374nm) at warfarin concentrations of above (8 x10^-6M). No similar enhancement of tryptophan fluorescence was observed for warfarin titration of BSA (crystalline, fraction V or defatted fraction V). Although HPA produced enhancement, much higher warfarin concentrations (20 x10^-6M) were required than for RSA. A similar shaped curve to that obtained with RSA was achieved with HPA, but at a ten-fold lower albumin concentration (1 x10^-6M). Undoubtedly the shape of these tryptophan quenching curves are affected by 'inner filter' effects
These effects are likely to be greater in BSA due to the presence of two tryptophan residues/mole of BSA as opposed to one with both HSA and RSA. The bathochromic shift in the albumins to the warfarin emission wavelength would also support the view that 'inner filter' effects are involved, however the differences between RSA and HSA, both of which contain one tryptophan/mole albumin and show only small differences in the enhancement of warfarin fluorescence (Fig. V, 5), would suggest that these observations cannot be completely explained by 'inner filter' effects. Such effects would also not explain the increase in tryptophan fluorescence in RSA, upon titration with warfarin. The observed increase in the tryptophan fluorescence in RSA and HSA would however be consistent with the tryptophan residues being moved into a more hydrophobic environment. Hypsochromic shift in tryptophan emission maximum would also have been expected to accompany the fluorescent enhancement, however, 'inner filter' effects may have obscured such changes. Tryptophan quenching followed by enhancement on subsequent additions of compounds to human and bovine albumins has been reported (Halfman and Nishida, 1971; Steinhardt et al., 1971). Further studies on other species of albumins may well provide insight into the relationship between degree of quenching produced by warfarin binding and number of tryptophans/mole albumin. Suitable species of albumin would be, for example:-- monkey (2-tryptophan/mole protein), dog(1 tryptophan/mole protein), most species of albumin contain 1 tryptophan/mole protein, although chicken albumin is unusual in being devoid of tryptophan.

The observed binding data and tryptophan quenching studies may be consistent with the primary binding on human albumin being in the region of the lone tryptophan residue. Swaney and Klotz (1970) have recently sequenced the immediate vicinity of the tryptophan as:

Lys - Ala-Trp-Ala-Val-Ala-Arg
If it is the locus of warfarin binding then the apolar character of this binding region may account for the observed hydrophobic nature of the warfarin fluorescence enhancement. Such a site would be compatible with the suggested involvement of hydrogen and hydrophobic bonds in the binding of warfarin to HPA (O'Reilly, 1969; Chignell, 1970a; O'Reilly and Motley, 1971). However, for bovine serum albumin the amino acid sequence in the neighbourhood of the tryptophans is thought to differ and only one of the tryptophans in BSA is considered to be embedded in the protein structure. The amino acid composition in the neighbourhood of the lone tryptophan in RSA is unknown.

A study of the effect of protein modification on warfarin binding was undertaken to provide further information on the nature of warfarin binding sites. Warfarin fluorescence when bound to acetylated crystalline BSA and HPA was shown to be significantly decreased (83 and 70%) when compared with native albumins (Fig. V, 8a and b). Similarly HPA treated with diethylpyrocarbonate (100-fold excess/mole albumin) produced a 64% reduction in warfarin fluorescence (Fig. V, 9). Determination of the intrinsic fluorescence spectrum of acetic anhydride and diethylpyrocarbonate treated albumins revealed hypsochromic shifts in the emission maximum of all albumins, quantum yields were also affected, but to different degrees (Table V, 5). The hypsochromic shift in the emission suggested a net change to a less polar environment, while the decreased quantum yields may be caused by a quenching of tryptophan fluorescence by the new groups introduced into the proteins.

The amino groups modified by acetic anhydride treatment included 49 lysines in HPA and 51 in BSA, plus 7 and 10 tyrosine hydroxyl groups respectively. An excess of 10 moles of diethylpyrocarbonate per mole of albumin resulted in 2 modified histidines and 15 modified lysines in HPA,
whereas an increase in the reagent/albumin ratio (i.e. 100-fold excess diethylpyrocarbonate) gave derivatives with 8 modified histidines and 26 modified lysines. Tyrosine, arginine and tryptophan residues remained intact in all diethylpyrocarbonate derivatives (Table V, 6). A 10-fold excess of diethylpyrocarbonate per mole of albumin produced a negligible effect on the fluorescence of warfarin in HPA (Fig. V, 9) whereas higher degrees of modification did. This suggested that in excess of 2 histidines and 15 lysines had to be modified before the binding of warfarin to HPA was affected. That lysine was involved in the binding of warfarin was supported by the decreased fluorescence of warfarin when bound to acetylated albumins, although the modified tyrosines may also be involved. Acetic anhydride has been shown to produce unfolding in the albumin molecule as a result of the increased net negative charge (Habeeb et al., 1958; Jonas and Weber, 1970; Jacobsen, 1972) thus disrupting the hydrophobic environment of the warfarin binding site. However the hypsochromic shift in warfarin fluorescence on addition to acetylated albumin, indicated that this effect was by no means total. The binding of several other anions; long chain fatty acids, (Spector et al., 1969), 2,4-dichlorophenolate (Teresi, 1950); 1-anilino-8-naphthalene sulphonate (Jonas and Weber, 1971); salicylate (Sturman and Smith, 1967); bilirubin (Jacobsen, 1973) have also been shown to be reduced in acetic anhydride modified albumins. Teresi (1950 suggested the involvement of lysine in the binding of methyl orange, and 2,4-dichlorophenolate to BSA. As a result of their studies Jonas and Weber (1971) have suggested that arginine may be involved in the binding site of ANS in BSA, similarly Jacobsen (1972) has indicated involvement of arginine in the binding of bilirubin to HSA. Unfortunately glyoxal (known to modify guanidino groups, Nakaya et al., 1967; Jonas and Weber, 1971), is strongly fluorescent and therefore could not be used in our fluorescent probe studies. However, the finding that warfarin interacts with ANS in a direct competitive manner for albumin binding sites.
This study and Jun et al., 1972) may suggest the involvement of arginine in the binding of warfarin, the determination of the binding of radioactively labelled warfarin to glyoxal treated albumin should resolve this. Also binding determinations of warfarin in tetranitromethane, n-acetylimidazole and o-nitrophenylsulfenylchloride modified albumins should clarify the possible role of tyrosine and tryptophan in the binding process.

