INTERACTIONS OF CARBENOXOLONE SODIUM
WITH OTHER PROTEIN-BOUND DRUGS

A thesis for the degree of
Doctor of Philosophy
in the University of Surrey
by
PHILLIP CHARLES THORNTON

March 1979
TO MY WIFE, ZITA

AND TO

MY PARENTS.

Poisons in small doses are the best medicines, and useful medicines in too large doses are Poisons.

William Withering

1785
SUMMARY

The binding of carbenoxolone to crystalline bovine and human albumin was measured \textit{in vitro} by equilibrium dialysis, ultrafiltration and fluorescent probe techniques. Comparison of the results of these methods did not provide a good correlation, although it was clear that marked species differences in binding were evident with each method. Carbenoxolone and phenylbutazone share a single class binding site on bovine albumin, but are bound at different sites on human albumin.

A fluorescent probe technique was chosen to compare the binding of carbenoxolone with that of other drugs. The binding of the probe, l-anilino-8-naphthalene sulphonate acid, to human albumin revealed two distinct binding sites on human albumin, which enabled the drugs to be classified into three groups. Two of the groups bind at only one site whilst the third group was bound at both sites.

Carbenoxolone was the most strongly bound member of its group which included: ciclooxolone, indomethacin, enoxolone and cholesterol. The second group of drugs comprised: flufenamic acid, phenylbutazone, warfarin, tolbutamide and imipramine. The remaining drugs, bound at both sites, consisted of prednisolone, aspirin, ibuprofen, chlorpropamide and phenytoin. It was inferred that carbenoxolone would displace the other members of its group, would not displace the drugs in group 2, and may displace the drugs in group 3 from their common binding sites.

Models of interaction \textit{in vivo} were established using the rat, whereby phenylbutazone was shown to potentiate the pharmacological activity of warfarin, tolbutamide and chlorpropamide. In the same models carbenoxolone was without effect on these drugs. Neither phenylbutazone
nor carbenoxolone affected the activity of phenytoin in the rat; probably due to the relatively low protein binding of phenytoin in the rat.

Further studies in the rat demonstrated a decrease in the plasma half-life of warfarin and an increase in the whole blood half-life of tolbutamide when phenylbutazone was co-administered. Carbenoxolone did not alter the pharmacokinetics of these two drugs.

Serum concentrations of carbenoxolone in both rat and man were unaltered by the concomitant administration of warfarin, tolbutamide, chlorpropamide or phenytoin.

In conclusion, carbenoxolone was shown to be free from many of the displacement interactions demonstrated with other drugs which are bound to plasma proteins.
ACKNOWLEDGEMENTS

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Chapter 1

INTRODUCTION

It has long been recognised that the binding of various drugs to plasma proteins may influence profoundly the free concentration of such agents and thus markedly alter the magnitude and duration of the pharmacological response. Carbenoxolone sodium is a potent ulcer-healing drug which is extensively bound to plasma proteins and has, as yet, received little study for possible drug interactions. This introduction attempts to give the general background of drug interaction, protein binding and the drug carbenoxolone.

1.1 DRUG INTERACTION

A drug interaction is said to have occurred when the presence of one drug modifies the pharmacological activity of another (Stockley, 1971). In this context the 'second drug' may include food, food additives, pesticides, industrial chemicals, or any 'xenobiotics' (Parke, 1972a; Sapeika, 1976).

Modern drugs per se are usually selectively-toxic chemicals which exert their mode of action by a variety of specific actions. For example, selective toxicity to an organism, in the case of antibiotics, to malignant cells with cytotoxic agents, or by influencing normal physiological function as with hypoglycaemic agents, anticoagulants, oral contraceptives, diuretics and β-adrenergic blockers. When the site of action of these drugs is limited to its pre-designated site, side-effects, or toxicity per se may be low. However, if the drug enters into an interaction with another drug, or chemical, one of a number of consequences may ensue which are briefly summarised in Table 1.1.

Introduction of the practice of 'polypharmacy' has resulted in an increased risk of drug interaction. From
Table 1.1  SUMMARY OF POSSIBLE CONSEQUENCES OF DRUG-XENOBIOTIC INTERACTIONS

<table>
<thead>
<tr>
<th>Mode of interaction</th>
<th>Possible consequences</th>
</tr>
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<tr>
<td>Chemical</td>
<td>Failure of drug absorption</td>
</tr>
<tr>
<td></td>
<td><em>e.g.</em> di- and tri-valent metal salts with tetracyclines</td>
</tr>
<tr>
<td>Prevention of absorption</td>
<td>Failure to reach site of action <em>e.g.</em> cholestyramine with acidic drugs</td>
</tr>
<tr>
<td>Increase in drug metabolising enzyme activity</td>
<td>Increased drug metabolism <em>e.g.</em> phenobarbitone with phenytoin</td>
</tr>
<tr>
<td>Prevention of metabolism and/or excretion</td>
<td>Potentiation, by failure to remove drug from site of action <em>e.g.</em> probenecid with penicillin</td>
</tr>
<tr>
<td>Displacement from plasma binding sites</td>
<td>Potentiation, by increase of drug concentration at its site of action <em>e.g.</em> phenylbutazone with warfarin</td>
</tr>
</tbody>
</table>
a recent survey (Editorial, 1973), it is evident that one in five British women of child-bearing age is taking an oral contraceptive agent, and one in six of the adult population is taking a psychotropic drug. From these two classes of drug alone it is shown that about one in sixty of the population is regularly taking two drugs. Even this rate of interaction does not include over-the-counter medications, methylxanthines in beverages, alcohol intake, cigarette smoking or environmental pollutants.

Thus studies of drug interactions are useful in that they may lead to the ability to predict and avoid them before they occur clinically in man.

The key factor in drug interactions may be seen, from the earlier definition, to be bioavailability. The bioavailability of a drug can be altered in many ways. Let us first consider the types and consequences of drug interactions:

1.1.1 CHEMICAL INTERACTIONS

This is essentially a pharmaceutical problem and has been considered under the guise of compatibilities.

The problem of compatibility arises less in present times than formerly, due to compound prescription 'powders', 'mixtures' and 'pills', being replaced by the unit dosage 'capsule', 'tablet' or 'proprietary mixture'. However, in the practice of ward pharmacy it is very relevant. The addition of drugs to intravenous fluids is considered a safe and convenient route of administration, especially in unconscious patients or those who are susceptible to vomit an orally administered drug. In a recent survey of ten hospitals, 39% of intravenous administrations over a period of one month (7,900 infusions) had had at least one drug included in the intravenous infusion (D'Arcy & Thompson, 1974). Interactions which occur tend to be the
result of an incompatible physico-chemical environment for the added drug. 'Salting out' of sparingly soluble antibiotics such as ampicillin can readily occur with the addition of other drugs or electrolytes. The mixing of drug solutions in the syringe is also subject to similar restrictions of changes in pH or in complex formation. Such an example is the mixing of soluble insulin and protamine zinc insulin whereby excess zinc ions combine with the soluble insulin to form the delayed complex and thus result in inadequate immediate glucose-lowering activity (Alstead, et al, 1971; Goodman & Gilman, 1975b).

The co-administration of di- and tri-valent metal ions such as aluminium, calcium, magnesium and iron, with tetracyclines greatly reduces serum levels of the antibiotic (Levy, 1970; Neuvonen, et al, 1970). The efficacy of early formulations of tetracyclines was reduced by the use of calcium phosphate, a common 'filler' of capsules.

1.1.2 DRUG ABSORPTION INTERACTIONS

Many factors may influence the absorption of drugs from the gastro-intestinal tract, a primary influence being tablet disintegration.

A reduction of gastric pH favours absorption of acidic drugs such as salicylates, phenylbutazone, warfarin and phenytoin by promoting the unionised lipid-soluble forms of the drug. Conversely the co-administration of antacids will reduce the absorption of such drugs as tetracyclines (Barr, et al, 1970).

Anticholinergics and opiates delay gastric emptying and may thus delay or reduce the absorption of other drugs. A reduced peristaltic activity may also retard tablet dissolution rates. Nimmo and colleagues' (1973) have shown that propantheline bromide delayed gastric emptying and
slowed the absorption of paracetamol. Again the converse situation is to be considered. An increased absorption of alcohol and paracetamol is seen with such drugs as metaclopramide which increase gastric emptying rates (Stockley, 1974).

Ionic complexes may be formed in the intestinal tract which prevent drug absorption, such as with calcium and tetracyclines (section 1.1.1). Cholestyramine is an ion-exchange resin with an affinity for acidic molecules and is used to increase the excretion of bile salts by ion-exchange in such conditions as pruritis of partial biliary obstruction. The consequent prevention of fat and cholesterol absorption may lead to steatorrhoea and inhibition of absorption of the lipophilic vitamins A, D and K. The malabsorption of vitamin K is of importance in anticoagulant therapy with warfarin (see later this section). Cholestyramine can also form complexes with acidic drugs such as aspirin, phenylbutazone and warfarin, thus interfering with their intestinal absorption (Gallo, et al, 1965; Koch-Weser & Sellers, 1971).

One drug may directly affect the absorption of another, and it is well known that barbiturates may inhibit the absorption of coumarin anticoagulants (Stockley, 1974). Whilst it is believed that induction of drug-metabolising enzymes by barbiturates (section 1.1.3) may be a factor in the reduced anticoagulant activity of the coumarins during co-administration of barbiturates, Aggeler and O'Reilly (1968) have shown that heptabarbitone will delay the absorption of bishydroxycoumarin. Similarly phenobarbitone will delay absorption of griseofulvin (Riegeleman, et al, 1970) and p-aminosalicylic acid will delay rifampicin absorption (Boman, et al, 1970). The latter interaction was of particular interest because of the nature of combined therapy in tuberculosis treatment. However, it is now known (Griffin & D'Arcy, 1975a) that it was not p-ami-
salicylic acid itself but bentonite in the granulation which was preventing absorption. This is of even greater significance in that bentonite is a constituent of many proprietary and ethical mixtures.

Increased lipid absorption, from a high dietary fat intake, leads to the more ready absorption of lipophilic drugs such as griseofulvin and the anthelmintic, trichlorethylene. The increased absorption may be beneficial in the case of griseofulvin but not with trichlorethylene (Crounse, 1961). Decreased intestinal absorption is often an advantage with such preparations as the anthelmintics bephenium hydroxynaphthoate and trichlorethylene which show increased efficacy and decreased toxicity if they are not absorbed.

Antibiotic therapy suppresses the production of vitamin K by intestinal microflora and is thus often considered to potentiate anticoagulant therapy. Neomycin is known to potentiate warfarin (Udall, 1965; 1970) but it is understood that both a reduction of intestinal bacteria and a reduced dietary intake of vitamin K are necessary to produce symptoms of vitamin K deficiency in man (Editorial, 1968). However, it is known that neomycin can induce steatorrhoea (Falcon, et al, 1966) and it is by this means that neomycin is likely to cause malabsorption of vitamin K, from whatever source. Other antibiotics may only reduce bacterial vitamin K production.

1.1.3 INTERACTIONS INVOLVING THE DRUG-METABOLISING ENZYMES

Induction of the drug-metabolising enzymes, which can lead to tolerance by a more rapid deactivation of the drug, is well known with such drugs as barbiturates, imipramine and phenylbutazone. When one drug induces drug-metabolising enzymes which also affect the metabolism of a second drug, then this constitutes an indirect interaction.
About 200 drugs are known to induce the microsomal hydroxylating enzymes of the liver, including the cytochrome P450 system (Griffin & D'Arcy, 1975b). The ability of cytochrome P450 to catalyse other metabolic processes including dealkylation, oxidative deamination and sulphoxidation leads to the ability of one drug to stimulate the metabolism of other drugs which are not structurally related (Pearson & Havard, 1974).

Cigarette smokers also have an increased enzymic ability to metabolise 3,4-benzpyrene or related polycyclic hydrocarbons and show an increased metabolism of nicotine (Conney, et al, 1957; Beckett & Triggs, 1967). Kerri-Szanto and Pomeroy (1971) not only demonstrated an increased metabolism of the analgesic pentazocine in smokers compared with non-smokers, but also in urban compared to rural dwellers. Other environmental xenobiotics, including chlorinated hydrocarbon pesticides, are also known to stimulate the drug-metabolising enzymes (Hart, et al, 1963). Barbiturates as a class are well known to induce liver microsomal enzymes and the combination of phenobarbital with phenytoin in epileptic patients is a well-established therapy. Phenobarbital will lower plasma levels of phenytoin and induce the more rapid excretion of the latter (Reynolds, 1967). The resulting lowered anticonvulsant activity of phenytoin is not clinically significant in that phenobarbital itself possesses similar anticonvulsant activity. However, if the phenobarbital is withdrawn, plasma levels of phenytoin increase and may lead to phenytoin toxicity. Similarly, barbiturates decrease the activity of warfarin (Levy, et al, 1970), griseofulvin (Riegeleman, et al, 1970) and oestrogens (Conney, 1967).

Conversely, drugs which inhibit liver microsomal enzymes are likely to potentiate the activities of other drugs, such as chloramphenicol, with oral hypoglycaemics
(Christensen & Skövsted, 1969) and monoamine oxidase inhibitors (MAOI) with barbiturates (Domino, et al, 1962). Most drugs which induce liver microsomal enzymes will initially demonstrate an inhibition prior to induction. In the case of MAOIs, potentiation of natural amines present in foods, such as cheese, yeast extracts and pickled herrings, may occur leading to hypertensive crises (Blackwell, 1963; Nuessle, et al, 1965; Blackwell & Marley, 1966; Cooper, 1967). It is thought probable that the mode of action of chloramphenicol in potentiating the anticoagulant activity of coumarol is by depression of coumarol biodegradation in the liver causing a 3- to 4-fold increase in both the plasma half-life and the activity of the anticoagulant (Christensen & Skövsted, 1969). Similarly, tolbutamide and phenytoin are potentiated by chloramphenicol.

1.1.4 DRUG INTERACTIONS INVOLVING EXCRETORY MECHANISMS

The principal routes of excretion of drugs and their metabolites are via the urine and bile (Parke, 1968a). Agents such as general anaesthetics may reduce renal blood flow and thus decrease the glomerular filtration rate, reducing drug excretion in general. Diuretics may, by increasing urine flow, cause an increased urinary excretion of drugs. For example, indomethacin efficacy is reduced by concomitant administration of frusemide (Brooks, et al, 1974a). Most drugs which are not protein-bound pass into the renal tubule lumen and are reabsorbed by simple diffusion or active transport.

As most drugs are weak electrolytes, only the unionised forms, being lipid soluble, are accessible to passive reabsorption. Weak bases such as amphetamine, imipramine and pethidine, are less easily reabsorbed from urine at a low pH and are thus excreted more readily. At a urine pH of 5.0, 50% of a dose of amphetamine is excreted by man within 16 hours, whereas at pH 8.0 only
3% is excreted in the same time (Beckett, et al, 1965). Many acidic drugs are actively secreted in the proximal tubules. Thus competition between drugs for this active transport mechanism may lead to a prolonged plasma life of the displaced drug. Phenylbutazone will prevent the renal excretion of hydroxyhexamide, an active metabolite of acetohexamide, leading to an enhanced hypoglycaemic activity (Field, et al, 1967).

Probenecid is used to reduce the renal tubular secretion of penicillin by competing for the active transport mechanism, probenecid itself being actively reabsorbed at a later stage to continue its potentiation of the antibiotic. This interaction with probenecid is particularly useful in the treatment of cases of cholecystitis, whereby the biliary excretion of penicillin and of the semisynthetic penicillins is increased (Sales, et al, 1972). Similarly, probenecid potentiates indomethacin activity by reducing its excretion (Brooks, et al, 1974b). Probenecid is also used as a uricosuric agent, exerting its activity by competing with uric acid for active reabsorption.

The extent of biliary excretion of a xenobiotic is influenced by physico-chemical factors including molecular weight (Smith, 1973). Biliary-excreted drugs are concentrated in the bile by active transport (Parke, 1968a) and, presumably, simultaneously-administered drugs may compete for this active process.

1.1.5 PHYSIOLOGICAL INTERACTIONS

Interaction between competitive blocking agents and endogenous substances, such as atropine and acetylcholine, mepyramine and histamine, bromolysergic acid and 5-hydroxytryptamine, are well known but are rarely considered with drug interactions because the consequences of such interactions are the object of the use of the
drugs. However, where the mode of action of one or more
drugs lies in the alteration of a physiological mechanism,
then the results of concomitant use may result in
interaction. Drugs which act on the adrenergic neurone
illustrate this principle well.

The guanide adrenergic blocking agents, such as
guanethidine and bretylium, exert their activity by
preventing the release of noradrenaline from the
adrenergic neurones. In order to exert this influence
they must be taken up into the neurone by the active
mechanism which takes up noradrenaline back into the
neurone. Tricyclic antidepressant agents, such as
imipramine and amitriptyline, are potent competitive
inhibitors of the noradrenaline uptake mechanism so that
these drugs will depress the hypotensive actions of the
guanides (Leishman, et al, 1963). There is some evidence
that the guanides, in reducing noradrenaline release,
render the adrenergic receptor more sensitive to
stimulation. Thus, if directly-acting sympathomimetic
drugs are given to guanide-treated patients they may
cause an increased adrenergic reaction which is prolonged
due to competition for noradrenaline uptake between the
guanides and the sympathomimetics (Boura & Green, 1962).
Similarly, indirectly acting sympathomimetics antagonise
the hypotensive activities of the guanides (Boura & Green,
1962; Sneddon & Turner, 1969). In reserpinised patients
or animals, where the noradrenaline stores are depleted,
directly-acting sympathomimetic agents have enhanced
activity due to increased receptor sensitivity. Conversely,
indirectly acting sympathomimetics, which rely on inducing
noradrenaline release, are reduced in activity (Moore &

1.1.6 INTERACTIONS AT PLASMA BINDING SITES

After absorption many drugs enter the blood and bind
to plasma proteins, usually albumin, and their tissue
distribution is often a function of their protein binding affinity. This binding to plasma proteins is reversible and in a constant state of equilibrium. It is the free or unbound portion that is generally considered to be the bioactive component of the drug (Rolinson, 1964). Where two drugs share a common binding site they will compete with each other for that site. A drug with a high affinity for a binding site will quantitatively displace a drug with a lower affinity, resulting in an increased level of free displaced drug. If the displaced drug is only bound to a small extent, say 50%, then a displacement of 5% is of little pharmacological consequence; whereas if a drug which is extensively bound, say 98%, is displaced so that only 96% is bound, this represents a two-fold increase in the free concentration of the drug and potentiation of its activity (Prescott, 1969). This type of interaction is demonstrated between warfarin and phenylbutazone.

Both warfarin and phenylbutazone are highly bound to plasma proteins at around 99% and 98% respectively, but phenylbutazone has the greater binding affinity and will displace warfarin, potentiating its anticoagulant effect by increasing the free concentration of the drug in the liver (Aggeler, et al, 1967; Udall, 1970; O'Reilly & Levy, 1970). It is interesting to note that the plasma half-life of warfarin is reduced as a consequence of more drug being available for hydroxylation in the liver, thus the potency is enhanced but not the duration of action. There has been a suggestion that enzyme induction by phenylbutazone is responsible for the decreased plasma concentration of warfarin, but this has only been demonstrated in animals and occurs only after the first few days of treatment (Welch, et al, 1969).