These findings give a partial insight into possible warfarin binding sites on albumin and indicate the role of lysine, histidine and tyrosine in the interaction. The fluorescent probe observations also indicated the hydrophobic nature of the binding sites.

A considerable number of drugs have been reported to displace warfarin, but few systematic studies have been presented (Solomon and Schrogie, 1967). The suitability of the fluorescent probe technique to study displacement is demonstrated by the competitive displacement found, for warfarin binding to HPA, by therapeutic concentrations of phenylbutazone (Fig. V, 10). Several acidic, neutral and basic drugs have been investigated for their displacement of warfarin from both human and bovine albumin binding sites (Table V, 7). The direct competitive nature of the displacement found for the acidic compounds ANS, clofibrate, sulphormethoxine would appear to be in agreement with the findings of other workers (Solomon and Schrogie, 1967; Solomon et al, 1968; Sellers and Koch-Weser, 1971; Jun et al., 1972). It has also been demonstrated that long-chain fatty acids compete with ANS for binding sites on albumin (Santos and Spector, 1972). The observation that the benzodiazepines, chlordiazepoxide and diazepam did not displace warfarin would appear to support the finding of Orme et al. (1972) that administration of chlordiazepoxide did not affect the steady-state plasma warfarin concentrations, the plasma half-life or anticoagulant control in patients. Few binding studies of the benzodiazepines to plasma proteins appear to have been performed (van der Kleijn, 1969; Müller and Wollert, 1973), however initial indications are that they may
be highly bound. The results presented here would suggest that they
bind to sites on albumin, other than those which bind warfarin. The
tricyclic antidepressant drug, iprindole was also found to have an
insignificant effect on the binding of warfarin to both human and bovine
albumins. Borg et al. (1969) have demonstrated the high percentage
binding to human plasma of therapeutic concentrations of several anti-
 depressants (amitryptiline, imipramine, nortryptyline and protryptylene).
They have also reported that a large number of drugs, including phenyl-
butazone and clofibrate, failed to displace the antidepressants from their
plasma protein binding sites. In general, however, it would appear from
the limited studies carried out that basic compounds are less well bound
than are acidic drugs and the percentage bound relatively independent of
drug concentration (Borg et al., 1969; Franksson and Anggard, 1970;

We attempted to utilize the fluorescent properties of two basic
compounds, iprindole and benzidine, to gain an insight into the nature
of their albumin binding site. Despite the fact that both these compounds
indicated the necessary fluorescent properties to function as fluorescent
probes, no enhancement of their fluorescence, or hypsochromic wavelength
shift, could be detected on binding to albumin. This finding may indicate
that their binding sites were located in relatively hydrophilic environments,
and would therefore appear to contrast distinctly with the hydrophobic
environment of the warfarin binding site. No displacement of warfarin,
from albumin binding sites, by any of the neutral carbamates studied was
observed, even though they are likely to bind to a hydrophobic site. The
insertion of an electron donating hydroxyl group into the biphenyl
structure (i.e. 2- and 4-hydroxy) appeared to produce increased displacement
of warfarin, although such displacement was non-competitive.

Clearly, insufficient structurally different compounds have been investi-
gated for their displacement potential to allow firm conclusions to be drawn
about the physico-chemical requirements for displacement. However, these findings would appear to confirm that acidic compounds form a major group of drugs which compete with warfarin for albumin binding sites. This study has demonstrated the potential of the fluorescent probe technique in studying drug interactions, although one important consideration is that the competitor should be relatively non-fluorescent, otherwise lengthy 'blank' corrections are required. Such studies need not be limited to albumin solutions and the technique is readily compatible with the use of serum samples.

**SUMMARY**

The application of the fluorescent probe technique in studying protein binding has been demonstrated. A novel fluorescent probe, warfarin, has been used to investigate its binding to several different species and preparations of albumin as well as drug competition studies. Inter-species differences were apparent for the binding of warfarin, and may suggest that crystalline bovine albumin is unsuitable for warfarin binding determination, if extrapolation to man is required. The marked differences in the binding of warfarin to crystalline and fraction V bovine albumin preparations further demonstrated the 'pitfalls' that may jeopardise meaningful correlation of in vitro binding data with in vivo studies. Good agreement was found between HPA and human serum for the binding of warfarin, however, agreement between the binding data obtained with serum/plasma and isolated albumin fractions may not always occur (Witiak and Whitehouse, 1969; Thorp, 1972). The use of chemically modified albumins has provided a partial insight into the nature of the warfarin binding site in both human plasma albumin and bovine serum albumin. The results would suggest that lysine, histidine and tyrosine residues may be involved, either directly or indirectly in the warfarin-albumin interaction,
although the possible involvement of other amino acids (e.g. arginine) cannot be ruled out. Possible differences in the polarity of the human, rat and bovine albumin binding sites for warfarin were also apparent. Whether the high affinity warfarin binding site on human plasma albumin is that surrounding the 'lone' tryptophan residue (Swaney and Klotz, 1970) cannot be unambiguously concluded. The fluorescent probe method has been shown to be valuable in studying drug interactions, and may present a relatively simple and rapid procedure for screening novel drugs for their displacement potential of the clinically important drug warfarin.
CHAPTER SIX

USE OF FLUORESCENT PROBES TO
TO INVESTIGATE HEPATIC MICROSOMAL
'DRUG' BINDING SITES
1. **Introduction**

The value of non-covalently bound fluorescent molecules as extrinsic probes of membrane structure and function is now well established, (Radda, 1971). In view of this it is surprising that few studies have appeared using such compounds to investigate microsomal-sites of drug interactions, (DiAugustine, et al., 1970, Eling and DiAugustine, 1971, Hawkins and Freedman, 1973). These sites have been classified as types I, II, and RI, (Remmer, et al., 1966, Imai and Sato, 1966, Schenkman, et al., 1967) according to the nature of the spectral change produced on addition of a compound to a microsomal protein solution. Previous applications of the fluorescent probe technique to the study microsomal binding sites has been restricted to the use of ANS, a type I fluorescent probe. (DiAugustine, et al., 1970, Eling and DiAugustine, 1971). However to derive satisfactory information from this technique, several probes with different physio-chemical and molecular properties are needed. Thus, in order to fully characterise microsomal binding sites, suitable types I, II and RI fluorescent probes are a pre-requisite.

The type of difference spectral change on interaction with microsomes, elicited by the common used fluorescent probes, with the exception of ANS, is unknown, (Brand and Gohlke, 1972). However, close structural resemblance of the large majority of these probes to ANS would suggest they are unlikely to be suitable for the investigation of types II and RI microsomal sites. The interaction of warfarin with rat hepatic microsomes has been variously reported to produce type II (DiAugustine, et al., 1970) or type RI (Deckert and Remmer, 1972) spectral changes. The results from the preceding chapter, (Chapter Five) demonstrated that warfarin exhibited the necessary fluorescent properties to be a suitable probe for albumin binding sites and its value for
probing microsomal binding sites was therefore investigated.

Cytochrome P-450 has not been obtained in a purified form and therefore little is known about the chemical bonds of the haem moiety or of the coordination bonds of the fifth and sixth ligands of the haem iron. Attempts to determine the nature of the binding sites of cytochrome P-450 have therefore been indirect and highly speculative (Mannering, 1971). Our present understanding of the nature of types I, II, and RI binding sites is thus poor.

We have attempted in this study to gain an insight into the nature of type I, II and RI microsomal interactions, through the use of appropriate fluorescent probes and conventional difference spectral techniques. The possible use of the protein albumin as a model for these 'drug'-microsomal protein interactions, to overcome the problems inherent with studies of cytochrome P-450, has been discussed.
2. Results
**Table VI, 1**

Fluorescence data of ANS, Warfarin and Benzidine in different solvents

<table>
<thead>
<tr>
<th>Solvent</th>
<th>ANS λ&lt;sub&gt;e&lt;/sub&gt; = 376 nm</th>
<th>Warfarin λ&lt;sub&gt;e&lt;/sub&gt; = 320 nm</th>
<th>Benzidine λ&lt;sub&gt;e&lt;/sub&gt; = 300 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Emission λ&lt;sub&gt;max&lt;/sub&gt;. (nm)</td>
<td>Relative fluorescence</td>
<td>Emission λ&lt;sub&gt;max&lt;/sub&gt;. (nm)</td>
</tr>
<tr>
<td>0.1 M-phosphate buffer</td>
<td>513</td>
<td>0.3</td>
<td>386</td>
</tr>
<tr>
<td>Ethanol</td>
<td>468</td>
<td>100</td>
<td>383</td>
</tr>
<tr>
<td>n-Hexane</td>
<td>447</td>
<td>66.1</td>
<td>354</td>
</tr>
<tr>
<td>Microsomes&lt;sup&gt;a&lt;/sup&gt; (rat or hamster)</td>
<td>464</td>
<td>69.2</td>
<td>374</td>
</tr>
</tbody>
</table>

<sup>a</sup> Microsomal protein concentration = 2 mg/ml. The concentrations of ANS, warfarin and benzidine were 3 μM, 25 μM, and 27 μM respectively.
Fig. VI, 1  Difference Spectral Interaction of R(+) Warfarin (type I) and S(-) Warfarin (type RI) with Hamster Hepatic Microsomes

2.4 x 10^{-4} M R(+) Warfarin  Type I

1.2 x 10^{-4} M S(-) Warfarin  Type RI

Microsomal Protein
Conc = 2 mg/ml

--- baseline
Fig. VI, 2.

Typical Lineweaver-Burke Plot for Determination of the Spectral Dissociation Constant ($K_s$) for the Interaction of $R(+)$ Warfarin with Rat Hepatic Microsomes

Each point is the mean of 4 determinations.
Table VI, 2. Relationship between the Spectral Dissociation Constant \( (K_s) \) and Type of Spectral Change for Rat and Hamster Microsomes

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rat</th>
<th>Hamster</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( K_s \times 10^{-5} \text{M} )</td>
<td>( K_s \times 10^{-5} \text{M} )</td>
</tr>
<tr>
<td>ANS</td>
<td>4.0±0.3</td>
<td>6.5±0.5</td>
</tr>
<tr>
<td>WARFARIN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R+</td>
<td>45.0±4.1</td>
<td>8.0±1.5</td>
</tr>
<tr>
<td>S-</td>
<td>16.0±1.4</td>
<td>11.0±1.2</td>
</tr>
<tr>
<td>Racemic</td>
<td>23.0±1.8</td>
<td>8.8±1.0</td>
</tr>
<tr>
<td>BENZIDINE</td>
<td>6.0±0.8</td>
<td>5.3±0.6</td>
</tr>
</tbody>
</table>

Microsomal Protein Concentration = 2 mg/ml.

Mean ± S.E.M. of 4 animals.
Data for Spectral Interaction of R(+) + S(-) Isomers of Warfarin
With Control and Phenobarbitone Pretreated Rat Microsomes

Microsomal Protein Concentration = 2 mg/ml
Mean of 4 animals

R(+) Warfarin - Type RI

Fig. V, 3a

\[
\frac{1}{\Delta E} vs \frac{1}{R + \text{Warfarin}} \times \text{mM}^{-1}
\]

S(-) Warfarin - Type RI

Fig. VI, 3b

\[
\frac{1}{\Delta E} vs \frac{1}{S(-) \text{ Warfarin}} \times \text{mM}^{-1}
\]
Fluorescence Titration of Hamster Microsomes and Phosphate Buffer with ANS.

Microsomal Protein
Concn. 1 mg/ml
Fluorescence excited at 376 nm with both microsomes and phosphate buffer.

Fluorescence Intensity in Hamster Microsomes ($\lambda_{em} = 464$ nm)

Fluorescence Intensity in 0.1 M Phosphate Buffer ($\lambda_{em} = 513$ nm)
Fluorescence Titrations of Hamster Hepatic Microsomes and Phosphate Buffer with (R+) Warfarin

Fluorescence excited at 320 nm
mean of 4 determinations

- Microsomal Protein, Conc. = 2 mg/ml
  \(\lambda_{em} = 374\) nm

- Phosphate buffer, 0.1 M
  pH 7.4
  \(\lambda_{em} = 386\) nm
Fig. VI, 6.

Titration of Hamster Microsomes with R(+) and S(-) Warfarin

- $[R(+)\text{ Warfarin}] = 320 \text{ n mole}$
- $[S(-)\text{ Warfarin}] = 374 \text{ n mole}$

Mean of 4 animals

$\lambda_{\text{ex}} = 320 \text{ nm}$
$\lambda_{\text{em}} = 374 \text{ nm}$

Microsomal Protein
$\text{Conc}^n = 2 \text{ mg/ml}$
Mean of 4 animals

---

Fluorescence Intensity

Warfarin Conc$^n$ (x $10^{-5}$ M)
Fig. VI, 7. Lineweaver-Burk plot for the Competitive Inhibition by ANS of the Binding of S(-) Warfarin to Hamster Microsomes

Microsomal Protein 2 mg/ml

$\lambda_{ex} = 320$ nm
$\lambda_{em} = 374$ nm
ANS = type I
S(-) Warfarin = type RI

$S(-)$ Warfarin $+ 7.4 \times 10^{-5}$M ANS

Fluorescence $\times 100$

$\frac{1}{S(-) \text{ Warfarin}} \times \text{mM}^{-1}$
Lineweaver-Burk Plot of the Effect of S(-) Warfarin (Type RI) on ANS-Hamster Microsome Fluorescence.