Similar displacement interactions have been reported from studies in vitro between phenylbutazone, warfarin,
indomethacin and tolbutamide, the binding affinities decreasing in the order given, thus each drug may be displaced by those preceding it (Assandri & Perazzi, 1976). Displacement interactions in vivo are seen between warfarin and mefenamic acid (Sellers & Koch-Weser, 1970; 1971), sulphonamides and sulphonylureas, phenylbutazone and sulphoxidine (Wardell, 1971).

A delayed interaction is precipitated after withdrawal of chloral hydrate from warfarin-treated patients. Chloral hydrate is metabolised to trichloracetic acid, which is strongly bound to plasma proteins and will displace warfarin from plasma binding sites. However, the anticoagulant effect of warfarin is not increased because chloral hydrate also induces a hydroxylating enzyme resulting in lower plasma warfarin concentrations. Thus whilst both drugs are given there is no pharmacological change but withdrawal of the hypnotic and maintenance of the same dose of anticoagulant has produced fatal haemorrhage (Cucinell, et al, 1966).

1.2 PROTEIN BINDING

Interaction of small organic ions with proteins has been the subject of multidisciplinary research for many years (Goldstein, 1949). Early investigations dealt largely with the qualitative aspects of organic binding on both acidic and basic sides of the protein iso-electric point. The organic anions investigated were often dyes, probably due to the relatively limited technology available to assay the anions themselves. However, quantification and interpretation of binding data incorporating the law of Mass Action was introduced in studies on bovine serum albumin fractions by Klotz and co-workers (1946; 1948) and Scatchard (1949). The field of study was expanding by this time and has been the subject of several comprehensive reviews commencing with the classical review of Goldenstein (1949), and followed.

1.2.1 DESCRIPTION AND DEFINITION

Protein binding is a reversible interaction between a small molecule and a protein. This binding is analogous to the enzyme-substrate interaction except that it does not result in degradation or formation of new products; it is also analogous to most drug-receptor complexes where covalent binding does not occur. This interacting small ion is sometimes referred to as a ligand.

The ionic interaction forces involve hydrogen bonding, hydrophobic bonding, electrostatic forces and van der Waal forces. The relative importance of these influences has been compared by Davison (1971), who reviewed various measurements on related structures (see Table 1.2). The exact contribution or importance of each type of inter-molecular force in protein-ligand binding is unknown but it has been debated that hydrogen bonding is not dominant whilst hydrophobic interactions are important (Klotz, 1973).

These suggestions were based on the positive entropies associated with hydrophobic ligands but were also found to be present with non-polar ligands where only electrostatic forces were involved (Klotz & Fiess, 1951; Kauzmann, 1959). Changes in enthalpy have been taken as indicators of van der Waal involvement in binding (Steinhardt & Reynolds, 1969). Hansch and co-workers (1965) derived a model of binding which was mechanistically similar to phenol partition between water and octanol and concluded that binding was non-specific and due to hydrophobic bonding.

However, only where studies using X-ray crystallography have been used can an evaluation of precise binding
Table 1.2  STRUCTURAL FACTORS AFFECTING PROTEIN BINDING

<table>
<thead>
<tr>
<th>Parent structure</th>
<th>Additional group</th>
<th>Binding change</th>
<th>Example</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>Hydrogen bonding</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzene</td>
<td>Ionising</td>
<td>increase</td>
<td>benzoic acid &gt;phenol&gt; benzamide</td>
<td>(Davison, 1971)</td>
</tr>
<tr>
<td></td>
<td>substituent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>Polar</td>
<td>increase</td>
<td>hydroxybenzoic&gt; benzoic</td>
<td>(Davison, 1971)</td>
</tr>
<tr>
<td></td>
<td>substituent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>Ortho-</td>
<td>increase</td>
<td>o-hydroxy&gt; m- &amp; p-</td>
<td>(Klotz, 1948)</td>
</tr>
<tr>
<td></td>
<td>substituent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrophobic bonding</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fatty acid</td>
<td>Lipophilic</td>
<td>increase</td>
<td>binding increases with chain length</td>
<td>(Teresi &amp; Luck, 1952)</td>
</tr>
</tbody>
</table>
locations be made. Such a study is that of Arnone (1972), on diphosphoglycerate binding to haemoglobin, where it was found that electrostatic forces were responsible for this binding.

As the binding of ligands is reversible, the complexes will obey the law of Mass Action and will be in a state of equilibrium.

\[ [P] + [D] \rightleftharpoons [PD] \]

where
- \([P]\) = free protein concentration
- \([D]\) = free drug concentration
- \([PD]\) = drug-protein concentration

Thus at equilibrium an apparent association constant \((K_a)\) may be formulated:

\[ (K_a) = \frac{[PD]}{[P][D]} \]

Thus it is clear that the proportion of a bound ligand will be related to the apparent affinity constant \(K_a\) and the number of binding sites per molecule.

1.2.2 FUNCTION OF PROTEIN BINDING

That protein binding exists as a specific function is highly probable because specific binding proteins have been discovered, in plasma, to bind hormones like testosterone and thyroxine and substances such as vitamins A and \(B_{12}\) and corticosteroids. The binding is likely to be a means of transportation in an inactive form and has been described at length (Desgrez & De Traverse, 1966; Steinhardt & Reynolds, 1969).

Of the blood proteins albumin is by far the most common substrate for binding of all classes of ligand. Albumin may have a low affinity for the specific ligands mentioned above but a high binding capacity; whereas the
specific binding proteins, such as corticosteroid-binding globulin, bind steroids like prednisolone with a high affinity but low capacity. When the binding sites on corticosteroid-binding globulin are fully occupied the steroids bind to albumin with a low affinity but a high capacity. In consequence, at low albumin levels (2.5g/dl), side-effects due to free prednisolone appear (Lewis, et al, 1971). Fatty acids and prostaglandins are known to bind well to albumin, which may prove to be the transport protein for these endogenous substances (Brodie, 1966; Gugler, et al, 1974; Gueriguian, 1976). It is also probable that protein binding plays a role in the dietary absorption and transport of such hydrophobic molecules as fatty acids. Most drug-protein binding studies investigate this binding and transport function which is usurped by the 'xenobiotics' and may affect the drug activity in some of the ways mentioned in the following section.

1.2.3 PROTEIN BINDING INFLUENCES

Drug absorption and distribution

It is widely accepted that, because macromolecules and macromolecular complexes pass across membranes only with difficulty, plasma protein binding can influence the distribution pattern of drugs. A drug of low aqueous solubility will have a higher plasma concentration if it binds to plasma proteins, thus increasing its apparent solubility gradient between the gut and blood (Goldstein, 1949; Brodie, 1966). This raised plasma concentration will thus increase the drug's availability to other tissues and hence its efficacy.

Martin (1965) produced a theoretical model of drug binding and correlated drug availability with various binding affinities. He stated that a drug is distributed within two aqueous compartments, plasma and tissue fluid, and that it is in a state of equilibrium between the two.
Thus a highly bound drug at low dose levels will be concentrated in the plasma compartment, whilst at the threshold of saturation a small increase in the dose level will greatly increase the concentration of available drug. Similarly a less strongly bound drug will achieve a higher tissue concentration. Martin concluded that a change in the protein binding will significantly alter the distribution of highly bound drugs only where the free concentration is small.

Metabolism and excretion

Where a drug is strongly bound to plasma proteins, the concentration in the liver may well be much less than the plasma concentration and, in consequence, the rate of metabolism may be slow. Levy and co-workers have demonstrated this effect with the coumarin anticoagulants. The ratio between liver and plasma drug concentrations reflects the degree of plasma binding and, for bishydroxycoumarin, is higher in the rat than in man (Nagashima, et al., 1968). In consequence the plasma half-life of bishydroxycoumarin is less in the rat than in man. The free fraction of warfarin in plasma is variable between individuals and is not related to total plasma concentration; however, the clearance rate is directly proportional to the free concentration of the drug (Levy & Yacobi, 1974). Furthermore, the pharmacological potency of warfarin was directly correlated with its biological half-life (Yacobi, et al., 1974). Similar results were obtained for dicoumarol (Jähnchen & Levy, 1974).

These results from animal studies have been confirmed clinically where a hypoalbuminaemic patient (1.6g/dl) proved to be resistant to the pharmacological action of warfarin and the plasma half-life of the drug was six hours in comparison with the normal two days (Lewis, et al., 1967). In a study of cardiovascular patients, who had been individually established on warfarin therapy, the
variation of free warfarin concentration was less than the variation of total warfarin concentration. However, no correlation was seen between prothrombin times and the free concentration of warfarin, indicating that, in man, variables other than protein binding were affecting the anticoagulant action (Yacobi, et al., 1976a). The intrasubject levels in these patients were unaltered after three to six months' therapy, indicating that intersubject variations were not due to enzyme induction (Yacobi, et al., 1976b).

Thus these findings with warfarin clearly demonstrate that its metabolism and pharmacological consequences are governed, in part, by the degree to which the drug is bound to plasma proteins.

Similarly, studies with propanolol have shown that its plasma half-life is directly proportional to its degree of plasma binding in animals and in man (Evans, et al., 1973; Evans & Shand, 1973). Further studies have shown the protein binding of phenytoin (Lund, et al., 1972), sulphonamides (Seydel, 1970) and naproxen (Runkel, et al., 1974) to influence the plasma half-life and/or clearance of each drug. It has also been suggested (Glassman, et al., 1973) that variations in the binding of imipramine, between 5 and 23%, may account for the inability to correlate plasma levels, plasma clearance and clinical response. However, with such a weakly bound drug it is possible that other factors are responsible.

The association-dissociation rates between albumin and ligands are so rapid that they probably do not directly affect the rate of metabolism of the ligand (Meyer & Guttman, 1968). For example, phenylbutazone is highly bound to plasma proteins in several animal species including man; whilst, in most species except man, it is rapidly excreted (Brodie, 1962).
Protein binding in diseased, neonatal and elderly patients

Albumin is the principal binding protein in plasma and its concentration in plasma may be lowered from normal in certain clinical conditions such as renal dysfunction, burns, post-surgery, bronchitis and other chronic diseases. Plasma albumin concentration may also be considered 'abnormally low' in neonatal and elderly subjects (Reidenberg, 1974; Jusko & Gretch, 1976; Vallner, 1977). If albumin concentrations are lowered then the capacity for drug binding is lowered, either leading to an increased concentration of free drug at normal dosage, or to an increased danger of displacement interaction. The rapid elimination of warfarin in a hypoalbuminaemic patient was mentioned previously (Lewis, et al, 1967).

Disease. The ratio between extravascular and intravascular albumin (normally 1.2 to 1.8) is altered in many diseased states, and in particular by prolonged bed rest. Five paraplegic patients confined to bed, post-operatively, for two months had an extravascular/intravascular albumin ratio (EV/IV) more than twice normal (Plantin, et al, 1971). Various surgical procedures, especially abdominal surgery, are known to reduce plasma albumin levels by as much as 0.5g/dl (Mourisden, 1967). Burn injury understandably gives rise to albumin loss, because the largest extravascular compartment of albumin is contained in the skin (Birke, et al, 1968). Albumin concentrations fall directly with the progress of neoplastic diseases with an average fall in one study of 1.0g/dl (Midler, et al, 1950; Steinfeld, 1960).

Patients with renal dysfunction may have a lowered albumin concentration but often have a raised plasma urea concentration which can reduce the binding capacity of the albumin, especially for acidic drugs. A reduced plasma binding of phenytoin in hyperuraemic patients was observed which was not directly dependent on albumin or
total protein concentrations, or related to the presence of dialysable products in the plasma (Reidenberg, et al., 1971). These results were confirmed by Blum and colleagues (1972) who also demonstrated that the principal metabolite of phenytoin was not responsible for a competitive inhibition. Andreasen (1973) has also observed a reduced binding of phenytoin in hyperuraemic patients but was later able to demonstrate an increased binding of phenytoin in vitro in dialysed plasma from both normal and hyperuraemic patients (Andreasen, 1974). These findings suggested either (a) the presence of a non-dialysable competitor; (b) the presence of a very highly bound ligand; or (c) a physical alteration in the albumin quaternary structure. The latter is also suggested by Shoeman and colleagues (1973). Both normal and hyperuraemic patients stabilised on phenytoin therapy have a similar plasma concentration of free drug (Odar-Cederlöf & Borgå, 1974), whilst a subsequent study has shown that the distribution of phenytoin between plasma and erythrocytes is altered in hyperuraemic patients (Ehrnebo & Odar-Cederlöf, 1975).

As the liver is the site of synthesis of albumin, liver dysfunction may be expected to result in low plasma albumin concentrations, associated with a decreased total drug-binding capacity. Cirrhosis is a common, complex disorder often associated with a history of chronic alcoholism (Kradjan, 1973). Although plasma albumin concentration is low in cirrhotic patients, the total albumin mass is only slightly reduced, due to an expanded plasma volume, whilst the EV/IV ratio is also slightly lowered (Dykes, 1968). Further studies on alcoholic liver disease patients have shown that, as a group, they bind basic drugs less well than normal patients, free concentrations of quinidine being increased three-fold. However, the plasma protein binding of acidic drugs varied, without a direct correlation with liver disease (Reidenberg, 1974; Affrime & Reidenberg, 1975).
Viral hepatitis is also responsible for lower albumin synthesis and consequent reduction in circulating albumin (Jusko & Gretch, 1976). Phenytoin binding is reduced by up to 30% in viral hepatitis patients (Blaschke, et al, 1975).

Age. Drug disposition in neonates is different to that in adults partly due to the greater body water content, and lower fat content, of neonates. Therefore, protein binding plays an important rôle in the regulation of pharmacokinetics in neonates (Yaffe & Juchau, 1974). Bound fractions of phenytoin and salicylate are lower in neonates than in adults. The increased concentrations of the free drugs were paralleled by an increase in serum bilirubin concentration, suggesting a possible competitive displacement of the drugs from serum albumin by bilirubin (Krasner, et al, 1973). Nafcillin was very much less bound in the neonate, although a subsequent study revealed that albumin was not the primary binding site of this drug (Krasner & Yaffe, 1975).

Although neonatal plasma contains less albumin there is evidence to conclude that the reduced binding of drugs is due largely to competition from free fatty acids and bilirubin (Fredholm, et al, 1975). Two studies comparing the binding of phenytoin, imipramine, diazoxide and cephalothin in neonates and adults demonstrated a correlation between the lower binding in the neonate with decreased plasma albumin concentration, and also revealed that neonatal hyperbilirubinaemia was a contributing factor (Pruit & Dayton, 1971; Ehrnebo, et al, 1971).

The elderly are known to have a lower plasma albumin concentration than younger subjects, possibly associated with a decreased mobility (Woodford-Williams, et al, 1965). This puts the elderly in the category of hypoalbuminaemic patients, mentioned earlier. Hayes and colleagues (1975a;
1975b) have demonstrated a decreased total binding capacity for warfarin and phenytoin in the blood plasma of the elderly. Changes in binding affinities were not altered in elderly patients and the decrease in clearance rate of phenytoin correlated with decreased albumin levels. Yacobi and colleagues (1976a) in a study of mixed young and elderly patients showed a similar correlation of warfarin clearance with the plasma concentration of free drug. However, in a subsequent study in elderly patients, the clearance rate of carbadoxolone sodium was decreased even though the total binding capacity was also decreased because of lower albumin concentration (Hayes, et al, 1977). Thus there appears to be no structural impedance to the binding of drugs to albumin in the elderly, only a reduced total capacity for binding. This, together with a possible decreased hepatic clearance, may lead to higher plasma concentrations of drugs on repeated dosage and thus adverse reactions.

Species difference

Differences in protein binding of drugs such as warfarin between young and elderly patients and between healthy individuals have been mentioned. It is not surprising therefore that correlation between animal species and man is often a non sequitur.

Phenytoin is bound more strongly to plasma proteins in man than in the rat, the difference being reflected in the longer plasma half-life in man (Conard, et al, 1971). It is interesting to note that the principal metabolite of phenytoin, p-hydroxyphenytoin, is bound less strongly in man than in the rat, the free levels of the metabolite being associated with gingival hyperplasia in man but not in the rat. Similarly the plasma half-lives of propranolol and diazepam, together with their metabolites, have been correlated with the plasma binding affinities in several species. In the species where the degree of binding is high the plasma half-life is long, so that only for some
species may these drugs be considered to be highly bound (Evans, et al, 1973; Klotz, et al, 1976). Rieder (1963) has reported a correlation between drug distribution and the unbound drug fraction for various sulphonamides in the rabbit. Ruiz-Torres and Meinig (1965) could find no such correlation in the rat.

Studies with bovine and human serum albumin in vitro have indicated that the presence of fatty acids minimised the observed differences in testosterone binding between the albumins of the two species (Ryan & Chopra, 1976). The number of binding sites for testosterone varied between 9 and 10 for untreated human albumin and between 5 and 6 for de-fatted albumin. In contrast, for bovine albumin there was only 1 site on the untreated material which became 5 for the de-fatted albumin. These results provide the unusual data that the presence of fatty acids increases the binding of testosterone in contrast to the frequently seen reductions in binding (Chen, 1967). Both imipramine and its metabolites are bound to bovine serum albumin to a greater extent than to human serum albumin (Weder & Bickel, 1970). Low concentrations of salicylate have a higher affinity for human serum albumin than for bovine serum albumin, leading to the suggestion that the difference in binding involves \( \epsilon \)-amino and possibly guanido groups (Davidson & Smith, 1961). Using a gel filtration technique to measure salicylate binding to plasma proteins, Sturman and Smith (1967) were able to differentiate between various species where either a low- or high-order binding affinity for salicylate was observed. Baboon, rat, mouse, horse, dog, toad, rabbit and turkey formed a group with a low-order affinity for salicylate, and man, monkey (macaca mulata) and guinea pig, possessed a high-order affinity, suggesting that those in the low-order group are deficient in \( \epsilon \)-amino and guanido groups on their plasma albumins. In contrast, Kucera and Bullock (1969) used equilibrium dialysis to measure the binding of salicylate to plasma
proteins and their results also enabled differentiation between species with high- and low-order affinity for salicylate. Rat and dog possessed a low-order affinity, while man, green monkey, rabbit and guinea pig had a high-order affinity. It was suggested that the differences in the results with rabbit albumin, between these authors and others (Sturman & Smith, 1967), were due to the different methodology, in that Sephadex Gel is known to bind acidic drugs such as salicylate.

In contrast, similar binding affinities for a range of prostaglandins have been observed for both human and bovine serum albumin (Gueriguian, 1976), and for prostaglandin E₂ with both rat and human plasma in vivo (Raz, 1972). However, the affinity for prostaglandin binding was relatively low in comparison with the drugs which demonstrated species differences above. Paracetamol is only weakly bound to plasma proteins (20%) and its binding characteristics are similar in man and pig (Gazzard, et al, 1973). A calcium-binding protein from the gastro-intestinal tract showed only small differences in affinity but was remarkably similar in chicken, rat, pig and man, showing surprisingly little difference between such varied species (Hitchman & Harrison, 1972).

**Displacement interaction**

Some aspects of displacement interactions were described under the drug interaction section (section 1.1.6). Whilst all the above factors influencing the protein binding of drugs can alter the pharmacological activity of the drug, it is displacement of drugs from plasma binding sites that receives the greatest clinically-orientated attention. There are very few new drug 'data sheets' issued, especially for anti-inflammatory or anticoagulant drugs, which do not give some warning of possible displacement interactions.

Earlier in this section the transport role of plasma proteins for fatty acids was mentioned. As a result of an
increased metabolism, after exercise, the plasma concentrations of free fatty acids are elevated. Diazepam was observed to be displaced by elevated free fatty acid concentrations, but only in studies in vitro (Tsutsumi, et al, 1975). A study of rats in vivo has demonstrated that free fatty acid concentrations, elevated by exercise, have displaced warfarin and phenytoin from plasma binding sites (Gugler, et al, 1974). However, such displacement was not demonstrated with phenytoin in vivo and warfarin in vitro in man (Borgå, et al, 1978).

Displacement from plasma binding sites is of considerable importance with antibiotics and sulphonamides, where the free drug is the active form (Witzgall & Boyens, 1964; Rolinson & Sutherland, 1965). A study of the ability of anti-inflammatory agents to displace urate from plasma proteins in vitro led to a clinical evaluation, as uricosuric agents, of four of the drugs studied (Whitehouse, et al, 1973). Subsequently an association between hypouricaemia and hyperbilirubinaemia was established demonstrating the ability of bilirubin to displace urate from plasma binding sites (Schlosstein, et al, 1973).

1.3 CARBENOXOLONE SODIUM

Carbenoxolone sodium is the first drug which has convincingly been shown to accelerate the healing of gastric ulcer (Avery Jones, 1968). This much-investigated drug has been the subject of six symposia (Robson & Sullivan, 1968; Baron & Sullivan, 1970; Avery Jones & Sullivan, 1972; Avery Jones & Parke, 1975; Beck, 1976; Avery Jones, et al, 1978). Carbenoxolone sodium itself is a pure, semi-synthetic compound (Fig. 1.1), the hemisuccinate ester of β-glycyrrhetinic acid (enoxolone), which is the aglycone of glycyrrhizic acid, one of the many constituents of liquorice root (glycyrrhiza glabra radix).
Fig 1.1 STRUCTURAL FORMULAE OF CARBENOXOLONE AND RELATED COMPOUNDS.
1.3.1 PHARMACOLOGY

The pharmacology of carbenoxolone was originally described by Finney and Tárnokey (1960) and by Khan and Sullivan (1968). The anti-inflammatory activity of carbenoxolone was one third of that of hydrocortisone when given parenterally in a cotton pellet-granuloma test in rats. This activity was reduced following adrenalectomy but was not present following oral administration. However, an oral anti-inflammatory activity of carbenoxolone has subsequently been demonstrated, using the Freund's Adjuvant arthritis test, with a potency about half that of phenylbutazone (Thornton, MacDonald, Sacra & Gottfried, unpublished observations). Carbenoxolone is known to be irritant when administered parenterally (Khan & Sullivan, 1968), thus it is possible that these earlier anti-inflammatory activities included a portion of adrenal stimulation by a counter-irritant principle (Cygiełman, 1963).

Carbenoxolone has an aldosterone-like anti-diuretic activity in rats, causing a retention of sodium and water and an increased excretion of potassium (Khan & Sullivan, 1968). Other workers (Baron & Nabarro, 1968; Hausman & Tárnokey, 1968) have demonstrated similar effects in man and stated that the effects were essentially resembling those of aldosterone. Porter (1970) observed that carbenoxolone lacked an intrinsic activity but potentiated the activity of aldosterone on sodium transport in the isolated toad bladder. However, no such potentiation of aldosterone by carbenoxolone on sodium excretion was observed in adrenalectomised rats. Moreover, at the highest level of carbenoxolone used some reduction of potassium excretion was seen (Porter, et al, 1974). Baron and colleagues (1969) demonstrated a reduced secretion rate of aldosterone, which was not renin-mediated, in a carbenoxolone patient and suggested that this might be due to a feedback inhibition of aldosterone secretion due to an intrinsic mineralocorticoid activity of carbenoxolone.
The intracellular distribution of $^3$H-aldosterone was studied in the adrenalectomised rat kidney (Chakraborty, et al, 1970). The distribution of polar metabolites was not significantly altered, although there was an increase in unchanged aldosterone concentration. However, no change in the plasma concentration of free aldosterone was observed. Carbenoxolone has been shown to displace aldosterone from chicken plasma in vitro at a concentration of 400 times the molar equivalent of aldosterone (Tárnyky, 1970). Hayes and Langman (1975) also demonstrated some displacement of aldosterone by carbenoxolone from human plasma in vitro, at a carbenoxolone concentration 10 times therapeutic levels, but found that even with plasma from elderly patients, this displacement was clinically insignificant. Subsequently, where plasma binding and hepatic clearance of carbenoxolone was reduced in elderly patients, it was suggested that an increased aldosterone-like activity of carbenoxolone in the elderly may be due, in part, to a greater free drug concentration and consequent displacement of aldosterone (Hayes, et al, 1977).

Apart from this aldosterone-like activity, carbenoxolone is a remarkably non-toxic drug, the acute toxicity orally and intravenously in rats and mice is relatively low (Khan & Sullivan, 1968). Long term administration and carcinogenicity tests in mice, rats and dogs produced no signs of toxicity. Reproductive fertility studies in mice, rats and rabbits have not shown any adverse effects.

1.3.2 EXPERIMENTAL ULCER ACTIVITY

Experimental ulcer formation in the gastro-intestinal tract of animals is an extremely capricious model, especially in rodents. The first studies with carbenoxolone on restraint-induced and 5-hydroxytryptamine-induced erosions were unsuccessful. However, Khan and
Sullivan (1968), in experiments on diathermy-induced gastric ulcers from a specially designed electrocautery device, were able to demonstrate the beneficial effect of carbenoxolone in rats at dose levels of 25 and 50mg/kg orally. At the same time other workers (Dean, 1968; Lipkin & Ludwig, 1968) were successful in treating 40/80-induced ulcers in rats, and restraint stress-induced ulcers in guinea pigs, with carbenoxolone. All these authors reported an increased gastric mucus production and suggested this as a possible mode of action of carbenoxolone. Subsequently Lipkin (1970) demonstrated an increased lifespan of the mucosal cells of the mouse gastric mucosa following treatment with carbenoxolone. The increased lifespan of the mucosal cells was thought to play a rôle in an increased mucus production.

The results with diathermy-induced ulcers have been confirmed recently, at a dose level of carbenoxolone of 20mg/kg, in a comparison with an analogue, cicloxolone sodium (Thornton, MacDonald, Sacra & Gottfried, unpublished observations). These authors also demonstrated a preventative effect of carbenoxolone on stress-induced erosions, when given intraperitoneally at 60mg/kg. When rats are dosed with this regimen there follows a short period of depression; it is possible that this prevention of stress-induced erosions is mediated via a central mechanism.

1.3.3 CLINICAL USE OF CARBENOXOLONE

In the first trial of carbenoxolone in gastric ulcer patients the drug was compared with the effects on in-patients of various traditional remedies, such as bed rest and cessation of smoking. Carbenoxolone was found to be as beneficial to ambulant out-patients as the bed rest to in-patients (Doll, et al., 1962). Subsequent trials confirmed the healing effect in man but demonstrated the side-effects of fluid retention and hypokalaemia in some
patients (Doll, et al., 1965). The incidence of side-effects in South African trials, which included long-term maintenance therapy, were less frequent than in British studies (Bank, et al., 1967; Bank & Marks, 1970; Bank, 1975). Treatment of the mineralocorticoid side-effects with the aldosterone-antagonist spironolactone reduced the fluid retention but also antagonised the beneficial effect of carbenoxolone (Doll, et al., 1968). In the same study thiazide diuretics proved to be capable of reducing the sodium and water retention without hindrance to the healing pattern. It is also interesting to note that the thiazide diuretics did not potentiate the potassium loss in this trial.

Treatment of duodenal ulcer with carbenoxolone as Biogastrone tablets was unsuccessful (Doll, et al., 1962). However, the development of the Duogastrone capsule produced the first studies of healing of duodenal ulcers (Craig, et al., 1967; Hunt, 1968). The capsule is tanned gelatin designed to swell over 2½-3 hours in the stomach and rupture in the pylorus to deliver the carbenoxolone topically to the duodenum (Galloway, 1968; Lindup, et al., 1970; Lessof, 1975).

Following these early studies, the healing effect of carbenoxolone on duodenal ulcers has been confirmed worldwide; Nigeria (Amure, 1970), Spain (Marcos Perez, 1970), Canada (Archambault, 1976), France (Villart, 1976), the United States (Hirschowitz, 1976) and Australia (Nagy, 1978).

Carbenoxolone has also been used to treat oesophagitis when formulated as an electuary (McAndrew & Foote, 1970; Silber, 1970; Atkinson, et al., 1970). When reformulated, for stability reasons, as a chewable tablet Pyrogastrone, carbenoxolone has proved effective in double-blind studies (Davies & Reed, 1975; Reed & Davies, 1978).
As stated earlier (section 1.3.1), carbenoxolone is relatively free from side-effects except for its aldosterone-like activity. Transient increases in hepatic transaminases have been observed in some patients and following long-term treatment in the rat (Cliff, 1968; Baron & Nabarro, 1968; Hausman & Tarnoky, 1968; Sullivan, 1972). These changes are not thought to be related to pathology and occur with other drugs excreted via the bile.

The retention of sodium leading to oedema was recognised from the earliest clinical trials (Doll, et al, 1965) but has been managed by concomitant therapy with thiazide diuretics (Doll, et al, 1968). The hypokalaemia does not appear to result from urinary loss and is not associated with total body potassium flux (Edmonds & Tomkins, 1975). These authors have suggested that the hypokalaemia is due to a small continuous loss from the gastro-intestinal tract which is demonstrated by changes in colonic potential difference (Tomkins & Edmonds, 1975). The administration of bendrofluazide with carbenoxolone did result in a marked loss of total body potassium, but not when amiloride was given as a diuretic. Failure of the kidney to excrete sodium from high plasma concentrations as an 'escape' mechanism is possibly the idiosyncratic origin of the occurrence of side-effects in only some patients (Edmonds & Tomkins, 1975). Side-effects are more prevalent in elderly patients and in those with renal or hepatic failure, being contraindicated in the latter two cases. Comparison between normal and elderly patients has demonstrated a reduced plasma clearance rate and total binding capacity, suggesting that an increase in free carbenoxolone may, in part, account for the increased incidence of side-effects in the elderly (Hayes, et al, 1977).

In cases of prolonged carbenoxolone therapy without
adequate patient monitoring, especially in elderly patients, the side-effects have consequently been severe (Davies, et al, 1974).

1.3.5 MODE OF ACTION OF CARBENOXOLONE

From the earliest clinical studies it was considered that carbenoxolone exerted its mode of action in healing peptic ulcer by augmenting the defensive factors rather than inhibiting the aggressive forces (Avery Jones, 1968). Carbenoxolone does not affect gastric motility (Connell & Loane, 1968) or acid and pepsin secretion (Ottenjann & Rösch, 1970; Berstad, et al, 1970; Baron, 1977).

Increase in gastric mucus secretion has been seen in cats, and in man, following treatment with carbenoxolone (Goodier, 1968; Johnson, 1968). Lipkin (1970) has demonstrated an increased lifespan of gastric mucosal cells, a possible factor in the increased production of mucus.

Torma and colleagues (1975) have also demonstrated the ability of carbenoxolone pretreatment, in the dog, to reduce the cell loss from the gastric mucosa in aspirin-induced gastritis in situ. Studies of gastric epithelial cells in carbenoxolone-treated patients have subsequently confirmed Lipkin's original findings in mice (Croft, 1973; Klein, et al, 1975). It is possible that the membrane stabilisation of carbenoxolone plays a rôle in the decreased rate of turnover of gastric epithelial cells (Parke, 1976; Symons, 1976).

Animal studies on glucuronyl transferase activity of the gastric mucosa and the content of hexosamine, fucose and sialic acid in mucus have provided some pharmacological evidence for the increase in mucus secretion following carbenoxolone treatment (Shillingford, et al, 1973). Studies on rat duodenal mucosa did not show
the same activity, but the animals were dosed orally so that most of the drug would have been absorbed from the stomach mucosa and thus not able to exert a topical effect in the duodenum. In a further study, the rate of incorporation of $^3$H-N-acetylglucosamine and other sugars into rat and ferret stomach glycoproteins \textit{in vitro} was increased following oral treatment with carbenoxolone for seven days prior to the study (Shillingford, \textit{et al}, 1974). Again carbenoxolone did not show any effect on the duodenal mucosa when given orally to these animals. The addition of carbenoxolone to stomach portions \textit{in vitro}, of previously untreated animals, inhibited the incorporation of the sugars into gastric glycoproteins. This is not surprising because free carbenoxolone is a potent uncoupler of oxidative phosphorylation (Whitehouse, \textit{et al}, 1987).

The increase in N-acetylglucosamine incorporation into the rat stomach mucosa has been confirmed where the animals received a bolus oral dose of carbenoxolone in addition to an equal dose via the drinking water (Thornton, MacDonald, Sacra & Gottfried, unpublished observations). In clinical studies a reduced rate of N-acetylglucosamine incorporation is associated with gastric ulcer and gastric cancer. Treatment with carbenoxolone has restored this rate of incorporation (Johnston, \textit{et al}, 1975).

The aldosterone antagonist, spironolactone, is known to inhibit the healing activity of carbenoxolone (Doll, \textit{et al}, 1968). In animal studies, pretreatment for seven days with spironolactone has been shown to inhibit the incorporation of N-acetylglucosamine and galactose into rat gastric mucosa, the opposite effect of carbenoxolone (Johnston, \textit{et al}, 1975).

Carbenoxolone protects the gastric mucosa against bile salt-induced damage in human patients and in gastric pouches in dogs (Cross & Rhodes, 1972; Ivey & Gray, 1973). Further studies in dog pouches have demonstrated the
ability of carbenoxolone to reduce the hydrogen ion back-diffusion across the gastric mucosa (Thompson, et al, 1975). Clinical studies have correlated the prevention of hydrogen ion back-diffusion with an increased mucus secretion as measured by N-acetylneuraminic acid content (Domschke, et al, 1975). Aspirin-induced damage to gastric mucosa results in an increased permeability to the back-diffusion of hydrogen ions which may be monitored by a fall in mucosal potential difference (Overholt & Pollard, 1968). Clinical studies have shown the beneficial effect of carbenoxolone in preventing the lowering of gastric potential difference induced by aspirin (Hossenbocus & Colin-Jones, 1975).

Although the principal action of carbenoxolone seems to be mediated via the gastric mucosa and mucus synthesis, some inhibition of pepsin activity has been demonstrated in vitro (Henman, 1970) and in vivo (Berstad, 1972). Birnbaum and Karmeli (1975) demonstrated a reduction of uropeptic secretion following carbenoxolone treatment of both rats and man. Similarly a reduction in pepsin secretion in man following carbenoxolone therapy was observed (Walker & Taylor, 1975). These authors demonstrated a fall in pepsin secretion in response to pentagastrin stimulation, a reduction in the proportion of Pepsin 1 and a reduction in the minimum secretion of pepsins.

### 1.3.6 DISPOSITION AND METABOLISM

Early studies of carbenoxolone in the rat with $^{14}$C in the succinate moeity revealed that the drug was hydrolysed to enoxolone after oral administration (Parke, 1968b). Subsequent studies were undertaken with $^3$H- enoxolone and with carbenoxolone doubly labelled, $^3$H in the triterpenoid and $^{14}$C in the succinate moieties. After hydrolysis to enoxolone this was absorbed rapidly from the stomach, conjugated in the liver as glucuronide and sulphate and excreted almost entirely in the bile. Further
hydrolysis occurred in the gastro-intestinal tract to leave enoxolone in the faeces (Parke, et al, 1963; Iveson et al, 1971). Enoxolone was shown to undergo enterohepatic circulation. When $^{14}$C-carbenoxolone was given orally, 60-75% of the $^{14}$C was excreted as carbon dioxide, presumably from the succinate hydrolysis followed by metabolism via the citric acid cycle (Iveson, et al, 1966). Less than 20% of the radioactivity was detected in the urine. There is evidence to suggest that the hydrolysis of carbenoxolone is due to the microflora of the rat gastro-intestinal tract and not to mammalian tissue hydrolysis, because $^{14}$CO$_2$ excretion is less following intraperitoneal administration and rat caecal contents have been shown to hydrolyse carbenoxolone (Iveson, et al, 1971). The major biliary metabolites following oral administration to the rat are the 3-sulphate, 30-glucuronide and 3,30-diglucuronide of enoxolone. However, when administered by the intraperitoneal route to rats, or orally to rats pretreated with orally-administered anti­biotics, the principal biliary metabolite is the 30-glucuronide of carbenoxolone. In man, ferret and the squirrel monkey the principal metabolite is the 30-glucuronide of carbenoxolone, the squirrel monkey excretes more $^{14}$C-carbenoxolone in the faeces and less $^{14}$CO$_2$ than man (Iveson, et al, 1971; Shillingford, Lindup & Parke, personal communication).

When carbenoxolone is orally administered to man it is rapidly absorbed from the stomach resulting in blood concentrations of about 20 µg/ml after 1 hour. This represents about 80% of the 100 mg dose in the plasma (Downer, et al, 1970). The rapid absorption of carbenoxolone may be interpreted as a measure of both the lipophilicity of this high molecular weight sodium salt, which is insoluble at gastric pH, and of its affinity for proteins (Parke, 1972). Recent rat studies in vitro and in situ have confirmed that carbenoxolone is absorbed in both ionised and unionised forms with extensive
absorption on to the proteins of the gastric mucosa (Bridges, et al, 1976).

Following absorption, carbenoxolone is virtually confined to the plasma, liver, bile and gastro-intestinal tract due to its high affinity for protein binding. With less than 2% appearing in the urine it is evident that there is considerable enterohepatic circulation (Downer, et al, 1970; Parke, et al, 1972). Studies with $^{14}$C-carbenoxolone in the rat have not shown association with kidneys, fat, brain or other tissues. Furthermore, when administered by the intraperitoneal route, the drug was found to be associated with the gastric mucosa, suggesting a special affinity for carbenoxolone at that site (Parke, 1972).

Investigations using plasma from various species have shown that carbenoxolone is almost totally bound to plasma proteins in circulation (Chakraborty, et al, 1970; Parke & Lindup, 1973). At concentrations of up to 91 µg/ml carbenoxolone was more than 99% bound to plasma proteins in the rat, dog, monkey and man. Clinical studies have since revealed maximum human plasma concentrations, after repeated therapy, to be around 100 µg/ml and up to 20 µg/ml after a single 100 mg dose (Baron, et al, 1975).

Carbenoxolone is bound principally to albumin but it is also associated to a small extent with $\alpha_1$ and $\alpha_2$ globulins (Parke & Lindup, 1973). The albumin binding has a high affinity of the order $10^7$ l/mol at the primary sites ($n_1 = 2$). A conformational change of the albumin is not suspected but further sites are available with an affinity of $2 \times 10^6$ l/mol ($n_2 = 6$).
Chapter 2

MEASUREMENT OF CARBENOXOLONE BINDING IN VITRO
Chapter 2

MEASUREMENT OF CARBENOXOLONE BINDING IN VITRO

2.1 INTRODUCTION

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MEASUREMENT OF CARBENOXOLONE BINDING IN VITRO

2.1 INTRODUCTION

2.1.1 METHODS OF MEASUREMENT OF PROTEIN BINDING

In all of the various physico-chemical techniques used to measure protein binding (Table 2.1) one of three basic parameters is being determined:

i) the concentration of free ligand;

ii) perturbation of the bound ligand;

iii) perturbation of the binding protein.