Microsomal Protein
2mg/ml

○ ANS in buffer

• ANS + S(-) Warfarin (7.4 x 10⁻⁵M) in 0.1M phosphate buffer pH 7.4

ANS fluorescence measured at λ<sub>ex</sub>=376 nm, λ<sub>em</sub>=464nm

Fluorescence Intensity x 100

1

1

0

10

20

30

40

50

60

1/ANS x mM⁻¹
Fig. VI, 9.

Titration of Control and Phenobarbitone Induced Hamster Microsomes with R (+) Warfarin

- Phenobarbitone Induced Microsomes
- Control Microsomes

Microsomal Protein
Conc\textsuperscript{n}. = 1 mg/ml
\( \lambda_{ex} = 320 \text{ nm} \quad \lambda_{em} = 374 \text{ nm} \)
mean of 4 animals
3. Discussion

From the studies presented (Table VI, 1), ANS would appear to be a suitable type I fluorescent probe for hepatic microsomes, thus confirming the work of Eling and DiAugustine (1971). Investigation of warfarin fluorescence in the presence of microsomes (Table VI, 1), revealed that it exhibited a similar 12nm hypsochromic shift in emission wavelength to that experienced in the presence of albumin (Chapter Five) although the enhancement of fluorescence was less. This finding validated its use as a fluorescent probe for hepatic microsomes.

Difference spectral studies of the hepatic microsomal interaction of warfarin revealed some interesting species differences. Whilst the racemate and S(-) isomer produced a RI spectral change with both rat and hamster microsomes, the R(+) isomer differed, producing a type RI change with rat, but a typical type I change with hamster microsomes (Fig. VI, 1).

The racemic and S(-) warfarins, therefore can be justifiably used as RI fluorescent probes for rat and hamster microsomes, whereas the R(+) warfarin can be employed as an additional type I fluorescent probe for studies involving hamster microsomes.

The search for potential type II fluorescent probe raised some intriguing problems. The main criteria required being a compound with suitable fluorescence intensity and wavelength changes in solvents of different polarity, and also one that would produce a type II spectral change on interaction with microsomes.

Several compounds were studied which met one, but not both, selection criteria. However, our attention was drawn to a paper describing the application of benzidine in the determination of small amounts of nitrite (Oshima and Nagasawa, 1970), in which it was demonstrated that benzidine exhibited increased fluorescence in ethanol compared with aqueous solution. On
more detailed investigation of the fluorescence properties of benzidine in several solvents, it was found that an increase in benzidine fluorescence and hypsochromic shift in emission wavelength (24 nm, aqueous to hexane, Table VI, 1) occurred on decrease in the solvent polarity. Investigation of the interaction of benzidine with both rat and hamster microsomes, by difference spectra, revealed a characteristic type II spectral change. The decrease in the magnitude of the type II spectrum due to benzidine, on the addition of aniline as a spectral modifier (Leibman, et al., 1969) to both reference and sample cuvettes, verified benzidine as a type II compound. Addition of biphenyl (type I) as a spectral modifier produced an increase in the magnitude of the type II spectral change with benzidine. Similar findings have been reported for aniline by several authors (Schenkman, 1970; Orrenius et al., 1972) who interpreted them as being due to the presence of a type I component in the aniline type II spectral change. However, their suggestion has not been verified experimentally and it is likely that other factors may be involved.

The above investigations have shown that ANS, benzidine and the warfarins can be used as suitable fluorescent probes for the three types of microsomal binding site. Difference spectral and fluorescent probe studies with these compounds were therefore performed, over the same concentration range, in both rat and hamster microsomes.

A typical Lineweaver-Burk plot for the difference spectral interaction of R(+) Warfarin with rat microsomes (Fig. VI, 2) showed that a single regression line could be adequately fitted to the experimental data, although a possible divergence is apparent at low substrate concentrations. Such divergence may indicate the involvement of high and low affinity binding sites (Walker, 1963; Burke, 1972). Similarly good correlations were obtained for each probe. The spectral dissociation constant ($K_s$), for ANS was similar for rat and hamster microsomes (Table VI, 2) and was in good
agreement with the determined fluorescence dissociation constant ($K_f$) derived from the fluorescent probe studies (e.g. $K_s = 6.5 \times 10^{-5}$, $K_f = 8.5 \times 10^{-5}$M, for hamster microsomes). The $K_s$ values for benzidine were also similar in both species, however the situation with warfarin was more complex. As well as the apparent species difference in type of spectral binding, the $K_s$ values for all three warfarins were found to be higher with rat microsomes than for hamster microsomes. This discrepancy was more noticeable for the R(+) isomer, than the S(-), while the racemate reflected the contribution from both R(+) and S(-) isomers. (Table VI, 2). The $K_s$ value for racemic warfarin in rat microsomes was in good agreement with that found by Deckert and Remmer (1972). Phenobarbitone pretreatment of both hamster and rat hepatic microsomes induced no significant changes in either $K_s$ or the shape of the difference spectrum for R(+) warfarin (with control hamster microsomes the $K_s$ was $45.0 \pm 4.1$ and for induced microsomes $38.0 \pm 2.0 \times 10^{-5}$M).

Similarly no differences were observed for ANS binding. These results are in agreement with those of Kato, et al., (1970) who reported no significant hexobarbital type I $K_s$ changes after phenobarbitone or 3-methylcholanthrene induction. However, for the S(-) warfarin, it was not possible to calculate the $K_s$ value because the magnitude of the spectral change was reduced with increase of S(-) warfarin concentration (Fig. VI, 3b). The shape of the Lineweaver-Burk plot may suggest that only binding to the low affinity site was affected, however, why the binding of the R(+) isomer (Fig. VI, 3a) was not similarly affected, has yet to be ascertained. However, this may reflect an important difference between the isomers and clearly requires further investigation, as does the nature of the high and low affinity sites. It may be that the low affinity site is purely hydrophobic in interaction, whereas the high affinity might reflect an electrostatic interaction with cationic phospholipid groups, possibly stabilised by hydrophobic forces. The presence of more than one class of site, as evidenced by the biphasic nature of Lineweaver-Burk plots for spectral data, has been reported for
both type I and II compounds (Schenkman, 1970).