Early or 'classical' methods of measurement of protein binding consisted of equilibrium dialysis, ultrafiltration and electrophoresis.

*Equilibrium dialysis* (Klotz, *et al*, 1946) is by far the most commonly used method to study protein binding (Bush & Alvin, 1973). The system consists of a semi-permeable visking membrane separating a protein solution from a drug solution. After a given period of time the drug equilibrates between the two compartments, whence the drug concentration in the protein-free compartment represents the level of free drug. This method relies on the assumption that the drug is in a state of dynamic equilibrium with the protein and that the free fraction of drug is in equilibrium between the two compartments. The disadvantages of the method lie within these assumptions, that a Donnan membrane effect may prevent equilibrium across the membrane being achieved or that the drug may bind to the membrane itself. In practice a considerable length of time (up to 48 hours) may be required to establish the equilibrium, introducing additional risks of bacterial contamination and degradation of the protein and/or drug.

*Ultrafiltration* (Rehberg, 1943) is an attempt to achieve the results of equilibrium dialysis more rapidly. The drug-protein complex is subjected to an increased pressure,
Table 2.1 SUMMARY OF COMMON METHODS OF MEASUREMENT OF LIGAND–PROTEIN BINDING

Estimation of free ligand concentration

a) Distribution between phases – equilibrium dialysis
   – ultrafiltration
   – dynamic dialysis
   – partition
   – gel filtration

b) Competition with bound indicator – optical spectroscopy
   – fluorescence
   – nuclear magnetic resonance

Perturbation of properties of bound ligand

a) Optical spectroscopy
b) Optical rotation
c) Fluorescence
d) Nuclear magnetic resonance

Perturbation of properties of binding macromolecule

a) As above
b) Electrophoresis
c) X-ray crystallography
either directly or by ultracentrifugation, across a semi-permeable membrane whereby a small aliquot of the vehicle, containing the free concentration of drug, is forced across the membrane. This method has obvious advantages of time saving over equilibrium dialysis but shares the problem of membrane binding and, furthermore, induces a change in the protein concentration which may alter the binding being measured.

*Electrophoresis* (Alberty & Martin, 1951) of plasma-ligand (or serum-ligand) complexes will produce a qualitative analysis of binding but does not lend itself readily to interpretation of quantitative data. For rapid attainment of qualitative data a crossing electrophoresis technique has been introduced (Bickel & Bovet, 1962).

These 'classical' methods have been improved since the advent of radio-chemically labelled drugs enabling studies to be carried out at therapeutic concentrations (Meyer & Guttman, 1968).

*Gel filtration*, a more recent method (Porath & Flodin, 1959; Flodin, 1961) uses cross-linked polysaccharides (Sephadex) to separate chromatographically the protein-ligand complex from the free drug. Results are similar to those from dialysis methods without the problems of membrane binding, Donnan effects and time factors. The disadvantages of gel filtration include an interaction between the drug and Sephadex, and a dilution of the protein-ligand complex (Kucera & Bullock, 1969; Meyer & Guttman, 1968). To prevent interaction between the ligand-protein complex and the Sephadex, elution is carried out with a solution of the drug in place of the usual buffer (Hummel & Dreyer, 1962).

The binding of ligands to protein can induce changes which are measureable by various *spectrophotometric methods*.
Solutions of albumin possess a native fluorescence which may be quenched by the binding of a ligand (Chignell, 1969). This quenching is proportional to the molar ratio between the ligand and protein, thus titration of the ligand with the protein enables the evaluation of free and bound drug concentrations (Chignell, 1972a). However, where more than one class of binding site is present, the sites with lower affinities may be masked (Chignell, 1972b). The use of this method is limited by the fact that fluorescence quenching measurements may only be made on single protein fractions.

When warfarin binds to human serum albumin, the fluorescence quantum yield of the drug increases eight-fold and with rat serum albumin thirteen-fold (Chignell, 1970). Thus the binding of warfarin and other fluorescent drugs, such as tetracyclines may be monitored by this method (Popov, et al, 1972). The obvious drawback of this technique is that the ligands must have native fluorescence when bound to proteins.

A fluorescent probe is a ligand which possesses negligible native fluorescence in the free state but strongly fluoresces when bound to a macromolecule (Weber, 1952). A fluorescent probe also has a low intrinsic binding affinity and is quantitatively displaced by a second ligand, in proportion to the affinity of the second ligand. This technique is rapid and does not suffer from any of the problems of dialysis or gel filtration. Fluorescent probes may also be used to reveal data on the nature of the binding sites (Chignell, 1972a). However, data which is obtained for drug binding is of an indirect nature and must rely on the drug binding to the same site as the probe.

An analogous series of measurements to the above fluorescence techniques may be made using ultraviolet light.
absorption. Westphal (1961) and Ryan (1968) have used the technique of ultraviolet light difference spectroscopy, whilst Moriguchi and colleagues (1968a, 1968b) have used an ultraviolet spectrophotometric probe. However, Zia and Price (1975) more recently introduced studies with a spectrophotometric probe based on the fluorescence work of Jun and colleagues (1971). The use of ultraviolet spectrophotometric measurements share both the advantages and disadvantages of the fluorescence methods but are superior under conditions whereby fluorescence of a ligand does not occur, or fluorescence changes are associated with photoxidation (Zia & Price, 1975).

Circular dichroism is a technique which relies upon the binding of certain ligands to protein which induces extrinsic Cotton effects (Blout, 1964) which are measurable on spectropolarimeters (Chignell, 1968). These changes may be utilised to follow ligand binding in a three dimensional form. However, in order to yield useful data the ligand needs to contain a chromophore which absorbs ultraviolet light, and must be bound to the protein in an asymmetrical fashion (Ikeda & Hamaguchi, 1969). This method shares the advantages of other spectrophotometric studies over the classical methods in that it is rapid and does not involve membrane binding or dilution changes. However, the method requires specific equipment which is expensive.

Nuclear magnetic resonance (nmr) spectroscopy is a refined technique which can provide qualitative data on the exact nature of the functional groups involved in binding (Jardetzky, 1964; Jardetzky & Wade-Jardetzky, 1965; Fisher & Jardetzky, 1965).
One drawback of the method is that the exact amino acid sequence of the protein under study must be evaluated and the peaks corresponding to protons in given amino acids established, whereby changes in these peaks are seen to correspond to binding interaction (Davison, 1971). It is only possible to obtain qualitative binding from NMR studies and, again, the necessary equipment is expensive.

Two more recent innovations of measurement are dynamic dialysis and dialysis in vivo, essentially modifications of the classical equilibrium dialysis.

Dynamic dialysis is an attempt to reduce the time factors of equilibrium dialysis. The method is based on the assumption that the rate of disappearance of the ligand from the external phase is proportional to the concentration of the unbound species (Meyer & Guttman, 1968a). Studies with dynamic dialysis have yielded results comparable to ultrafiltration and equilibrium dialysis (Meyer & Guttman, 1970). However, although the technique is described as 'rapid', the apparent permeability rate for the ligand has to be determined (2h) prior to use and the model is then observed for up to 9 hours (Meyer & Guttman, 1970). The other disadvantages are similar to equilibrium dialysis, being visking tube binding and Donnan membrane effects. The method has been little used to date.

All of the previous methods evaluate ligand-protein binding in vitro and are thus non-physiological. However, equilibrium
dialysis has been adapted to a situation in vivo using an intraperitoneally implanted sac, allowing aliquots to be withdrawn for determination of the free ligand concentration (McQueen, 1968). However, this technique may only be useful for repeated measurements in large animals such as sheep (McQueen & Wardell, 1971).

Various other methods of measuring protein binding have been tried but have received limited use and are thus not established. Measurement of changes in biological activity are suitable only for antibacterial agents, similarly increases in solubility, changes in pH, refractive index, conductivity and EMF are only suitable for certain ligands and will yield only limited binding data (Goldstein, 1949; Meyer & Guttman, 1968; Steinhardt & Reynolds, 1969; Davison, 1971; Klotz, 1973).

2.1.2 INTERPRETATION OF DATA

Methods of presentation of ligand-macromolecule data are diverse. A simple graphical plot of degree of binding versus ligand or protein concentration reveals the saturation point of binding only. However, where a protein mixture such as plasma is used it may be the only valid interpretation. As Goldstein (1949) pointed out, the statement that a given fraction of drug is bound is meaningless without reference to the unbound concentration and protein concentration.

Where a pure protein fraction has been used at an accurate concentration then estimations of three basic parameters may be made (i) the number of classes of binding site; (ii) the number of molecules of ligand bound at each class of site; (iii) the apparent affinity of the ligand for the binding sites.

The law of Mass Action may be applied to parameters measured, thus the affinity constant, k, of a ligand for a protein may be defined as:
\[ k = \frac{[P] [D_f]}{[P] [D_f]} \quad \text{or} \quad [P] [D_f] = k [P] [D_f] \]  

(1)

Where \([P]\) is the protein concentration, \([D_f]\) is the free ligand concentration and \([P\] [D\]) is the bound ligand-protein complex, then

\[ r = \frac{\text{moles bound drug}}{\text{total moles protein}} = \frac{[P\] [D\]}{[P\] [D\] + [P\]} \]  

(2)

\[ = \frac{k [D_f]}{1 + k [D_f]} \]  

(3)

Where more than one binding site exists and where \(n\) equals the number of identical independent sites,

\[ r_1 + r_2 = \ldots = r_n \]  

\[ = \frac{nk [D_f]}{1 + k [D_f]} \]  

(4)

thus equation (4) may be rearranged to give

\[ \frac{1}{r} = \frac{1}{n} + \frac{1}{nk [D_f]} \]  

(5)

A direct plot of \(r\) versus \([D_f]\) is analogous to the Michaelis-Menton plot and is a hyperbola with \(r = n\) at the plateau and \([D_f] = 1/k\) when \(r = n/2\).

A reciprocal plot of \(1/r\) versus \(1/D_f\) is analogous to a Lineweaver-Burke plot and is a linear transformation from which \(n\) and \(k\) can be estimated from the slope \((1/kn)\) and intercept \((1/n)\). However, low values of \(1/r\) are spread poorly.

Scatchard (1949) performed an alternative rearrangement of equation (4) to

\[ r/ [D_f] = nk - rk \]  

(6)

By plotting \(r/ [D_f]\) versus \(r\), the abscissa intercept is \(n\), the ordinate intercept \(nk\) and the slope \(k\). The Scatchard plot spreads the data well and is generally the method of choice (Meyer & Guttman, 1968). It is certainly the most
commonly used method of analysis which facilitates comparison between workers.

Rosenthal (1967) produced a rearrangement of equation (4) for use where the protein concentration was not known and described a plot similar to that of Scatchard (1949).

\[
\frac{[D_b]}{[D_t]} = nk[P] - k[D_b]
\]

(7)

Where \([D_b]\) is the bound ligand concentration. A plot of \([D_b]/[D_t]\) versus \([D_t]\) (total ligand concentration) is linear and allows the estimation of \(n[P]\) from the abscissa intercept, \(nk[P]\) from the ordinate intercept and \(k\) from the slope. The Rosenthal method is still not as widely used as that of Scatchard.

All of these methods of determining binding parameters rely on a linear transformation, but where more than one class of binding site is present then differing affinities for each site will result in a curvi-linear plot. Because of the reliance of these methods on a standard uniform binding pattern, extrapolation in the presence of more than the one class of site does not bear the same validity as with one class system (Madsen & Robertson, 1974).

2.1.3 PRESENT EXPERIMENTS

Estimation of the protein binding of carbenoxolone was undertaken using three methods. Equilibrium dialysis and ultrafiltration were chosen as classical methods to allow comparison with previous studies (Lindup, 1971). Fluorescent probe studies were carried out to investigate a newer methodology capable of rapidly yielding data on several ligands without requiring the use of radio-labelled materials. Both bovine and human crystalline albumins were used to allow correlation with other workers and to provide a basis for comparison with the clinical situation. Interpretations of binding data were made using the Scatchard equation, again for reasons of comparison.
2.2 EXPERIMENTAL

The carbenoxolone (BIOREX Laboratories Ltd.) used throughout this and subsequent chapters was the disodium salt. Phenylbutazone (BIOREX Laboratories Ltd.) and warfarin sodium (Gehardt Penick) were also used. The protein fractions used were crystalline Cohn fraction V bovine (BDH) and human (Calbiochem) serum albumins.

All samples containing $^3$H-carbenoxolone were determined by scintillation counting in Triton-toluene scintillant using a Packard Tri-carb 3320 scintillation counter. Verification of counting efficiency was made and the specific activity and radiochemical purity of the $^3$H-carbenoxolone determined prior to, and during, experimentation by scintillation counting and thin layer chromatography.

2.2.1 EQUILIBRIUM DIALYSIS

Factors affecting equilibrium dialysis. Pilot tests were carried out to establish (i) whether carbenoxolone was bound to the dialysis tubing, and if so procedures to reduce it, (ii) length of time necessary to obtain equilibrium, and (iii) internal phase volume size.

Visking tubing (Scientific Instruments Ltd.) was used at $\frac{1}{4}$ inch and $\frac{1}{2}$ inch widths in 12 cm lengths. Carbenoxolone binding to the membrane was estimated as follows: dialysis sacs were cut into small pieces and boiled in 25 ml 0.1M NaOH for 30 minutes. The mixture was filtered and the portions of visking sac rinsed. The filtrate was evaporated to dryness and the precipitate was taken up in 1 ml distilled water whence aliquots of 0.2 ml were used to estimate the carbenoxolone concentration.

The following solutions were used to pretreat the visking sacs: NaHCO$_3$ as a 5% w/v solution; buffers; pH 7.0 BDH tablets; pH 7.4 0.1 M phosphate. Di potassium ethylene
diamine tetra acetic acid ($K_2$EDTA) was used as a 10 mM aqueous solution.

Pretreatment of visking tubing commenced with
i) storing at 90°C in sodium bicarbonate for 1 hour, rinsing in distilled water and storing in pH 7.0 buffer overnight at 4°C; ii) bicarbonate as above, then soaking in $K_2$EDTA for 1 hour prior to storage at pH 7.4 overnight at 4°C; iii) washing procedure as in ii) with storage in phosphate buffer containing 50 µg/ml unlabelled carbenoxolone.

Dialysis was carried out for 18 and 24 hours with stationary internal and external phases. This was repeated with air agitation of the external phase for 24 hours. Internal phase volumes of 5.0 and 0.5 ml were used. Dialyses with water, saline and 0.1 M phosphate buffer were carried out to determine the equilibration time.

**Binding of $^3$H-carbenoxolone to bovine serum albumin.** Dialysis experiments were performed in duplicate. The internal phase was 0.5 ml bovine serum albumin at $10^{-4}$M in 0.1 M phosphate buffer at pH 7.4. The external phase was 5 ml of buffer only. Visking tubing was 1/2 inch width. The tubing was pretreated by storing at 90°C in 5% NaHCO$_3$ for 1 hour, then in 10 mM aqueous $K_2$EDTA for 1 hour and stored overnight at 4°C in 0.1 M phosphate buffer at pH 7.4. $^3$H-Carbenoxolone had a specific activity of 7.36 mCi/g and was used as a methanolic solution.

Aliquots of the carbenoxolone solution were placed in teflon-stoppered tubes and evaporated in a water bath. The external phase was added to each tube and mixed for 30 minutes to establish complete dissolution. Samples of the external phase were taken for determination of the initial carbenoxolone concentration. Carbenoxolone concentrations of 10 to 500 µg/ml were used. The internal phase was then pipetted into the dialysis sacs which were
placed in the stoppered tubes on a rotary mixer for 24 hours at 4°C. After the period of dialysis, 0.2 ml aliquots of each phase were used to determine the carbenoxolone concentration.

The influence of phenylbutazone on carbenoxolone binding.
Equilibrium dialysis was repeated with unlabelled phenylbutazone added to the external phase at an equimolar concentration to the carbenoxolone. Determinations were carried out in duplicate.

2.2.2 FLUORESCENT PROBE TECHNIQUE

These studies were based on the technique of Jun and colleagues (1972). The fluorescent probe used was 1-anilino-8-naphthalene sulphonic acid (ANSA, Sigma) as a $10^{-3} \text{M}$ solution in methanol. Bovine and human serum albumins were used in 0.05 M, and initially in 0.1 M, phosphate buffer at pH 7.4. Carbenoxolone, phenylbutazone and warfarin were used as 0.1 M methanolic solutions. The methanol was redistilled AR grade and the water was double-distilled from glass.

The fluorescence intensities of the bound probe, ANSA, were measured on a Baird Atomic FP100 Fluoripoint spectrofluorimeter $\lambda$ activation 380 nm and $\lambda$ fluorescence 470 nm (uncorrected instrumental values). The instrument was set on high sensitivity with 10 nm slit widths. The serum solutions (2.0 ml) in a quartz cuvette were titrated with the fluorescent probe solution (1-10 $\mu$l aliquots) using an automatic micropipette (Finnpipette). Titrations were performed with two protein concentrations, 0.1 and 1.0 mg/ml and with the buffer alone. Where drug binding was investigated the drug was added at $10^{-4} \text{M}$ to the albumin solution prior to titration with the fluorescent probe.

The fraction of probe bound was calculated by the method of Brand and colleagues (1967).
\[ x = \frac{(I_o / I_f) - 1}{(I_b / I_f) - 1} \]

where the fraction of probe bound, \( I_b \), is the fluorescence intensity of the probe in the high protein concentration, \( I_o \) is the fluorescence intensity of the probe in the low protein concentration and \( I_f \) is the fluorescence intensity of the probe in the buffer alone. Subsequently the Scatchard (1949) equation was applied.

ANSA was titrated with both bovine and human serum albumins in 0.05 M phosphate buffer alone, and in the presence of carbenoxolone sodium, phenylbutazone and warfarin.

2.2.3 ULTRAFILTRATION TECHNIQUE

This method was basically that of Keller and colleagues (1966). Bovine and human serum albumins were used at \( 10^{-4} \) M and \( 1.49 \times 10^{-4} \) M (1%) in 0.1 M phosphate buffer at pH 7.4. Dialysis tubing was 24/32 inches (Scientific Supplies Ltd.) and was pretreated as for equilibrium dialysis (section 2.2.1). For centrifugation Toribara tubes (Toribara, et al., 1957) were used as modified by Lindup (1971). These consisted of 10 cm Pyrex tubes with a sintered disc (porosity 1) at 2.5 cm. Phenylbutazone and warfarin were used as 0.1 M solutions in methanol.

Measurements of binding of \( \text{H-carbenoxolone} \) to bovine serum albumin were made at concentrations of albumin between 0.1 and 10%. Carbenoxolone was used up to 500 \( \mu \)g/ml. The binding of carbenoxolone to the dialysis sacs was determined as in section 2.2.1.