Recent work in our Department would support the role of hydrophobic interactions in type I microsomal binding. A linear correlation has been found between the log. $K_s$ and log. partition coefficient for the binding of aliphatic carbamates (butyl to decyl) to rat microsomes, (B. Houston, personal communication), and for aromatic ring systems containing alkyl side chains (K. Al-Galainy, personal communication). That such correlations are not restricted to type I compounds, has been shown with type II n-alkylamines (Jefcoate, et al., 1969). Whether this latter correlation is a reflection of the type II site, or simply indicates a type I component in the observed type II spectra (Orrenius, et al., 1972) is uncertain.

Binding of ANS to hepatic microsomes produced a 49nm hypsochromic shift in emission wavelength maximum of ANS, accompanied by an increase in its fluorescence intensity. A typical curve for the titration of microsomes with ANS is shown in Fig. VI, 4. Similar fluorescence changes were obtained for both rat and hamster microsomes, indicating that the ANS site (type I) is located in a hydrophobic region of cytochrome P-450. The binding of R(+) warfarin to microsomes was accompanied by a 12nm hypsochromic shift in emission wavelength, and an increase in fluorescence intensity (Fig. VI, 5), which was similar for both rat and hamster microsomes. Although the increase in the observed fluorescence is less than that obtained with serum albumin (Chapt. Five) it is nevertheless adequate to reveal the nature of type RI site's environment. It is important to note that the fluorescence of S(-) (type RI) and R(+) (type I) warfarins in hamster microsomes (Fig. VI, 6) gave identical fluorescence increases and produced similar shifts of emission maxima. Likewise the fluorescence of racemic S(-) and R(+) Warfarins were indistinguishable in rat microsomes. These results would suggest that the type I and RI microsomal binding sites are located in similarly lipophilic environments.
Competitive studies, between ANS and the warfarin isomers using the fluorescent probe technique, were performed in both rat and hamster microsomes. ANS (type I) and racemic or S(-) warfarin (type RI) as well as R(+) warfarin (type I), appeared to interact in a direct competitive manner. (Figs. VI, 7 and 8). ANS and both Warfarin isomers appeared to be mutually effective in displacing each other from their microsomal binding site, similar findings were observed with albumin. DiAugustine, et al., (1970), suggested that racemic Warfarin competitively displaced ANS from high but not low, affinity binding sites in rat hepatic microsomes.

Following phenobarbitone pretreatment both rat and hamster microsomes did not display any increase in the intensity of fluorescence expressed per mg. of microsomal protein, with either ANS, racemic, S(-) or R(+) Warfarins, (Fig. VI, 9). This suggests that the environment of the type I and type RI binding sites are not significantly altered by phenobarbitone pretreatment in either species. It is difficult to resolve the suggestions of Schenkman, et al., (1969) and Diehl, et al., (1970), that the type RI binding site is due to the displacement of an endogenous type I compound, with the above finding that there are marked differences in the form of spectral binding for very closely structural related compounds namely the two warfarin isomers in hamster microsomes (i.e. R(+), type I; S(-), type RI). Furthermore, using hamster microsomes no significant difference in the spectral dissociation constant \(K_s\) could be detected for these isomers (Table VI, 2). An affinity change would be expected if only the S(-) isomer were able to displace the endogenous type I compound. Neither would the findings support the view of Orrenius, et al., (1972), that the type RI spectrum might be a composite spectrum arising from the superimposition of a type I with a type II spectrum, since no change in the type of spectral binding, or fluorescent probe characteristics, were observed over a wide concentration range of warfarin used. Some detectable changes would appear
likely if a type I contributor were present in the type RI spectral change. Although Orrenius, et al., (1972), could not demonstrate a type I component in the type RI spectra of ethanol, they were however, able to show it for the mono-oxygenase substrates, tryptophan and agroclavine. They suggested that the lack of a type I component in the type RI spectra might distinguish type RI non-substrates from type RI substrates, however this would not explain the differences for the warfarins, which are known substrates of the mono-oxygenase system. Our results would appear to support the suggestion that the type I site is in a hydrophobic environment, probably the phospholipid region of the microsomal membrane (Imai and Sato, 1967; DiAugustine et al., 1970; Mannering, 1971). From the studies with the isomers of warfarin and also the competitive studies with ANS, the type RI site would appear to be either the same site as the type I, or a similarly lipophilic site in close proximity to it. Further work is required to establish whether this latter suggestion is compatible with the view of Schenkman, et al., (1972), that the type RI site is a site on the 'substrate-bound' form of cytochrome P-450 other than the type I site. However, the noteworthy differences between the type of spectral change encountered with the R(+) and S(-) warfarins in rat and hamster microsomal fractions, implies that very subtle changes in the stereochemical configuration may profoundly influence the nature of the spectral change accompanying cytochrome P-450 'drug' interactions.

Benzidine, as reported earlier, was shown to change its fluorescence wavelength and intensity with variations in solvent polarity (Table VI, 1). Although benzidine exhibited a low $K_s$ value for both rat and hamster microsomes (Table VI, 2), lower even than aniline (the classical type II substrate) it showed insignificant fluorescent enhancement and no emission wavelength shift when bound to hepatic microsomes (Table VI, 1). It thus exhibited fluorescence characteristics typical of a hydrophilic environment. Furthermore, these fluorescence observations would not appear
to support the presence of a type I component in the type II spectral change of benzidine. Titration of either serum (rat and hamster) or albumin solutions with benzidine, also failed to produce any significant alteration in fluorescence characteristics.

Our finding that the type II site appears to be in a relatively hydrophilic environment, whilst the type I site is in a hydrophobic region, is nevertheless consistent with the view that the two sites may be located in close proximity (Netter, et al., 1969). It may be that the haem moiety projects from the hydrophobic environment of the phospholipid membrane, into a relatively polar environment. Coordination complex formation between the nitrogen of a type II compound and the haem iron is likely to be favoured by a more hydrophilic surrounding (Hill et al., 1970). Recent electron paramagnetic resonance studies on purified cytochrome P-450_{cam} from *Pseudomonas putida* (Peterson and Griffin, 1973), have shown that the ferric ion of the haem group when bound to metyrapone (type II) was in a more hydrophilic environment than when bound to camphor (type I).

Our study has provided a partial insight into the nature of the hepatic microsomal binding sites, and demonstrated the potential of fluorescent probes in providing information on cytochrome P-450-drug interactions. However, the significance of the contribution of this technique depends largely on the discovery or synthesis of further appropriate probes. These incorporated with low temperature and fluorescence lifetime studies, should contribute significantly to our understanding of drug-P-450 interactions.

The possible use of albumin as a model for these drug-microsomal interactions has been investigated. The results would suggest that the albumin and microsomal binding sites for the probes, are characterised by similarly hydrophobic (for ANS and warfarin) or hydrophilic (for benzidine) environments. Although, it should be pointed out that the extent to which
benzidine is bound to albumin remains to be determined. It is therefore, not possible to fully evaluate whether the fluorescence characteristics in albumin were due to a hydrophilic binding site or that the degree of binding was very low. Nevertheless, the finding that ANS and the warfarin isomers, interact in a direct competitive manner in both albumin and microsomes, may suggest that the probes are bound by similar forces (possibly hydrophobic and electrostatic) in both systems. However, more extensive studies are required before an assessment of albumin as a model for microsomal binding studies can be made.
CHAPTER SEVEN

FINAL DISCUSSION
This last chapter contains a brief discussion on the implications of the experimental findings presented in this thesis and suggestions for future investigations.

The comparative evaluation of the three widely used techniques of equilibrium dialysis, ultrafiltration and frontal analysis, gel chromatography (Chapter Three) has shown that the binding data obtained by these three techniques, under the experimental conditions employed, is directly comparable over given variations in the percentage bound and the physico-chemical properties of the drug. These techniques would therefore appear, on the basis of the data obtained, to be equally applicable to the study of the protein binding of other small molecules. Clearly, however, certain experimental considerations may predispose towards the selection of one technique in preference to another. For example, if studies are to be made using plasma samples in which pH control by O₂/CO₂ gas mixture rather than foreign buffer is required, ultrafiltration (e.g. using Toribara tubes) will probably be the most suitable. However, both ultrafiltration and dialysis will not be suitable for drugs which bind to the dialysis membrane. The finding of good agreement between the techniques is reassuring, and may imply that where difference in the literature for the binding data of a particular compound exists, then factors other than the experimental method are responsible.

Most drug-plasma protein interaction investigations have been concerned with determination of binding in drug-protein systems at equilibrium, however, such determinations may tend to obscure the kinetic
nature of the interaction, particularly as occurring in vivo. The kinetics of binding are seldom investigated, however Froese, et al., (1962) studied the interaction between an acidic azo dye and bovine serum albumin, and showed that dissociation was extremely rapid (e.g. half-life of 20 milli-seconds). Interestingly both these workers and Robbins et al., (1965), who examined the interaction of thyroxine with albumin, showed that the dissociation process could be resolved into two half-lives. If the rate of association or dissociation of drug-plasma protein complexes is of a similar order then it will scarcely limit transport of drugs into or out of the plasma. The paucity of quantitative information on the kinetics of binding emphasises the need for fundamental studies on drug-protein interactions. Such studies are likely to require techniques more familiarly associated with the study of enzyme-substrate interactions, e.g. stopped flow spectrophotometry and fluorimetry, temperature and pressure jump studies, flash photolysis, and fluorescence lifetime studies, which could also be used for investigating the kinetics of drug displacement.

It is often tacitly assumed that the protein bound drug is always in complete equilibrium with unbound drug and that the removal of unbound drug from the plasma (e.g. by excretion, metabolism) will result in the immediate release of bound drug until equilibrium is restored. However, this may not always be the case. Extrapolation of in vitro determined equilibrium binding results to in vivo conditions may be of little practical relevance, particularly when binding at only one drug concentration, often unrelated to the therapeutic level, is determined. Several non-equilibrium dialysis methods have been introduced in recent years which do circumvent some of the problems apparent with the equilibrium techniques, but they are by no means ideal as they require large volumes and still employ a membrane. The replacement of the dialysis membrane by a partitioning system (e.g. organic solvents; hexane or octanol) could be validly
incorporated into a non-equilibrium system, and might be an improved model for the in vivo situation. Meyer and Guttman (1970) have reported good agreement between their non-equilibrium dialysis technique and equilibrium systems (i.e. equilibrium dialysis and ultrafiltration). It is perhaps ironic that even though the criticisms of equilibrium techniques are well established, these and other authors still justify the validity of a new method by the similarity of the data produced to that obtained with established equilibrium methods.

The finding in this study of a good agreement between the binding of salicylate to rat serum by in vitro ultrafiltration, and in vivo dialysis, may suggest that these in vitro techniques are able to reflect the in vivo binding. However, only a small serum salicylate concentration range was obtained in vivo, under the dosage regime employed, thus it cannot be unambiguously concluded whether such an agreement would still pertain at higher serum levels. It remains to be established whether this technique would be suitable for drugs of relatively short half-life where the serum concentration is rapidly changing. The successful application of this in vivo approach will necessitate more precise information regarding the relative rates of change of the drug in the serum, peritoneal fluid and dialysis sac contents. It may have considerable potential in both pharmacokinetic and pharmacodynamic studies especially if either regular sampling, or continual recirculation of the dialysis sac contents, possibly employing flow-through monitoring devices, can be obtained. The suggestion (Keen, 1971) that the concentration in the dialysis sac does not represent tissue fluid, because the effective diameter of the dialysis membrane pores is very much less than that of capillary pores, must however be considered, and experiments employing membranes with larger pore size should be performed. Adaptation and development of a technique recently described (Aziz and Dennhardt, 1973) of the continuous in vivo ultrafiltration of circulating blood may also have considerable potential in binding studies. The in vivo dialysis technique
may however be of considerable value, as demonstrated in our study with tryptophan, for investigating competitive binding effects of drugs with endogenous and other exogenous compounds and their concomitant clinical implications. The displacement of endogenous nutrients by drugs and other exogenous compounds is an area which merits far more attention.