The drug-protein complex was made up in bulk and 5 ml aliquots were equilibrated in 15 ml teflon-stoppered tubes on a rotary mixer for 30 minutes. Aliquots of 4 ml were then pipetted into 20 cm lengths of dialysis tubing which were
rested on the glass sinter in a U shape with the ends uppermost. The Toribara tubes were placed in 100 ml polypropylene tubes for centrifugation in an MSE 'Mistral' centrifuge at 4°C for 2 hours at 680 g. The ultrafiltrate was approximately 5% of the original volume. The original carbenoxolone concentration was determined from the remaining 1 ml aliquot of drug-protein complex from the teflon-stoppered tubes. Where a second drug was used, it was added to the teflon-stoppered tubes as a methanolic solution and the solvent evaporated prior to the addition of the protein solution.

The concentration of unbound carbenoxolone \( [D_f] \) was calculated from the equation (Keller, et al, 1966) where \( [D_t] \) is the initial molar concentration of carbenoxolone, and dpm the radioactive disintegrations per minute.

\[
[D_f] = [D_t] \times \frac{\text{net dpm/ml filtrate}}{\text{net dpm/ml sac contents}}
\]

The results were plotted using the method of Scatchard (1949).

The influence of phenylbutazone and warfarin on carbenoxolone binding. The carbenoxolone concentrations used were based on the maximum therapeutic blood level and a concentration which was 50% bound in this system. At the lower \(^3\)H-carbenoxolone level of 20 \( \mu \)g/ml, phenylbutazone and warfarin were added at a concentration of ten times the molar equivalent of carbenoxolone but at the higher carbenoxolone level of 450 \( \mu \)g/ml, equimolar concentrations of the interacting drugs were used.
2.3 RESULTS

Measurement of the counting efficiency of $^3$H was confirmed using standard $^3$H$_2$O. The radiochemical purity of the $^3$H-carbenoxolone used was 94.1% with 5% being identified as 18-β glycyrrhetinic acid. This was of a similar purity to the carbenoxolone standard used. The radiochemical purity of $^3$H-carbenoxolone was unchanged by storage as an aqueous solution at 4°C for 3 months.

2.3.1 EQUILIBRIUM DIALYSIS

Factors affecting equilibrium dialysis. Untreated visking membrane bound significant levels (12.3 μg/sac) of carbenoxolone from a saline solution, although this was reduced (5.9 μg/sac) when protein was present (0.01% bovine albumin). However, treatment of the visking with bicarbonate and EDTA also significantly reduced the binding (4.8 μg/sac, $P < 0.02$), whilst pretreatment with unlabelled carbenoxolone was unsuccessful.

Even within various stages of agitation equilibrium was not achieved within 24 hours until a rotamix was used with an internal phase volume of 0.5 ml and an external phase of 5 ml.

Binding of $^3$H-carbenoxolone to bovine serum albumin (Table 2.2, Fig. 2.1). A linear concentration-dependant binding was observed between carbenoxolone and bovine serum albumin at $10^{-4}$ M (correlation coefficient = 0.994). Maximal binding was only 80-85% at a carbenoxolone concentration of 10-50 μg/ml, whilst at 500 μg/ml carbenoxolone was only 50% bound. A Scatchard plot was linear (correlation coefficient = 0.973) which may be interpreted as representing only one class of binding site with approximately 9 binding sites per molecule and an affinity constant of the order of $8.9 \times 10^3$ l/mole.

The bound concentration of carbenoxolone was slightly decreased in the presence of an equimolar concentration of phenylbutazone, the effect being greatest at the highest
TABLE 2.2 BINDING OF $^3$H-CARBENOXOLONE SODIUM TO BOVINE SERUM ALBUMIN

<table>
<thead>
<tr>
<th>Initial concentration (µg/ml)</th>
<th>Final concentration</th>
<th>Degree of binding (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Internal ($10^{-4}M$)</td>
<td>External ($10^{-5}M$)</td>
</tr>
<tr>
<td>10</td>
<td>1.5</td>
<td>0 *</td>
</tr>
<tr>
<td>50</td>
<td>7.1</td>
<td>1.1</td>
</tr>
<tr>
<td>100</td>
<td>13.5</td>
<td>2.5</td>
</tr>
<tr>
<td>250</td>
<td>29.3</td>
<td>11.4</td>
</tr>
<tr>
<td>500</td>
<td>47.2</td>
<td>34.2</td>
</tr>
</tbody>
</table>

* not detectable within background limits

Equilibrium dialysis was carried out for 24 hours at 4°C with $10^{-4} M$ bovine serum albumin in 0.1 M phosphate buffer at pH 7.4. Internal phase volume was 0.5 ml and external volume 5 ml. Results are the mean of duplicate observations.
Fig 2.1 SCATCHARD PLOT OF $^3$H-CARBENOXOLONE BINDING TO BOVINE SERUM ALBUMIN, FROM EQUILIBRIUM DIALYSIS.

Where $\bar{V}$ = mole of bound carbenoxolone per mole of albumin

$A$ = molar concentration of free ligand.

Dialysis was carried out in 0.1M phosphate buffer at pH 7.4 and 4°C for 24 hours.

Linear correlation coefficient = 0.973   \( n_1 = 9 \)

\( k_a = 8.9 \times 10^3 \) l/mole

Each point represents the mean of 6 observations.
concentration of carbenoxolone (Fig. 2.2). The Scatchard plot reveals a linear relationship for each group but with differing slopes. The presence of phenylbutazone has effectively reduced the number of binding sites available to carbenoxolone from 9 to 6 but resulted in an apparent increase in affinity from 9.0 to $13.3 \times 10^3$ 1/mole.

2.3.2 FLUORESCENT PROBE TECHNIQUE

The effect of buffer molarity. The fluorescence intensity of the probe ANSA, when bound to bovine serum albumin at a concentration of 1.0 mg/ml, was higher in 0.1 M phosphate buffer than in 0.05 M phosphate buffer. Despite the fact that the concentration of albumin was constant in both cases, the probe was apparently bound to a larger extent in the buffer of greater molarity, producing an increased fluorescence intensity, suggesting that more binding sites were available. The buffer molarity was maintained at 0.05 M throughout the remaining studies.

The binding of carbenoxolone, phenylbutazone and warfarin to bovine serum albumin (Figs. 2.3 - 2.5). Titration of ANSA with bovine serum albumin at a concentration of 1.0 mg/ml revealed a linear relationship between probe concentration and fluorescence intensity, indicating an excess of binding sites. However, titration of the probe with bovine serum albumin, at a concentration of 0.1 mg/ml, demonstrated a saturation of the binding sites, indicated by a plateau on the curve of fluorescence versus probe concentration (Fig. 2.3). Addition of the three drugs, carbenoxolone, phenylbutazone and warfarin, to the lower protein concentration caused displacement of the probe demonstrated by the reduced fluorescence in Fig. 2.4. The greatest displacement was caused by carbenoxolone and phenylbutazone, with warfarin displacing the probe to a lesser extent. Scatchard plots (Fig. 2.5) were linear for each drug and for the probe itself, which may be interpreted as evidence of the presence of only a single class of binding
Fig 2.2 SCATCHARD PLOT OF $^3$H-CARBENOXOLONE BINDING AND THE INFLUENCE OF PHENYL BUTAZONE

Carbenoxolone (O) and in the presence of phenylbutazone (©)

Where $\bar{V} = $ mole of bound carbenoxolone per mole of protein

$A = $ molar concentration of free ligand

Dialysis was carried out at 4°C for 24 hours with $10^{-4}$ M bovine serum albumin in 0.1 M phosphate buffer at pH 7.4. Phenylbutazone was present in an equimolar ratio to carbenoxolone. Results are the mean of 6 observations.
Fig 2.3 VARIATION OF FLUORESCENCE INTENSITY WITH FLUORESCENT PROBE CONCENTRATION, FOR BOVINE SERUM ALBUMIN.

Two ml of albumin solution, at 0.1 mg/ml (Θ) and 1.0 mg/ml (O), was titrated with 1-anilino-8-naphthalene sulphonic acid (ANSA) at a concentration of 0.1 M in methanol. The fluorescence intensity was measured at 380/470 nm.
Fig 2.4 THE EFFECT OF DRUGS ON THE FLUORESCENCE OF ANSA BINDING TO BOVINE SERUM ALBUMIN

ANSA was titrated against 2 ml of bovine serum albumin, 0.1 mg/ml in 0.05 M phosphate buffer at pH 7.4, alone (O) and in the presence of $10^{-4}$ M warfarin sodium (O), phenylbutazone (△) and carbenoxolone sodium (△). Fluorescence was measured at 380/470 nm.
Fig 2.5 SCATCHARD PLOT FOR THE BINDING OF ANSA TO BOVINE SERUM ALBUMIN IN THE PRESENCE OF VARIOUS DRUGS

Where $\bar{V}$ = mole of bound probe per mole of protein
$A$ = molar concentration of free probe

ANSA was titrated with bovine serum albumin in 0.05M phosphate buffer at pH 7.4 alone (O) and in the presence of $10^{-4} M$ warfarin sodium (O), phenylbutazone (△) and carbenoxolone (A).
The intercept of the abscissa was common for each plot, indicating binding at the same single class site. The reduced slope in the presence of drugs is a result of the competition between the drugs and the fluorescent probe, being reduced almost equally for phenylbutazone and carbenoxolone, thus a similar affinity for each drug is suggested. The affinity of warfarin for the site is less than the other drugs, indicating that this drug would be competitively displaced by both carbenoxolone and phenylbutazone. When ANSA was titrated in the presence of both warfarin and phenylbutazone there was no additive reduction in the fluorescence and this is interpreted as additional evidence that only one class of binding site was involved.

The binding of carbenoxolone, phenylbutazone and warfarin to human serum albumin. Titration of ANSA with 1.0 and 0.1 mg/ml human serum albumin gave similar results to those with bovine albumin. However, a higher level of fluorescence was seen with the human albumin than with bovine albumin (Fig. 2.6). The presence of the three drugs, carbenoxolone, phenylbutazone and warfarin resulted in a reduced fluorescence of the probe (Fig. 2.7) although the shape of the curve was not similar for all three drugs as was the case with bovine serum albumin (Fig. 2.4).

A Scatchard plot of the fluorescent probe binding to human serum albumin (Fig. 2.8) was non-linear but may be interpreted as representing three classes of binding site \( n_1 = 1, n_2 = 3, n_3 = 4 \). Carbenoxolone was bound at the first class of site whilst warfarin and phenylbutazone were bound at the second, where phenylbutazone had the higher affinity. From these results it may be inferred that carbenoxolone would not compete with the other two drugs for a common binding site on human serum albumin, which is in contrast to the findings with bovine albumin. Both phenylbutazone and warfarin were bound at the same class of site on human albumin as was the case with bovine albumin. Phenylbutazone showed a higher affinity than warfarin in
Fig 2.6 Fluorescence intensity of ANSA with human and bovine serum albumin

ANSA was titrated with 2 ml human (o) and bovine (△) serum albumin at 1.0 mg/ml in 0.05 M phosphate buffer at pH 7.4. Fluorescence was measured at 380/470 nm.
Fig 2.7 THE EFFECT OF DRUGS ON THE FLUORESCENCE OF ANSA BINDING TO HUMAN SERUM ALBUMIN

ANSA was titrated with 2ml human serum albumin at 0.1 mg/ml in 0.05M phosphate buffer at pH 7.4, alone (O) and in the presence of $10^{-4}$M warfarin sodium (Δ), phenylbutazone (Δ) and carbenoxolone (©). Fluorescence was measured at 380/470nm.
Fig 2.8 SCATCHARD PLOT FOR THE BINDING OF ANSA TO HUMAN SERUM ALBUMIN IN THE PRESENCE OF VARIOUS DRUGS

Where $\bar{V} =$ mole of bound probe per mole of protein

$A =$ molar concentration of free probe

ANSA was titrated with human serum albumin in 0.05M phosphate buffer at pH 7.4, alone (O) and in the presence of $10^{-4} \text{ M}$ carbenoxolone (©), phenylbutazone (△) and warfarin sodium (△).
each case, leading to the suggestion that the competitive displacement of warfarin by phenylbutazone would occur.

2.3.3 ULTRAFILTRATION TECHNIQUE

The influence of protein concentration. At the lower bovine serum albumin concentrations carbenoxolone was present in a molar excess and, in consequence, binding sites were saturated. However, at the 1.0% albumin concentration $12.0 \times 10^{-5} M$ carbenoxolone was near maximally bound, whilst $52.8 \times 10^{-5} M$ was only 61.8% bound, whereas the latter carbenoxolone level was maximally bound at a concentration of 10% albumin. It is interesting to note that at higher protein concentrations less binding of carbenoxolone to the visking dialysis sac was seen, as was the case with equilibrium dialysis.

Binding of carbenoxolone to bovine serum albumin. With bovine serum albumin at a concentration of $10^{-4} M$, maximal carbenoxolone binding was seen up to 250 $\mu g/ml$, when determination of the free concentration was only just within measurable limits.

However, a Scatchard plot of the data (Fig. 2.9) was linear, suggesting the presence of only one class of binding site ($n = 9$) with an affinity of $8.1 \times 10^{-3} 1/mole$. These results agree favourably with those obtained by equilibrium dialysis.

Binding of carbenoxolone to human serum albumin. Carbenoxolone was bound to a slightly lesser extent to human serum albumin than to bovine albumin. At a concentration of 200 $\mu g/ml$ only 92% was bound to human albumin, whilst 97% was bound at 250 $\mu g/ml$ to bovine albumin.

A Scatchard plot of the binding (Fig 2.10) was non-linear suggesting (i) a significant electrostatic interaction between binding sites; (ii) the binding of carbenoxolone in different ionic states (it is a divalent ion); or more likely (iii) more than one class of binding site exists in the albumin molecule. The primary binding site had a high
Ultrafiltration was carried out at 4°C in 0.1 M phosphate buffer at pH 7.4.

$V_1 = 9$ mole of free carbenoxolone per mole of protein

$K_1 = 8.1 \times 10^3$ L/mole

Where $V$ = mole of bound carbenoxolone per mole of protein

$A$ = molar concentration of free carbenoxolone

Fig. 2.9 SCATCHARD PLOT OF $^3$H-CARBENOXOLONE BINDING TO BOVINE SERUM ALBUMIN, FROM ULTRAFLTRATION STUDIES

$\bar{V}/A \times 10^{-4}$
Fig 2.10 SCATCHARD PLOT OF $^3$H-CARBENOXOLONE BINDING TO HUMAN SERUM ALBUMIN, FROM ULTRAFLTRATION STUDIES

Where $\bar{V}$ = mole of bound carbenoxolone per mole of albumin

$A$ = molar concentration of free carbenoxolone

Ultrafiltration was carried out at 4°C in 0.1 M phosphate buffer at pH 7.4.

$n_1 = 3 \quad k_1 = \text{at least } 10^6 \text{ l/mole}$

$n_2 = 7 \quad k_2 = 7.1 \times 10^3 \text{ l/mole}$
affinity of at least $10^6$ l/mole for carbenoxolone where $n_1 = 3$, whilst the second class of site had a lower affinity of $7.1 \times 10^3$ l/mole where $n_2 = 7$. The existence of more than one class of site is in contrast to the results with bovine albumin, but confirms the previous results in this chapter.

The influence of phenylbutazone and warfarin on the binding of carbenoxolone to human albumin. At the lowest carbenoxolone concentration of 20 µg/ml, maximal binding was seen, thus rendering measurements of the free drug difficult (Table 2.3), whilst at the higher level of 450 µg/ml, the level of binding was 62%. The small changes induced by the presence of phenylbutazone and warfarin were within 12% of the original carbenoxolone levels suggesting that no real displacement had occurred.
<table>
<thead>
<tr>
<th>Carbenoxolone concentration (µg/ml)</th>
<th>Phenylbutazone concentration (µg/ml)</th>
<th>Free concentration (M ± s.d.)</th>
<th>Degree of carbenoxolone binding (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>-</td>
<td>$0.8 \times 10^{-7} \pm 0.17$</td>
<td>99.7</td>
</tr>
<tr>
<td>20</td>
<td>100</td>
<td>$1.0 \times 10^{-7} \pm 0.10$</td>
<td>99.6</td>
</tr>
<tr>
<td>450</td>
<td>-</td>
<td>$28.5 \times 10^{-5} \pm 3.2$</td>
<td>62.1</td>
</tr>
<tr>
<td>450</td>
<td>225</td>
<td>$32.3 \times 10^{-5} \pm 2.4$</td>
<td>58.3</td>
</tr>
</tbody>
</table>

Results are the means of 6 observations.

Ultrafiltration of $10^{-4} M$ human serum albumin in 0.1 $M$ phosphate buffer at pH 7.4 was carried out at 4°C. 20 µg/ml carbenoxolone represents therapeutic albumin/carbenoxolone levels and 100 µg/ml phenylbutazone is a 10-fold molar equivalent. 450 µg/ml carbenoxolone was a level approximate to 50% binding and 225 µg/ml of phenylbutazone is an equimolar level.
2.4 CONCLUSIONS

The present experiments with equilibrium dialysis have shown that the methodology is not entirely satisfactory for the evaluation for carbenoxolone binding. Significant binding of carbenoxolone to the visking membrane was encountered not only with equilibrium dialysis but also with ultrafiltration. Even though steps were taken to reduce the binding, it could not be abolished in the absence of protein, although it was further reduced in the presence of high concentrations of protein. Binding to membranes is a known impediment of these methods (Davison, 1971). Lindup (1971), using ultrafiltration techniques, did not encounter membrane binding difficulties, although in the presence of whole serum the binding of carbenoxolone may be minimal. The difficulties in obtaining equilibrium with carbenoxolone in the absence of protein may well be due to this extraneous binding.

Results from the equilibrium dialysis studies of carbenoxolone binding to bovine serum albumin showed that, in contrast to previous studies using human serum albumin, the drug was binding to one class of site only (Chakraborty, et al., 1970; Lindup, 1971; Parke & Lindup, 1973). These authors studied the protein binding of carbenoxolone in other species but only used whole serum rather than isolated serum protein. The present studies using a fluorescent probe have confirmed that there is only one single class of binding site for carbenoxolone on bovine serum albumin, although the actual number of binding sites per protein molecule are different according to the experimental method used. The present findings concerning the binding of phenylbutazone and warfarin to bovine serum albumin agree with those of Jun and colleagues (1972) so that the present results using a fluorescent probe are validated. A possible explanation for the difference found in the present work between equilibrium dialysis and fluorescent probe methods may be due to differences in the ionic strength of the buffer used, since ionic strength did influence the
probe fluorescence and is known to alter protein binding (Davison, 1971). The buffer molarity was 0.1 \( M \) for equilibrium dialysis and ultrafiltration studies but 0.05 \( M \) for the fluorescent probe method. Alternatively, the presence of binding sites on the visking membrane may constitute a second difference between these methods of measurement. Whatever small differences exist between the results obtained from the different methods, it is clear that only one class of binding site exists on bovine serum albumin for carbenoxolone, phenylbutazone and warfarin.

Thus the inference may be made that both carbenoxolone and phenylbutazone are likely to displace warfarin from binding sites on bovine albumin. In contrast, only phenylbutazone will displace warfarin from human albumin. The latter interaction is well known from clinical and animal studies \textit{in vitro} (Sigg, \textit{et al}, 1956; Aggeler, \textit{et al}, 1967; O'Reilly & Levy, 1970; Jun, \textit{et al}, 1972). In addition, phenylbutazone has been shown to displace carbenoxolone from bovine albumin binding sites. It is interesting to note that the presence of phenylbutazone reduces the number of binding sites for carbenoxolone from 9 to 6 which may, in part, be the mechanism of displacement. In reducing the number of binding sites it is possible that phenylbutazone is (a) binding at 3 sites per molecule, thus reducing the number available to 6, or (b) inducing a conformational change in the albumin so that 3 carbenoxolone sites are no longer available, or (c) preventing a conformational change induced by carbenoxolone to increase the binding sites by 3. Parke and Lindup (1973) suggested that a conformational change was not likely to be induced by carbenoxolone on human serum albumin.

From the fluorescent probe study with bovine and human serum albumins, the results reveal a species difference. With human serum albumin more than one class of binding site was present for the fluorescent probe itself. Carbenoxolone
was bound at the first class of site, whereas phenylbutazone and warfarin were bound at the second class of site, leading to the suggestion that carbenoxolone was not likely to interact with the other two drugs by competitive displacement.

Measurement of carbenoxolone binding by ultrafiltration confirmed the species difference, and the Scatchard plot of carbenoxolone binding was similar to that of Lindup (1971) but with lower affinities. The present experiments suggest binding affinities of at least $10^6$ l/mole and $7.1 \times 10^3$ l/mole, for the two sites. Whilst Chakraborty and colleagues (1970) found $10^7$ l/mole and $2 \times 10^6$ l/mole, Parke and Lindup (1973) agreed with the former but interpreted the latter as $3 \times 10^6$ l/mole. The numbers of binding sites per molecule were also slightly different to previous workers, $n_1 = 3$, and $n_2 = 7$ in the present experiments, whilst previous results were $n_1 = 3$, $n_2 = 6$ (Chakraborty, et al, 1970) and $n_1 = 2$, $n_2 = 4$ (Parke & Lindup, 1973). These differences may be due to slight changes in the methodology such as temperature, since it is known that protein binding may be temperature-dependant (Davison, 1971). The present experiments were performed at $4^\circ$C for comparison with the equilibrium dialysis. Lindup did not report the use of any de-fatting procedures for his crystalline albumin fractions, whilst in the present study such procedures did not alter the binding characteristics of the bovine or human serum albumin used. It is known that the presence of fatty acid contamination can cause alteration of binding characteristics (Chen, 1967). However, as most interpretations of binding data depend upon linear transformations and various constant assumptions, the method used to evaluate $n$ and $k$ by different authors may influence the actual values obtained (Madsen & Robertson, 1974; Swillens & Dumont, 1975). Similar views on comparison between results of different authors have been expressed by Vallner and colleagues (1976) who have illustrated the point by a literature survey of
dicoumarol binding affinity whose range showed a 30-fold difference between authors.

The present ultrafiltration studies also confirmed another prediction from the fluorescent probe study, in that phenylbutazone and warfarin were not seen to displace carbenoxolone from binding sites on human serum albumin either directly or by altering the available binding sites by conformational changes.

It is interesting to note that carbenoxolone, up to a concentration of 250 µg/ml, was maximally bound to human serum albumin at a concentration of $10^{-4} M$. In whole blood this represents a binding capacity of carbenoxolone to albumin solution of 1.68 mg/ml, when assuming a serum albumin concentration of 4.5 g/dl. In turn this represents, for a blood volume of 5 litres and a dose level of carbenoxolone of 300 mg per day, a binding capacity for 28 days accumulated dose. Therefore the capacity of albumin for the binding of carbenoxolone greatly exceeds the concentrations of carbenoxolone observed clinically.

Thus the present experiments in vitro have confirmed the high degree to which carbenoxolone sodium binds to albumin, maximal binding at therapeutic drug/albumin ratios being observed. A species difference in the binding of carbenoxolone and other drugs was clearly seen, suggesting that for extrapolation to the clinical situation, human serum albumin should be used. Some differences between experimental methods and authors were also indicated, suggesting that comparison between experiments in vitro should be carried out on one experimental model under identical conditions to enable suggestions for clinical interaction to be made. Studies along these lines are reported in Chapter 3.
Chapter 3

THE BINDING OF CARBENOXOLONE AND OTHER DRUGS

BY FLUORESCENT PROBE TECHNIQUE
Chapter 3
THE BINDING OF CARBENOXOLONE AND OTHER DRUGS
BY FLUORESCENT PROBE TECHNIQUE

3.1 INTRODUCTION

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3.1.2 Present studies

3.2 EXPERIMENTAL

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3.2.2 Studies with a second fluorescent probe (BANS)
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Chapter 3

THE BINDING OF CARBENOXOLONE AND OTHER DRUGS
BY FLUORESCENT PROBE TECHNIQUE

3.1 INTRODUCTION

Drug interactions are often complex in mechanism and cannot always be reliably predicted from studies in vitro. However, it is unrealistic to examine for all possible drug interactions in vivo (Modell, 1964). Investigation of the possible interactions of carbenoxolone, involving displacement from protein binding sites, followed careful selection of drug candidates. These included other protein-bound drugs which may be encountered in the clinical use of carbenoxolone, drugs which have shown 'classical' displacement interactions, representatives of drugs administered on a long-term basis and analogues of the drug, carbenoxolone, itself. The following introduction attempts to give the background to these drug candidates which are listed in Table 3.1.

3.1.1 DRUG CANDIDATES FOR INTERACTION STUDY

Drugs which are detrimental to the gastro-intestinal tract

Carbenoxolone is a drug which heals gastric and duodenal ulcers and is therefore likely to be used concomitantly or after other drugs which may induce dyspepsia or ulceration, the most common of which are the non-steroidal anti-inflammatory agents. There are five main categories of non-steroidal anti-inflammatory agents, (i) salicylates, (ii) pyrazoles, (iii) anthranilic acids, (iv) phenylalkanoic acids, and (v) indoleacetic acids.

The salicylates. In 1899 Dreser synthesised aspirin (Fig. 3.1) which is still the most extensively used analgesic-antipyretic and anti-inflammatory drug. In
Table 3.1 DRUG CANDIDATES FOR INTERACTION STUDY
IN VITRO WITH CARBENOXOLONE

1) Drugs detrimental to the gastro-intestinal tract

Non steroidal anti-inflammatory agents:
    aspirin
    phenylbutazone
    flufenamic acid
    ibuprofen
    indomethacin

Corticosteroids:
    prednisolone

2) Drugs which have shown interaction by displacement from
plasma binding sites

    warfarin sodium
    tolbutamide
    chlorpropamide

3) Drugs employed in long-term therapy

    phenytoin
    imipramine

4) Analogues of carbenoxolone

    enoxolone (parent triterpenoid)
    cicloxolone (cyclohexane di-carboxylic acid replaces succinic acid)
common with other anti-inflammatory agents aspirin irritates the gastro-intestinal mucosa leading to haemorrhage and ulcer formation in man and animals (Paulus & Whitehouse, 1973). It is readily absorbed from the stomach and is extensively bound to plasma proteins, mainly albumin, in the circulation (Goodman & Gilman, 1975b). Together with other salicylates, aspirin is known to displace many substances from plasma binding sites.

**Pyrazoles.** The pyrazoles as a class have been known for many years and were in use before the synthesis of aspirin, although phenylbutazone (Fig. 3.1) was introduced in 1949. Phenylbutazone is rapidly absorbed from the stomach and extensively bound to plasma proteins (Burns, et al, 1953; 1955). Its toxicity includes gastro-intestinal irritation (Steinberg, et al, 1953), although a significant portion of the drug's potential toxicity lies in its ability to displace and potentiate many drugs from plasma binding sites (Goodman & Gilman, 1975b).

**Anthranilic acids.** The members of this group are the amine analogues of the salicylates, and include flufenamic acid (Fig 3.1). The analgesic potency of flufenamic acid is equal to that of the salicylates but the anti-inflammatory activity is less (Winder, et al, 1963; 1969). Flufenamic acid is rapidly absorbed from the stomach and is extensively bound to plasma proteins (Martindale, 1977). It is also known to displace and potentiate other drugs bound to plasma proteins (Goodman & Gilman, 1975b).

**Phenylalkanoic acids.** This group of drugs was initiated with ibufenac and its methyl analogue, ibuprofen, which were synthesised in 1964 (Hart & Boardman, 1965). Ibuprofen (Fig 3.1) possesses anti-inflammatory, antipyretic and analgesic activity but is less potent than aspirin (Adams, et al, 1969a; 1969b). Oral doses are well absorbed and extensively bound to plasma proteins, being capable of
Fig 3.1 - NON-STERoidal ANTI-INFLAMMATORY AGENTS
displacing other protein-bound drugs (Martindale, 1977). Gastro-intestinal irritation is considered to be less with ibuprofen than with aspirin but has produced peptic ulceration in combination with aspirin and exacerbated ulceration already present (Goodman & Gilman, 1975b).

Indoleacetic acids. This group was initiated in 1963 with indomethacin (Fig. 3.1) which is a potent anti-inflammatory and antipyretic agent, being more potent than aspirin (Winter, et al, 1963). Indomethacin is well absorbed from the gastro-intestinal tract and is about 90% bound to plasma albumin (Duggan, et al, 1972). Antagonism of its anti-inflammatory activity by aspirin has been demonstrated with \(^{14}\)C-indomethacin both in animal models and clinically (Jeremy & Towson, 1970; Yesair, et al, 1970), although these results were not confirmed with non-radioactive indomethacin (Champion, et al, 1972). It is thought that the oral anticoagulants are not displaced by indomethacin (Goodman & Gilman, 1975b), whereas sulphonamides are displaced and potentiated (McIver, 1967). Indomethacin is irritant to the whole gastro-intestinal tract, not only leading to dyspepsia and peptic ulceration, but to ulcerative lesions of the bowel (Lövgren & Allender, 1965; Boardman & Hart, 1967; Duggan, et al, 1975). These effects are readily demonstrable in small animals, especially following adrenalectomy (Thornton & Sacra, unpublished observations).

In common with the above anti-inflammatory agents, steroids with anti-inflammatory actions are also detrimental to the gastro-intestinal tract.

Corticosteroids, both natural and synthetic, are used in many disorders ranging from replacement therapy, following adrenalectomy, to immunosuppression and anti-inflammatory therapy (Wilson & Schild, 1968). It is recognised that the side effects of corticosteroid therapy are multiple and it has been suggested that they are enhanced by an
increase in free plasma concentrations (Lewis, et al, 1971). Prednisolone (Fig. 3.2) was one of the early corticosteroids synthesised in 1959 and possesses glucocorticoid, immunosuppressive and anti-inflammatory actions. The drug is readily absorbed orally and is bound to plasma proteins only to the extent of about 55% (Lewis, et al, 1971). The same authors correlated increased prednisolone toxicity in elderly patients, with reduced plasma albumin levels and, presumably, reduced drug binding capacity.

**Drugs which have shown displacement interactions**

**Coumarin anticoagulants.** The oral anticoagulants are so called because, although they have no activity in vitro, they affect coagulation by competing with vitamin K during synthesis of prothrombin. One of the earliest recognised drug-drug interactions was that of phenylbutazone with oral anticoagulant agents (Sigg, et al, 1956) although the mechanism of displacement from plasma binding sites was not elucidated until later (Aggeler, et al, 1967; O'Reilly & Levy, 1970). The coumarin anticoagulants, as a group, are subject to interaction changes of potency by several mechanisms, although displacement from plasma binding sites is a major one (Goodman & Gilman, 1975c). Furthermore, the plasma half-life of chlorpropamide, tolbutamide and phenytoin may be increased by concomitant therapy with coumarin anticoagulants (Koch Weser & Sellers, 1971).

Warfarin (Fig. 3.2) is the most widely used of the coumarin anticoagulants (Mackie & Douglas, 1978). The drug is readily absorbed from the gastro-intestinal tract and is highly bound to plasma proteins, with an average plasma half-life in man of 44 hours (Aggeler & O'Reilly, 1968). It is well known that several anti-inflammatory drugs will displace warfarin from plasma binding sites, and that warfarin, in turn, will displace sulphonylurea hypoglycaemic agents (Stockley, 1974). Warfarin has thus become a 'classical' drug in the study of drug interactions.
Fig. 3.2 DRUGS USED IN THE FLUORESCENT PROBE STUDY
Sulphonylurea oral hypoglycaemic agents. These agents stimulate the release of insulin from the Islets of Langerhans in both man and many animal species, but not in the dog (Root & Anderson, 1956). The synthesis of chlorpropamide (Fig. 3.2) in 1958 soon followed that of tolbutamide in 1956 (Fig. 3.2), these two agents being the most commonly used sulphonylureas.

Both tolbutamide and chlorpropamide are readily absorbed from the gastro-intestinal tract and are extensively bound to plasma proteins (Martindale, 1977; Crooks & Brown, 1974). The main difference in activity between these compounds lies in their different rates of metabolism. The plasma half-life of tolbutamide in man is about 5 hours, whilst that of chlorpropamide is about 36 hours (Goodman & Gilman, 1975a). Coumarin anticoagulant drugs, together with many other agents, will displace the sulphonylureas from plasma binding sites (Sise, 1967; Tannenbaum, et al, 1974; Brown & Crooks, 1976).

Drugs used in long-term therapy

Phenytoin (Fig. 3.2) was first introduced in 1938, (Merritt & Putnam, 1938a; 1938b) and is still one of the most widely used anticonvulsant drugs (Woodbury & Kemp, 1971). It is a basic drug which has only a limited aqueous solubility and is thus slowly absorbed from an oral or parenteral dose (Glazko, 1972). The drug is 70-95% bound to plasma proteins in circulation, with a half-life of about 24 hours in man, although the half-life is dose-dependant at high dose levels. The plasma half-life of phenytoin is decreased by concomitant therapy with other drugs, such as phenobarbitone, which induce hepatic microsomal enzymes. In contrast, drugs such as disulfiram and dicoumarol will increase plasma levels of phenytoin by decreasing the metabolism of the anticonvulsant (Reynolds, 1967; Christensen & Skövsted, 1969). Displacement from plasma
binding sites has been demonstrated in vitro by tolbutamide and correlated with studies in vivo (Wesseling & Mols-Thürkow, 1975).

**Imipramine** (Fig. 3.2) is a basic drug belonging to the group of tricyclic antidepressant agents and was first evaluated as an antidepressant by Kuhn (1958). The drug is well absorbed from an oral dose and one of its metabolites, desmethylinipramine, is also pharmacologically active. The binding of imipramine to plasma proteins has been described as weak, whereas its principal metabolites are more highly bound (Glassman & Perel, 1973; Glassman et al, 1973). The pharmacological activities of phenylbutazone and coumarin anticoagulants are potentiated by imipramine, due to an inhibition of liver microsomal enzymes, and hence the metabolism of the other drugs (Remmer & Mercker, 1965). A similar mechanism is thought to be the mode of the potentiation of phenytoin by imipramine (Borgå, et al, 1969). However, the absorption of phenylbutazone is delayed by desmethylinipramine, possibly by its anticholinergic activity (Consolo, 1968).

**Carbenoxolone analogues**

**Enoxolone** (Fig. 3.3) is the parent triterpenoid of carbenoxolone, the hemi-succinate ester of enoxolone. It has some anti-inflammatory activity similar to that of carbenoxolone, furthermore its metabolism and excretion are similar in the rat (Finney & Somers, 1958; Finney, et al, 1958; Parke, et al, 1963). Little study has been made of the protein binding of enoxolone.

**Ciclooxolone** (Fig. 3.3) is the cyclohexane di-carboxylic acid analogue of carbenoxolone. This compound has a greater anti-inflammatory activity than carbenoxolone, with a similar effect on glycoprotein synthesis (Thornton, MacDonald, Sacra & Gottfried, unpublished observations). The metabolism of ciclooxolone is also similar to that of carbenoxolone (Gilbert & Rhodes, personal communication).
Fig 3.3 ANALOGUES OF CARBENOXOLONE

- enoxolone: $R = \text{H}$
- carbenoxolone: $R = -\text{CO-(CH}_2\text{)}_2\text{-COOH}$
- cicloxolone: $R = -\text{CO}$

![Diagram of analogues of carbenoxolone](image)
3.1.2 PRESENT STUDIES

The binding of the fluorescent probe, 1-...-naphthalene sulphonic acid (ANSA), to serum albumin was reviewed in the previous chapter. However, Jun and Luzzi (1971) have indicated that ANSA is probably binding to tryptophan residues on bovine serum albumin whilst a second experimental probe, N-benzyl-(...-naphthalene)-1-sulphonamide (BANS), was more hydrophobic and therefore likely to bind at alternative sites. The fluorescent probe technique is a useful method of evaluating possible displacement interactions \textit{in vitro} of carbenoxolone sodium. Studies with the second probe, BANS, are proposed here in addition to the use of ANSA.

The list of drugs to be used has been reviewed above. In an attempt to assist correlation with studies \textit{in vivo}, evaluation of the fluorescent probe binding to diluted whole serum is proposed, whereby the serum is diluted to the same albumin levels as that used in previous studies with crystalline albumins.
3.2 EXPERIMENTAL

3.2.1 STUDIES OF THE BINDING OF CARBENOXOLONE AND OTHER DRUGS BY THE FLUORESCENT PROBE ANSA

The probe, 1-anilino-8-naphthalene sulphonic acid (ANSA), human serum albumin, buffer and solvents were used as in the previous study (section 2.2.2). Carbenoxolone sodium, cicloxiolone, phenylbutazone, aspirin, imipramine, prednisolone (all from Biorex Laboratories Ltd.), warfarin (Gehardt-Penick), tolbutamide and chlorpropamide (both from Berk Pharmaceuticals Ltd.), phenytoin sodium (Phase Separations), flufenamic acid (Aldrich) and ibuprofen (Boots Ltd.) were used as 0.1 M solutions in methanol. Enoxolone (Biorex Laboratories Ltd.) and indomethacin (Merk, Sharpe & Dohme Ltd.) were used at 5 x 10^-2 M and cholesterol (BDH) at 2.5 x 10^-2 M in methanol.

Fluorescence titrations and treatment of fluorescence data were performed as described previously (section 2.2.2).

Native fluorescence and interference with ANSA spectrum. Aliquots of 2 ml human albumin solution, with drug added, were placed in quartz cuvettes and scans taken to investigate native fluorescence around the specific wavelengths of the probe (380/470 nm). Excitation scans were taken from 310-420 nm and emission scans from 440-510 nm.

To investigate a possible shift in the excitation or emission spectra of the probe, the latter was added to the drug-albumin complex at a level of 10^-5 M and the excitation and emission scans repeated.

Samples of human serum albumin were subjected to the defatting technique of Chen (1967) and the fluorescence titrations repeated.
INVESTIGATION OF A SECOND FLUORESCENT PROBE (BANS)

BANS (N-[5-dimethylaminoapthalene]-1-sulphonamide) was synthesised by reacting dansyl chloride with excess benzylamine (Fig. 3.4).

Binding studies. The probe, BANS, was used as a $10^{-3} M$ solution in methanol. Protein and drug solutions were as in the ANSA study above (section 3.2.1) and the fluorescence titrations performed as with ANSA. Fluorescence scans of the excitation and emission wavelengths were carried out with BANS in 0.05 M phosphate buffer at pH 7.4. Scans were taken of the free probe and when bound to human serum albumin at a concentration of 1.0 mg/ml. The effect on the fluorescence spectrum of the displacement of the probe, by phenylbutazone at a concentration of $10^{-4} M$, was also investigated.