When considering drug-plasma protein interactions, it is often tacitly assumed that the equilibrium is completely reversible, and the possibility of some irreversible binding is invariably ignored. Ironically the zonal gel chromatography technique, discarded in this study as unsuitable for reversible binding, could be of value for the measurement of irreversible binding. Exhaustive dialysis could be an alternative to this method. The presence of a small portion of the drug concentration irreversibly bound may potentially have several effects, for example, it may possess antigenic properties and this is presumably the case for morphine which binds irreversibly to γ-globulins (Ryan et al., 1971). Similarly the in vivo acetylation of human albumin by acetylsalicylic acid is suggested to produce antibodies against acetylsalicylic acid and acetylated albumin, as well as alter albumin's binding capacity for acetrizoate (Farr, 1971).

It has become relatively common to express in vitro binding data in terms of the number of binding sites \((n)\) on the protein and the association constant \((k)\) for the interaction. While this is obviously more informative than expression of the binding data as simply fraction or percentage bound, it is nevertheless not free from pitfalls. It is generally accepted that the graphical plot of choice for obtaining such information is that due to Scatchard (1949), however, while this method of data expression is ideally suited when the data can be fitted into a single linear plot, when curvature of this plot occurs then interpretation can be precarious. For accurate estimation of the binding parameters from the Scatchard plot it is essential that binding be determined over a wide drug concentration range. It is
particularly important when examining drug-protein interactions of high
affinity and involving a small number of binding sites, that a number of
determinations be made at low r values (moles drug bound/mole albumin) e.g. <1.
Even when this is accomplished it is frequently difficult to obtain sufficient
data points in the asymptotic slope corresponding to the primary binding site
(high affinity). Similarly for the secondary site (low affinity) at relatively
high r values. Whilst curved Scatchard plots can be analysed 'by eye', or by
'trial and error' solution of quadratic equations, the methods are tedious
and in the case of the former, liable to considerable error; solution by computer
analysis is preferable. The validity of some of the quoted n and k values in
the literature is highly questionable. Similarly controversy still exists as
to whether n values should be expressed as integer or non-integer values.
Possibly alternative graphical plots could be devised without the ambiguities
apparent with the Scatchard plot (e.g. log-plots). However, if the Scatchard
plot continues to be widely used then some attempt at standardisation of data
evaluation must be made. Perhaps all quoted values for n and k should be
qualified by the relevant r values over which the determinations were made,
other variables such as temperatures and pH must of course be quoted.
Alternatively, the use of thermodynamic parameters, enthalpy and entropy
may eradicate some of the problems associated with data evaluation.

The value of the establishment of binding parameters in assessing the
contribution of a particular physico-chemical parameter of the drug to the
binding was demonstrated in this study for the binding of a homologous series
of aliphatic carbamates to bovine serum albumin. This study also facilitated
an assessment of the importance of hydrophobic bonding in small molecule-
protein interactions. The carbamates were selected after careful consideration
of the requirements necessary for a study of hydrophobic bonding. Both the
percentage bound and binding constant (nk) for the carbamate-BSA interaction,
were found to be linearly correlated with the lipophilic character of carbamates. The presence of an aromatic ring (benzyl carbamate) did not detract from this relationship and possible steric effects, due to branching of the aliphatic side chain, could not be conclusively demonstrated. This linearity represents the fundamental relationship between the particular binding parameter and lipid solubility and departure from linearity thus must result from the superimposition of other variables. The slopes of these lines should be similar for other series of compounds whose binding is largely hydrophobic in nature, providing that similar experimental conditions prevail. However, the relationship between association constant ($k$) and lipid solubility was not so well correlated. The finding that the number of binding sites on albumin for the carbamates varied with chain length emphasised the need to consider both the parameters ($n$ and $k$) when investigating suspected correlations between binding and physico-chemical parameters. Where considerable variation in $n$ is apparent for a homologous series the parameter $nk$ is to be preferred.

The calculated free energy change for a single $\text{HC}_2 \cdots \text{CH}_2$ interaction showed reasonable agreement with that reported in the literature from studies on the aqueous solubility of hydrocarbons and would suggest that hydrophobic bonding is analogous to a partitioning effect. Nagwekar and Kostenbauder (1970), have suggested that estimation of the thermodynamic contribution of hydrophobic groups to form a drug-protein complex, may sometimes be obscured by the simultaneous contribution of protein molecules due to their 'conformational adaptability'. If this is true then the estimated free energy ($\Delta G$) values for hydrophobic bonding from the carbamate binding data may be of little quantitative significance, however, no evidence of conformational changes in the protein in our studies could be detected by uv. difference or fluorescence spectrophotometry. The calculated free energy change may be used as an approximate indication of the contribution of hydrophobic bonding in, for example, the interaction of long chain compounds. Determination
of the free energy of interaction for other functional groups (e.g. aromatic rings) may allow a theoretical assessment of the affinity of binding of a novel compound from a consideration of its chemical structure. The feasibility of such extrapolation awaits further systematic data collection.

At the relatively high carbamate-albumin ratios employed it is possible that other classes of binding site may have been missed. While no evidence for this was apparent for the lower homologues, some evidence of curvature was apparent for hexyl carbamate. Binding studies at lower carbamate concentrations using radioactively labelled carbamate should elucidate this. Although the association constants for the binding of carbamate to BSA were low (i.e. $10^3 - 10^4 \text{ M}^{-1}$), for long chain alkyl molecules the affinity of binding could be considerable. Comparison of the binding data of the carbamates with published values for the equivalent chain length carboxylic acids suggested that the presence of an ionizable group had little effect, however, this should be confirmed by determination of the fatty acid binding under the experimental conditions employed in our study. Similarly it would be of value to know whether carbamates bind to the same site as fatty acids and displacement studies should reveal this. Indirect evidence that they may bind to different sites is suggested by the inability of the carbamates to displace warfarin, whereas fatty acids are known to displace this drug. While hydrophobic bonding may be a major contributor to the binding of many drugs, it may not solely contribute to the binding affinity. Thus for warfarin hydrogen, hydrophobic and electrostatic bonds may be involved in its interaction with albumin.

Instead of investigating the binding of a drug or series of structurally related compounds directly, displacement could be employed. This approach has been demonstrated in the present work where the use of the fluorescent probe properties of warfarin has been made. The complete characterisation of the warfarin binding site on albumin would obviously considerably enhance the information available from such studies. The extent of displacement can of course be used to derive values for the association constant of the displacing
agent. Similarly, provided the displacer drug does not quench the intrinsic fluorescence of the protein, an association constant can be calculated if it competitively displaces another compound which does quench the fluorescence of the protein. Displacement studies can also be studied by non-spectroscopic techniques, especially if radioactively-labelled compounds can be employed. Such studies could be particularly valuable for potential displacing agents for which no simple means of assessment are available. It should be remembered, however, that quantitative information derived from such displacement studies only concerns those sites at which competition occurs, and no direct information is obtained regarding other sites. This fluorescence probe technique is particularly suitable for such studies in view of the speed with which studies can be performed, although the possible intrinsic fluorescence of the displacing agent must be accounted for in order to avoid erroneous conclusions. Such a technique may provide a valuable and rapid means of screening novel drugs, or combinations, for their displacement potential of warfarin. Other biologically important compounds namely; tetracyclines, bilirubin and kynurenine have also been reported to be potential fluorescent probes and these compounds could considerably extend the potential of this approach.