3.2.3 FLUORESCENCE STUDIES WITH DILUTED RAT AND HUMAN SERA

Attempts were made to correlate the fluorescence titrations of albumin fractions with those of whole serum diluted to produce the same albumin concentrations (0.1 and 1.0 mg/ml). Human and rat sera were used.

Serum albumin assay. This manual method is based on the automated assay in use in Biorex Laboratories using bromocresol green binding (Technical Bulletin No. 11, October 1967. Association of Clinical Biochemists Scientific Technical Committee). Citrate buffer (1M) was used at pH 3.8 whilst the colour reagent stock solution contained bromocresol green and 0.1 M NaOH. An EEL Colorimeter (Spectra 197) was used with measurements made at 635 nm. Blanks were used with citrate buffer and serum whilst 10 µl of serum in 2 ml bromocresol green reagent was assayed. The reagent quantitatively developed a blue colour when protein-bound, the absorption
N-Benzyl

Fig 3.4 SYNTHESIS OF \((5\text{-DIMETHYLAMINONAPHTHALENE})-1\text{-SULPHONAMIDE} (\text{BANS})\)

Dansyl chloride and excess benzylamine were mixed and left overnight at room temperature. The reaction mixture was poured into water and the precipitated solid filtered, washed with water and dried overnight in an oven at 50°C. The yield was 99%. 
of which was measured at 635 nm. Crystalline human serum albumin was used for calibration and a standard serum, 'Monitrol', included to verify the assay.

**Fluorescence titrations.** Human blood was collected from two male volunteers, placed in a polypropylene tube and allowed to clot at 4°C for 30 minutes. The serum was collected, after centrifugation, and stored at -20°C until used. Rat serum was prepared in a similar way but collected from the abdominal aorta under ether anaesthesia from 350 g male Biorex Wistar rats. The serum was assayed for albumin content, then diluted with 0.05 M phosphate buffer at pH 7.4 to give 0.1 and 1.0 mg/ml solutions of albumin. The diluted sera were titrated with the probe ANSA in the same way as for the crystalline albumin fractions (section 3.2.1).
3.3 RESULTS

3.3.1 THE BINDING OF CARBENOXOLONE AND OTHER DRUGS BY THE FLUORESCENT PROBE ANSA

Titration of the fluorescent probe, ANSA, with human serum albumin at a concentration of 0.1 mg/ml demonstrated a saturation of binding sites, but at 1.0 mg/ml a linear increase in fluorescence with probe concentration was evident (Fig. 3.5). The Scatchard plot of the probe binding was non-linear, suggesting the presence of 3 classes of binding sites on human serum albumin (Fig. 3.6) where $n_1 = 1$, $n_2 = 3$, $n_3 = 4$, being confirmed by regression analysis of the 3 linear portions (coefficients 0.93, 0.99 & 0.96).

The presence of various drugs reduced the fluorescence by competitive displacement of the probe. With the present series of drugs, binding was clearly differentiated into three groups:

i) The group which exhibited binding at the class I binding site only ($n = 1$) comprise the first category, group 1 (Fig. 3.7). The group includes carbenoxolone, enoxolone, ciclolexolone, indomethacin and cholesterol.

ii) Those drugs which were bound to the class II site only ($n = 3$) comprise the second category, group 2 (Fig. 3.8). The group includes flufenamic acid, phenylbutazone, warfarin, tolbutamide and imipramine.

iii) Those drugs which displayed binding with a weak affinity to both class I and class II sites comprise group 3 (Fig. 3.9) and include phenytoin, prednisolone, aspirin, chlorpropamide and ibuprofen.

Native fluorescence and interference with the ANSA spectrum.

None of the above drugs showed any interference with the fluorescence spectrum of the probe, except by reducing the fluorescence intensity. Native fluorescence was absent in most of the drugs with the following exceptions: flufenamic acid fluoresced in ethanolic solution with $\lambda_{activation}$ 400 nm and $\lambda_{fluorescence}$ 475 nm. This fluorescence underwent a change when bound to human albumin, becoming
Fig 3.5 VARIATION OF FLUORESCENCE INTENSITY WITH FLUORESCENT PROBE CONCENTRATION, FOR HUMAN SERUM ALBUMIN.

Two ml of albumin solution, at 0.1mg/ml (O) and 1.0mg/ml (O), was titrated with 1-anilino-8-naphthalene sulphonic acid (ANS) at a concentration of 0.1 M in methanol. The fluorescence intensity was measured at 380/470 nm.
Fig 3.6 SCATCHARD PLOT FOR ANSA WITH HUMAN SERUM ALBUMIN

Where $V$ is the mole ratio of bound ligand per mole of albumin and $A$ is the molar concentration of free ligand.

Two ml of human serum albumin solution, at concentrations of 1.0 and 0.1mg/ml in 0.05M phosphate buffer at pH7.4, were titrated with 1-anilino-8-naphthalene sulphonic acid (ANSA) at a concentration of 0.1M in methanol.

The abscissa intercepts reveal three classes of binding site, $n_1=1$, $n_2=3$, $n_3=4$. 

Fig 3.7 SCATCHARD PLOT FOR DRUGS BINDING TO HUMAN ALBUMIN

Where $V$ is the mole of bound ligand per mole of albumin and $A$ is the molar concentration of free ligand.

Results are from the titration of the probe, ANSA, with human serum albumin in 0.05M phosphate buffer at pH 7.4 in the presence of the following drugs at $10^{-4}M$: carbenoxolone ($\mathbb{H}$), ciclooxalone ($\mathbb{O}$), indomethacin ($\Delta$), enoxolone ($\mathbb{O}$) and cholesterol($\Delta$).
Fig 3.8 SCATCHARD PLOT FOR DRUGS BINDING TO HUMAN ALBUMIN

Where $\bar{V}$ is the mole of bound ligand per mole of albumin and $A$ is the molar concentration of free ligand.

Results are from the titration of the probe, ANSA, with human serum albumin in 0.05M phosphate buffer at pH 7.4 in the presence of the following drugs at $10^{-4}M$: flufenamic acid (□), phenylbutazone (○), warfarin (△), tolbutamide (⊗) and imipramine (▲).
Fig. 3.9 SCATCHARD PLOT FOR THE BINDING OF DRUGS TO HUMAN ALBUMIN

Where \( \bar{V} \) is the mole of bound ligand per mole of albumin and \( A \) is the molar concentration of free ligand.

Results are from the titration of the probe, ANSA, with human serum albumin in 0.05M phosphate buffer at pH 7.4 in the presence of the following drugs at 10\(^{-4}\)M: prednisolone (O), aspirin (A), ibuprofen (□), chlorpropamide (©) and phenytoin (△).
350 and 440 nm respectively. However, this native fluorescence was negligible and did not interfere with that of the bound probe at the respective activation and fluorescence wavelengths of 380 and 470 nm. Similarly warfarin in ethanolic solution demonstrated native fluorescence at 360/400 nm which changed to 320/440 nm when bound to human albumin. This did not interfere with the probe fluorescence. Imipramine demonstrated two episodes of native fluorescence when bound to human serum albumin at wavelengths of 380/430nm and 410/470 nm, although again this fluorescence was negligible at 380/470 nm.

Defatting of human albumin. Following defatting of the human serum albumin by the method of Chen (1967), no changes in the fluorescence of the probe ANSA, or the Scatchard plot of binding, were seen (Fig. 3.10). This finding confirmed that the sample of albumin used was of sufficient quality not to require further purification.

3.3.2 STUDIES WITH A SECOND FLUORESCENT PROBE (BANS)

The purity of BANS was evaluated by an infra-red scan of a nujol mull preparation yielding absorptions in the appropriate regions corresponding to the desired structure. The melting point was 141-142°C.

Binding studies

Fluorescence spectrum. A scan of the fluorescence of BANS in methanolic solution revealed a native fluorescence at 385/520 nm which was also present, although weak, in a solution of buffer at pH 7.4 (Table 3.2). However, when bound to human serum albumin the fluorescence was some 30 times higher and underwent a blue shift to 350/480nm. Addition of phenylbutazone to give a concentration of $10^{-4}M$ reduced the fluorescence to zero but caused the reappearance of the fluorescence at 385/540 nm. This suggested that BANS had been displaced from the albumin by phenylbutazone.
Table 3.2 FLUORESCENCE SPECTRUM OF BANS

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Activation wavelength (nm)</th>
<th>Fluorescence wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Methanol</td>
<td>385</td>
<td>520</td>
</tr>
<tr>
<td>(2) $0.05M$ phosphate at pH 7.4</td>
<td>350</td>
<td>540 (weak)</td>
</tr>
<tr>
<td>(3) $0.1$ mg/ml human serum albumin in (2)</td>
<td>355</td>
<td>495 (strong)</td>
</tr>
<tr>
<td>(4) $10^{-4}M$ phenyl-butazone in (3)</td>
<td>350</td>
<td>540 (weak)</td>
</tr>
</tbody>
</table>

The probe concentration was $2.0 \times 10^{-5}M$ having been added to $2$ ml of the solvent as a $10^{-3}M$ methanolic solution. Fluorescence was measured on a Baird Atomic Fluoripoint FP100 spectrofluorimeter.
Fluorescence intensity (arbitrary units)

Fig 3.10 COMPARISON OF THE FLUORESCENCE OF NORMAL AND DE-FATTED HUMAN ALBUMIN, WITH THE FLUORESCENT PROBE ANSA

Two ml of albumin solution, normal (O) and de-fatted by the method of Chen (1967) (©), at 1.0 mg/ml were titrated with the fluorescent probe 1-anilino-8-naphthalene sulphonic acid (ANSA) at a concentration of 0.1 M in methanol. The fluorescence was measured at 380/470 nm.
Fluorescence titration. The fluorescence of BANS, when bound to human serum albumin, was much lower than that for ANSA, and was reduced in the presence of warfarin and phenylbutazone, both at a concentration of \(10^{-4}M\). In contrast, the presence of carbenoxolone did not displace the probe. However, the Scatchard plot of binding was parallel to the abscissa perhaps indicating a non-specific binding.

3.3.3 FLUORESCENCE STUDIES WITH DILUTED RAT AND HUMAN SERA

Albumin assay. Standards of human serum albumin gave a curvilinear plot but were consistently reproducible and in agreement with the 'Monitrol' Standard.

Fluorescence titrations. Titrations of both diluted rat and human serum produced a fluorescence which was similar to that of the crystalline albumin fractions. However, there was a slight tailing off of fluorescence at an albumin concentration of 1.0 mg/ml. A Scatchard plot of the binding of both rat and human serum revealed only one class of binding site for ANSA \((n = 1)\) (Fig. 3.11). The presence of displacing drugs caused only minimal reduction of fluorescence and did not significantly alter the Scatchard plot.
Fig 3.11 SCATCHARD PLOT FOR ANSA WITH DILUTED RAT AND HUMAN SERUM

Where \( \bar{V} \) is the mole ratio of bound ligand per mole of albumin and \( A \) is the molar concentration of free ligand.

Two ml of rat (\( \text{A} \)) and human (\( \text{O} \)) sera, diluted to 0.1 and 1.0 mg/ml albumin concentration in 0.05M phosphate buffer at pH 7.4, were titrated with 1-anilino-8-naphthalene sulphonic acid at a concentration of 0.1M in methanol.
3.4 CONCLUSIONS

The fluorescent probe technique is an indirect assessment of protein binding but gives a good qualitative correlation between the protein binding affinities of the drugs used.

The results of this study allow the arrangement of the drugs into three groups, and the prediction of drug-drug displacement interactions (Table 3.3). From group 2 it may be inferred that phenylbutazone would displace warfarin and both, in turn, would displace tolbutamide; indeed, such interactions have been demonstrated in vivo (Aggeler, et al, 1967; O'Reilly & Levy, 1970; Assandri & Perazzi, 1976). Within group 1 it is possible to suggest that carbenoxolone would displace not only indomethacin but its own analogues and cholesterol. Interaction between these drugs from groups 1 and 2 is not considered likely because the drugs are binding at differing classes of site. This suggestion has been inferred elsewhere, in that indomethacin is thought not to potentiate the coumarin anticoagulants (Goodman & Gilman, 1975b).

The drugs from group 3 were bound at the class I and II sites but with a weaker affinity than those drugs which were bound to only one of the sites. These findings lead to the prediction of possible displacement by drugs from both group 1 and group 2 of those in group 3. Again, some of these displacement interactions have been demonstrated both in vivo and in vitro. Indomethacin is known to displace aspirin from one of its two binding sites in vitro, although the reverse effect is seen in vivo, with the possible explanation being due to the prevention of indomethacin binding at its primary site by the presence of aspirin at the secondary site (Mason & McQueen, 1974). Furthermore, both phenylbutazone and warfarin are known to displace tolbutamide (group 2) and chlorpropamide (group 3) (Stockley, 1974; Assandri & Perazzi, 1976).
Table 3.3 BINDING RESULTS FROM ANSA FLUORESCENCE

<table>
<thead>
<tr>
<th>Group</th>
<th>Class of binding site</th>
<th>Order of binding affinities</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I</td>
<td>carbenoxolone &gt; cicloxolone &gt; indomethacin &gt; enoxolone &gt; cholesterol</td>
</tr>
<tr>
<td>2</td>
<td>II</td>
<td>flufenamic acid &gt; phenylbutazone &gt; warfarin &gt; tolbutamide &gt; imipramine</td>
</tr>
<tr>
<td>3</td>
<td>I &amp; II</td>
<td>prednisolone &gt; aspirin &gt; ibuprofen &gt; chlorpropamide &gt; phenytoin</td>
</tr>
</tbody>
</table>

Binding affinities were indicated from the Scatchard plots.
Thus from the various known interactions between the drugs included in this study, there is good evidence to suggest that interpretation of drug displacement interactions from this model are valid. However, as was demonstrated with indomethacin and aspirin, \textit{in vitro} drug interactions are not always in accordance with the situation \textit{in vivo} (Mason & McQueen, 1974).

Further attempts to validate this fluorescent probe technique with ANSA were carried out with the second probe BANS, which is a more hydrophobic molecule. However, the usefulness of BANS, under the conditions used, did not merit its suggested rôle by other workers (Jun & Luzzi, 1971). The exact conditions used by these authors were not stated, but were possibly different to those used in the present series, in that a variation of the pH or buffer ionic strength may be necessary for the use of this probe. The pH of 7.4 was chosen in the present experiments as the average blood pH in order to obtain the closest possible correlation \textit{in vitro} with normal conditions \textit{in vivo}. It is also pertinent to note that the original authors used bovine and not human serum albumin as in the present experiments; even so, satisfactory results were not obtained in the present study using bovine serum albumin.

Fluorescence titrations of ANSA with diluted human serum revealed only one class of binding site and thus did not correlate with the studies using a crystalline albumin fraction. There are several reasons why the binding of the fluorescent probe may be different for the two protein preparations. The preparation and reconstitution of Cohn fraction V albumin may alter the secondary protein configurations and, in consequence, the binding sites, because even such small changes as the ionic strength of buffer will alter the binding characteristics of protein (Davison, 1971). A further complication may arise in the assay of the albumin portion of serum, in that the number of binding sites per molecule for bromocresol green may
differ slightly from those for the fluorescent probe ANSA. If an excess of albumin were present then only the primary, high affinity, site would be occupied, accounting for the single class of binding seen with diluted human and rat sera. A more likely explanation is that the presence of free fatty acids, or cholesterol, in the diluted sera prevented the fluorescent probe from binding to the secondary and subsequent sites. The presence of other proteins such as globulin may also be considered to alter the binding pattern of the probe.

In conclusion from these findings in vitro it is possible to postulate that carbenoxolone will not displace such drugs as warfarin and tolbutamide from their plasma binding sites in vivo. However, it is also obvious that any study in vitro cannot adequately reproduce the conditions encountered in vivo. Even the study in vitro with dilute serum did not correlate with the use of albumin fractions. Therefore, in order to obtain confirmation of these predictions from studies in vitro, further studies in vivo should be carried out. Such studies are presented in the following chapter.
Chapter 4

DRUG INTERACTIONS OF CARBENOXOLONE IN VIVO
Chapter 4

DRUG INTERACTIONS OF CARBENOXOLONE IN VIVO

4.1 INTRODUCTION

4.2 EXPERIMENTAL

4.2.1 Warfarin-carbenoxolone interaction
4.2.2 Tolbutamide-carbenoxolone interaction
4.2.3 Chlorpropamide-carbenoxolone interaction
4.2.4 Phenytoin-carbenoxolone interaction

4.3 RESULTS

4.4 CONCLUSIONS
4.1 INTRODUCTION

To assess the effect of carbenoxolone on the displacement of drugs from plasma binding sites in vivo, drugs with readily detectable pharmacological activities were selected from each of the representative groups of binding patterns discussed in the previous chapter. The selected drugs were warfarin, tolbutamide, chlorpropamide and phenytoin. The pharmacological activities of these drugs were monitored initially alone, then in the presence of a known displacing agent, phenylbutazone, and finally in the presence of carbenoxolone. The binding activities and pharmacological actions of the selected drugs are summarised below, together with the rationale of the methodology for estimation of their pharmacological potency.

Warfarin is bound to human serum albumin at a single class of site, identical to that of phenylbutazone but different to that of carbenoxolone. Phenylbutazone is known to displace warfarin from plasma binding sites, thus increasing the free concentration and potentiating the pharmacological activity of warfarin (O'Reilly & Levy, 1970). Warfarin exerts its mode of action because of its structural similarity to vitamin K, with which it competes during the synthesis of prothrombin (O'Reilly & Aggeler, 1968) and also reduces the extrinsic Factors VII and X. Prothrombin, or Factor II, forms part of the final stages of both the extrinsic and intrinsic pathways of coagulation at the stage of thrombin formation in the presence of Factor V and calcium ions (Simmons, 1968). Prothrombin times measure Factors II, V, VII and X and are thus an adequate estimation of warfarin activity.
Tolbutamide is bound to human serum albumin in a similar manner to warfarin, and is also potentiated by phenylbutazone (Assandri & Perazzi, 1976). The pharmacological action of this sulphonylurea is believed to be due to the stimulation of insulin release from the pancreas (Root & Anderson, 1956). Measurement of blood glucose levels in glucose-loaded rats will estimate the pharmacological potency of tolbutamide (Loubatières, 1964).

Chlorpropamide, in contrast to tolbutamide, is bound to human serum albumin at two classes of site, namely those of carbenoxolone and of phenylbutazone. As its pharmacological action is similar to that of tolbutamide, measurement of blood glucose levels in glucose-loaded rats will give an assessment of chlorpropamide activity.

Phenytoin is bound to human serum albumin in a similar manner to chlorpropamide and is also displaced from binding sites by phenylbutazone in vivo (Borgå, et al, 1969; Lunde, et al, 1970). Although its mode of action is unclear, the pharmacological activity of phenytoin may be measured by the inhibition of electroshock-induced convulsion (Toman, et al, 1946).

Phenylbutazone is bound to human serum albumin at the same sites as warfarin and tolbutamide, and shares one of the two binding sites of chlorpropamide and phenytoin. Phenylbutazone is known to displace and potentiate the pharmacological activities of the preceding drugs and is included in the present study to validate the experimental models.
4.2 EXPERIMENTAL

Dose levels expressed as mg/kg refer to mg/kg body weight throughout this and subsequent chapters.