Attempts to characterise the warfarin binding site on albumin by modification of the protein structure have suggested that lysine, histidine and tyrosine may be involved in the binding of warfarin. Further studies on 'modified' albumins are needed to fully elucidate the nature of the sites. The primary, high affinity, warfarin binding site in human albumin may be compatible with the amino acid sequence in the neighbourhood of the lone tryptophan in human serum albumin, with hydrogen, hydrophobic and electrostatic bonding possibly participating in complex formation. Bonding may possibly occur with the lysine residue, although if this is the binding site for warfarin then interaction with the arginine may also occur. Determination of the binding of warfarin to glyoxal modified albumins could elucidate whether arginine is involved in this binding process. The warfarin binding site can
also bind other anions as witnessed by its competitive displacement of, for example, phenylbutazone and clofibrate. In our study, no competitive displacement of warfarin could be detected by any of the neutral or basic compounds investigated.

It has been suggested that studies of drug-albumin interactions might serve as useful models for more fundamental understanding of drug-protein interactions. We selected cytochrome P-450 as a suitable reference tissue protein to investigate this hypothesis because of the ease with which its substrate interactions could be identified. Our results suggest that a similarity in the nature of the environments of the binding sites on albumin and microsomal cytochrome P-450 does exist, and may imply that drug-albumin interactions would be suitable models for a more detailed characterisation of the hepatic microsomal interactions. Further characterisation of the similarities between albumin and microsomal systems should involve evaluation of numbers of binding sites and affinities of interaction, and more extensive displacement studies using either the fluorescent probe technique or conventional non-spectroscopic methods should be carried out. The potential of albumin-drug interactions for providing fundamental information on other protein-drug interactions including enzyme-substrate interaction, cannot be validly assessed at the present time. Information of the absolute configuration of a drug would be invaluable in typifying binding sites on a protein and enable models of binding sites to be constructed, this could lead to the design of better drug molecules. It is apparent that understanding the protein-drug interaction on a molecular level is essential to our understanding of drug action, as well as understanding the transport function of the plasma proteins for nutrients. Characterisation of the physico-chemical parameters influencing the binding of a drug to plasma and tissue proteins are essential to our prediction of the protein binding of a novel drug. For drugs in which the degree of binding is likely to be of importance (i.e. highly bound drugs) binding to several species including human albumin and human serum should be determined.
It may well prove possible in the near future, using either a practical or theoretical assessment of the binding of a drug to plasma proteins to predict, by the use of computer models, the effect of a drug's protein binding on its pharmacological action and duration.
Still round the corner there may wait
   A new road or a secret gate,
And though we pass them by today,
   Tomorrow we may come this way.

J.R.R. Tolkien.
REFERENCES


Chignell, C.F. (1969b) ibid 5, 455.
Chignell, C.F. (1970a) ibid 6, 1.


Crothers, D.M. (1968) Biopolymers 6, 575.


Cunningham, R.S. (1920) Amer. J. Physiol. 53, 488.


Curry, S.H. (1970b) ibid 22, 753.

Davis, B.D. (1946) Amer. Scientist 34, 611.
Dearden, J.C. and Tomlinson, E. (1971) ibid 23, Suppl. 68S.
Dettlebach, H.R. and Ritzmann, S.E., editors (1968) "Laboratory Notes for Medical Diagnostics". Published by Hoechst Pharmaceuticals Ltd., Brentford, Middlesex.
Donnan, F.G. (1924) Chem. Rev. 1, 73.
Fischer, J.J. (1971) in "Methods in Pharmacology" (A. Schwartz, editor), vol. 1, (pp.431-453)
Förster, T. (1951) in "Fluoroszervz Organischer Verbindungen" p.85 Vandenhoeck u Ruprecht, Gottingen.

Goldbaum, L.R. and Smith, P.K. (1954) ibid 111, 197.

Haaf, E. (Editor) "Laboratory Notes for Medical Dignostics" Hoechst Pharmaceuticals, Brentford, Middlesex.


Hansch, C. and Dunn, W.J. (1972) ibid 61, 1.


Hansch, C., Kiehs, K. and Lawrence, G.L. (1965) ibid 87, 5770.


Hertzler, A.E. (1919) "The Peritoneum" vol. 2, Mosby, St. Louis, Mo.


Jirgensons, B. (1962) ibid 96, 321.


Judis, J. (1972) J. Pharm. Sci. 61, 89.


J. Pharm. Soc. (Japan) 62, 536.


Karush, F. (1950b) ibid 72, 2714.


Keen, P.M. (1971) in "Handbook of Experimental Pharmacology"
(Brodie, B.B. and Gillette, J.R., editors) Vol. XXVII (Part i)


Keller, N., Sandelbeck, L.R., Richardson, U.I., Moore, C. and Yates, F.E.

Anal. Biochem. 48, 80.


Klotz, I.M. (1953) in "The Proteins (Neurath, H. and Bailey, K.,

Laiken, N. and Nemethy, G. (1970b) ibid 74, 4431.
Lavietes, P.H. (1937) J. Biol. Chem. 120, 267.
Marks, V. (1972) Teach-in (several articles, Aug., p.641; Sept., p.711; Nov. p,861; Dec. p.909)
Martin, B.K. (1965b) ibid 207, 959.


McQueen, E.G. (1969) ibid 36, 29.


Nemethy, G. and Scheraga, H.A. (1962b) ibid 36, 3401.


Osborne, W.A. (1906) J. Physiol. 34, 84.


Raz, A. (1972c) Life Sciences, 11, 965.


Scholtan, W. (1968) ibid 18, 505.


Thorp, J.M. (1964) in "Absorption and Distribution of Drugs" (Binns, T.B., editor), pp.64-76, E.S. Livingstone, London.
Verwey, W.F. and Williams, H.R. Jr. (1962b) Antimicrobial Agents and Chemotherapy, 484.