4.2.1 WARFARIN-CARBENOXOLONE INTERACTION

This interaction was assessed by monitoring the effect on warfarin-prolonged prothrombin times.

Prothrombin estimations. These were carried out using the method of Quick (1935) with modifications introduced by Simmons (1968). Blood samples were obtained, under ether anaesthesia, using citrate/hepes buffer (0.13 M trisodium citrate and 0.05 M N-2-hydroxyethylpiperazine-N-2 ethanesulphonic acid) as anticoagulant (0.1 ml per ml of collected blood). Plasma was obtained by centrifugation at 1,150 g in an MSE bench centrifuge. Freeze-dried rabbit brain thromboplastin was used (Diagnostic Reagents Ltd.) and reconstituted prior to use.

Pyrex tubes (0.4 cm x 4 cm) were placed in a water bath at 37°C and aliquots of thromboplastin (0.1 ml) and plasma (0.1 ml) were added to each tube. Prewarmed 0.025 M aqueous calcium chloride (0.1 ml) was then added to each tube and a stop-watch was started immediately. The time taken for the appearance of a fibrous clot was recorded. Estimations of clotting times were performed in duplicate and, if the difference between the duplicated times was greater than 0.5s, a second duplicate estimation was carried out.

The effect of warfarin, carbenoxolone and phenylbutasone on prothrombin times. Groups of 8 female Biorex Wistar rats (200 ± 10g body weight) were fasted for 16 hours prior to treatment and were fed 8 hours after treatment. Warfarin was administered, by intubation, as an aqueous solution in a volume of 5ml/kg. A dose-response study of warfarin was carried out using dose levels between 1 and 20 mg/kg, after
which a single dose of 10 mg/kg was used for subsequent studies. Phenylbutazone and carbenoxolone were administered, subcutaneously in 0.9% NaCl (5ml/kg) at dose levels of 20 and 40 mg/kg respectively (twice the molar equivalent of warfarin) to assess any inherent activity on prothrombin times. Measurements of prothrombin times were made 1, 2, 4, 6, 8 and 24 hours after treatment.

A second series of tests was carried out with concomitant administration of either phenylbutazone or carbenoxolone with the warfarin.

4.2.2 TOLBUTAMIDE-CARBENOXOLONE INTERACTION

This interaction was assessed by monitoring the drug-induced fall of blood glucose concentrations in hyperglycaemic rats. Measurements of whole blood glucose concentrations were performed by the glucose oxidase method of Trinder (1969a, 1969b) using a Technicon Autoanalyser II.

Groups of 8 female Biorex Wistar rats (200 ± 10g body weight) were fasted for 16 hours prior to being rendered hyperglycaemic by the method of Sacra and Adamkiewicz (1965), which uses an oral load of 6 m mol glucose/100g body weight administered as a 3M solution. All drug treatments were given orally in 0.01% aqueous Tween 80 solution at a volume of 5ml/kg. A dose-response study of tolbutamide was carried out using dose levels between 50 and 200 mg/kg, after which a level of 50 mg/kg was used for subsequent studies.

Two models of interaction were studied, the first in which the interacting drug (phenylbutazone or carbenoxolone) was given before the tolbutamide, and the second in which the interacting drug was given in divided doses both before and after the tolbutamide (Table 4.1).

In the first model of interaction, tolbutamide was given alone 1 hour after the glucose load. Phenylbutazone or carbenoxolone were given 30 minutes after the glucose
Table 4.1 SCHEDULE OF TREATMENTS DURING SULPHONYLUREA-DRUG INTERACTION STUDIES

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Model 1</th>
<th>Model 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Glucose load</td>
<td>Interacting drug (equimolar)</td>
</tr>
<tr>
<td>1⁄2</td>
<td>Interacting drug (twice molar)</td>
<td>Sulphonylurea</td>
</tr>
<tr>
<td>1</td>
<td>Sulphonylurea</td>
<td>Glucose load</td>
</tr>
<tr>
<td>2</td>
<td>Blood sample</td>
<td>Interacting drug (equimolar)</td>
</tr>
<tr>
<td>2½</td>
<td>---</td>
<td>Blood sample</td>
</tr>
<tr>
<td>4</td>
<td>Blood sample</td>
<td>---</td>
</tr>
<tr>
<td>4½</td>
<td>---</td>
<td>Blood sample</td>
</tr>
<tr>
<td>6</td>
<td>Blood sample</td>
<td>---</td>
</tr>
<tr>
<td>6½</td>
<td>---</td>
<td>Blood sample</td>
</tr>
</tbody>
</table>

All treatments were oral. In Model 1 the interacting drug, carbenoxolone or phenylbutazone, was given at a dose level twice the molar equivalent of tolbutamide or chlorpropamide. In Model 2 this dose was divided into two equal portions.
load, at dose levels of 114 and 227 mg/kg (twice the molecular equivalent of tolbutamide). Blood samples were taken 2, 4 and 6 hours after the glucose administration. This regimen was repeated for carbenoxolone with bilaterally adrenalectomised rats, 21 days post-operatively. At termination the rats were examined to verify complete adrenalectomy.

The second model of interaction involved the treatments with phenylbutazone, or carbenoxolone, being given in two equimolar portions 1 hour before the glucose load and 1 hour after. Tolbutamide was administered 30 minutes before the glucose load and blood samples taken 1\frac{1}{2}, 3\frac{1}{2}, and 5\frac{1}{2} hours after the load.

4.2.3 CHLORPROPAMIDE-CARBENOXOLONE INTERACTION

This interaction was assessed by the second model above (section 4.2.2) in which the interacting drugs, phenylbutazone and carbenoxolone, were given in two administrations of 56 and 111 mg/kg respectively (the molar equivalents of chlorpropamide). Chlorpropamide was administered at 50 mg/kg.

4.2.4 PHENYT0IN-CARBENOXOLONE INTERACTION

Interaction with phenytoin was assessed by measurement of the effect on the protection of phenytoin against electroshock-induced convulsion.

Electro-shock convulsion was induced by the method of Toman and colleagues (1946). Two electrodes were placed sub-cutaneously, just posterior to the ears, to deliver pulses at a rate of 100 per second for a duration of 1 second at 80 volts and 200 mA. The pulse width was 5msec. A dose–response study of phenytoin was carried out, between 30 and 100 mg/kg, to establish an \( ED_{50} \), the level required to inhibit tonic seizure in 50% of the rats. The effect of concomitant doses of phenylbutazone (100 mg/kg) and carbenoxolone (200 mg/kg) with phenytoin (39 mg/kg) was then investigated. Dose levels of phenylbutazone and carbenoxolone were twice the molar
equivalent of phenytoin. Phenylbutazone and carbenoxolone were evaluated, at 200 and 400 mg/kg respectively, for intrinsic activity on electroshock-induced convulsions.
4.3 RESULTS

4.3.1 WARFARIN-CARBENOXOLONE INTERACTION

Treatment of rats for two days with warfarin, at a dose level of 4 mg/kg, produced a ten-fold increase in prothrombin time 48 hours after treatment. No effects of warfarin treatment were observed on the prothrombin time within an 8 hour period following treatment. At a dose level of 10 mg/kg, warfarin caused an increase in prothrombin time of nearly five-fold (p<0.001) 24 hours after treatment (Table 4.2). Maximal increases in prothrombin times were seen with dose levels greater than 20 mg/kg. Phenylbutazone and carbenoxolone were without inherent activity on prothrombin times, at the dose levels used (Table 4.2).

Phenylbutazone showed a significant (p<0.005) potentiation of warfarin activity, by causing an increase in the prothrombin time from 73.2 to 109.7 s. In contrast, the co-administration of carbenoxolone did not alter the prothrombin time from that following treatment with warfarin alone.

The inference from these findings must be that carbenoxolone does not displace warfarin from plasma binding sites leading to potentiation of its pharmacological activity, as is the case with phenylbutazone.

4.3.2 TOLBUTAMIDE-CARBENOXOLONE INTERACTION

Treatment with tolbutamide, at a dose level of 50 mg/kg, produced a significant reduction in blood glucose concentrations 2 and 4 hours after the glucose load. The reduction was not maximal, but this dose level was chosen to enable both potentiation and inhibition to be detected.

Phenylbutazone was without intrinsic activity on blood glucose concentrations (Table 4.3) but did significantly
Table 4.2 PROTHROMBIN TIMES IN RATS TREATED WITH VARIOUS DRUGS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose level (mg/kg)</th>
<th>Route of administration</th>
<th>Prothrombin time (s)</th>
<th>Number of rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Control</td>
<td>--</td>
<td>oral</td>
<td>17.0 ± 0.3</td>
<td>24</td>
</tr>
<tr>
<td>2 Warfarin</td>
<td>10</td>
<td>oral</td>
<td>73.2 ± 9.7(a)</td>
<td>24</td>
</tr>
<tr>
<td>3 Carbenoxolone</td>
<td>40</td>
<td>s.c.</td>
<td>17.4 ± 0.3</td>
<td>8</td>
</tr>
<tr>
<td>4 Phenylbutazone</td>
<td>20</td>
<td>s.c.</td>
<td>16.6 ± 0.9</td>
<td>8</td>
</tr>
<tr>
<td>5 Warfarin + Phenylbutazone</td>
<td>10</td>
<td>oral</td>
<td>109.7 ± 7.1(a)(b)</td>
<td>8</td>
</tr>
<tr>
<td>6 Warfarin + Carbenoxolone</td>
<td>10</td>
<td>oral</td>
<td>77.2 ± 3.5(a)</td>
<td>8</td>
</tr>
</tbody>
</table>

Female BX Wistar rats, 200g body weight, were used. Blood was collected 24 hours after treatment. Individual results are the mean of duplicate prothrombin estimations. Results are the mean ± s.e. mean. (a) $P < 0.001$ from control (b) $P < 0.005$ from group 2 by Student $t$ test.
Table 4.3 BLOOD GLUCOSE CONCENTRATION IN RATS FOLLOWING TREATMENT WITH TOLBUTAMIDE AND PHENYL BUTAZONE

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time after glucose load (h)</th>
<th>Blood glucose levels (mg/dl ± s.e. mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Control</td>
<td>-</td>
<td>108 ± 4 107 ± 3 85 ± 4</td>
</tr>
<tr>
<td>2 Phenylbutazone (114)</td>
<td>½</td>
<td>111 ± 4 110 ± 4 80 ± 4</td>
</tr>
<tr>
<td>3 Tolbutamide (50)</td>
<td>1</td>
<td>115 ± 2 88(a) ± 6 67(a) ± 6</td>
</tr>
<tr>
<td>4 Phenylbutazone (114) +</td>
<td>½</td>
<td>103(c) ± 3 72(bd) ± 5 60 ± 4</td>
</tr>
<tr>
<td>Tolbutamide (50)</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

(a) P < 0.01; (b) P < 0.001, from control; (c) P < 0.01; (d) P < 0.05 from group 3, Mann Whitney U test. Groups of 8 female BX Wistar rats received 6 m mol /100g oral glucose (0h). Phenylbutazone was given at twice the molar equivalent of tolbutamide.
potentiate the tolbutamide response at 2 and 4 hours 
\(p < 0.05\) after the glucose load. This potentiation 
confirms the findings \textit{in vitro} and clinically of other 
and validates the model system.

Treatment with carbenoxolone, at a dose level of 
227 mg/kg, reduced blood glucose concentrations at 4 
hours by 7% with respect to controls (Table 4.4) which, 
due to the small spread of results, was just a significant 
difference \(p < 0.05\). However, at 6 hours the glucose 
concentration in the carbenoxolone group was 6% higher 
than controls \(p > 0.05\), and was reflected in the group 
receiving both tolbutamide and carbenoxolone, where the 
glucose concentration was significantly higher than in 
the group which received only tolbutamide \(p < 0.01\). 
There was also an elevation in blood glucose concentrations 
\((14\%, \ p > 0.05)\) at the 2-hour stage following combined 
treatment with carbenoxolone and tolbutamide. Despite 
these changes carbenoxolone did not potentiate the 
activity of tolbutamide at 2 or 4 hours after the glucose 
load, as was the case with phenylbutazone treatment.

Because of the elevation of blood glucose concentrations, 
following concomitant therapy with tolbutamide and carben- 
oxolone, the treatment was repeated in adrenalectomised 
rats. The responses to the glucose load and tolbutamide 
treatments were not greatly altered by adrenalectomy 
(Fig. 4.1). Treatment with carbenoxolone did not alter 
either the normal blood glucose concentration or the 
response to tolbutamide in adrenalectomised rats (Fig. 4.2).

In the second series of tests, when phenylbutazone 
and carbenoxolone were given both before and after the 
glucose load phenylbutazone significantly potentiated the effect
## Table 4.4 BLOOD GLUCOSE CONCENTRATION IN RATS FOLLOWING TREATMENT WITH TOLBUTAMIDE AND CARBENOXOLONE

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Time after glucose load (h)</th>
<th>Blood glucose levels (mg/dl ± s.e. mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2h</td>
<td>112 ± 4</td>
</tr>
<tr>
<td>Carbenoxolone (227)</td>
<td>4h</td>
<td>118 ± 5 (a)</td>
</tr>
<tr>
<td>Tolbutamide (50)</td>
<td>6h</td>
<td>105 ± 4</td>
</tr>
<tr>
<td>Carbenoxolone (227)</td>
<td>4h</td>
<td>128 ± 12</td>
</tr>
<tr>
<td>Tolbutamide (50)</td>
<td>6h</td>
<td>91 (b) + 3</td>
</tr>
</tbody>
</table>

(a) *p*<0.05; (b) *p*<0.01; (c) *p*<0.001, from control; (d) *p*<0.01 from group 3, Mann Whitney U test. Groups of 8 female BX Wistar rats received a 6 m mol/100g oral glucose load (Oh). Carbenoxolone was given at twice the molar equivalent of tolbutamide.
Fig 4.1 BLOOD GLUCOSE CONCENTRATION IN NORMAL AND ADRENALECTOMISED FEMALE RATS, FOLLOWING AN ORAL GLUCOSE LOAD

The rats were fasted for 16 hours and given an oral glucose load of 6 mmol/100g (as a 3M solution) followed 1 hour later by tolbutamide treatment (T) at an oral dose level of 50mg/kg. The adrenalectomised rats were used 21 days post-operatively. The groups were; normal rats, control (O) and tolbutamide-treated (Δ); adrenalectomised rats, control (©) and tolbutamide-treated (Δ).
Fig 4.2 CONCENTRATION OF BLOOD GLUCOSE IN ADRENALECTOMISED RATS FOLLOWING AN ORAL GLUCOSE LOAD AND TREATMENT WITH TOLBUTAMIDE

The rats were fasted for 16 hours and given an oral glucose load of 6 mmol/100g (as a 3M solution). Controls (O) received vehicle only, carbenoxolone (Θ) was given 30 min. after the load (a) and tolbutamide (□) 1 hour after the load (b). Combined treatment (□) was given at the same time intervals. Tolbutamide was given at a dose level of 50 mg/kg and carbenoxolone at 227 mg/kg, twice the molar equivalent of tolbutamide.
of tolbutamide (Table 4.5) thus validating this model. Carbenoxolone treatment at a dose level of 227 mg/kg (Table 4.6) again resulted in elevated blood glucose levels which were significantly higher than controls at 6½ hours ($p < 0.05$). Furthermore, the response to tolbutamide was reduced by carbenoxolone at 6½ hours ($p < 0.05$) although no changes were seen at the earlier stages.

### 4.3.3 Chlorpropamide-Carbenoxolone Interaction

Concomitant administration of phenylbutazone and chlorpropamide (Table 4.7) resulted in a greater potentiation than was seen with phenylbutazone and tolbutamide. Treatment with carbenoxolone again resulted in glucose levels which were slightly higher than controls (Table 4.8). However, no change in the activity of chlorpropamide was evident.

The inference from these findings is that carbenoxolone is not displacing either tolbutamide or chlorpropamide from plasma binding sites, leading to potentiation, as is the case with phenylbutazone. The apparent inhibition of tolbutamide activity by carbenoxolone is likely to be due to an intrinsic activity of carbenoxolone rather than a direct effect upon tolbutamide itself.

### 4.3.4 Phenytoin-Carbenoxolone Interaction

Phenytoin inhibited the electroshock seizure by 50% at a dose level of 39 mg/kg when administered 2 hours prior to the shock. Neither phenylbutazone at 200 mg/kg nor carbenoxolone at 400 mg/kg induced any change in the electroshock response (Table 4.9).

Concomitant treatment with phenylbutazone at a dose level of twice the molar equivalent of the $ED_{50}$ of phenytoin did not cause a significant change in the protection of the anticonvulsant (Table 4.10). Although phenylbutazone is known to displace phenytoin from plasma binding sites in vitro, an alteration of
Table 4.5. BLOOD GLUCOSE CONCENTRATION IN RATS RECEIVING TOLBUTAMIDE AND PHENYLBUZTAZONE IN DIVIDED DOSES

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Time of treatment (h)</th>
<th>Blood glucose levels (mg/dl ± s.e. mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2½h</td>
<td>4½h</td>
</tr>
<tr>
<td>Control</td>
<td>127±4</td>
<td>120±3</td>
</tr>
<tr>
<td>Phenylbutazone (57)</td>
<td>0±2</td>
<td>125±3</td>
</tr>
<tr>
<td>Tolbutamide (50)</td>
<td>½</td>
<td>106(a)±9</td>
</tr>
<tr>
<td>Phenylbutazone (57)</td>
<td>0±2</td>
<td>94(c)±4</td>
</tr>
</tbody>
</table>

(a) P<0.05; (b) P<0.01; (c) P<0.001, from control; (d) P<0.05 from group 3.
Mann Whitney U test. Groups of 8 female BX Wistar rats received an oral glucose load of 6 m mol /100g at 1 hour. Phenylbutazone was given in a total of twice the molar equivalent of tolbutamide.
Table 4.6 BLOOD GLUCOSE CONCENTRATION IN RATS RECEIVING TOLBUTAMIDE AND CARBENOXOLONE IN DIVIDED DOSES

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Time of treatment (h)</th>
<th>Blood glucose levels (mg/dl ± s.e. mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2 1/2h</td>
</tr>
<tr>
<td>1 Control</td>
<td>-</td>
<td>116 ± 4</td>
</tr>
<tr>
<td>2 Carbenoxolone (114)</td>
<td>0&amp;2</td>
<td>126 ± 6</td>
</tr>
<tr>
<td>3 Tolbutamide (50)</td>
<td>1/2</td>
<td>89(b) ± 6</td>
</tr>
<tr>
<td>4 Carbenoxolone (114) + Tolbutamide (50)</td>
<td>1/2</td>
<td>91(b) ± 5</td>
</tr>
</tbody>
</table>

(a) \( P < 0.05 \); (b) \( P < 0.01 \); (c) \( P < 0.001 \), from control; (d) \( P < 0.05 \) from group 3

Mann Whitney U test. Groups of 8 female BX rats received an oral glucose load of 6 m mol/100g at 1 hour. Carbenoxolone was given in a total of twice the molar equivalent of tolbutamide.
Table 4.7  BLOOD GLUCOSE CONCENTRATION IN RATS RECEIVING CHLORPROPAMIDE AND PHENYLIBUTAZONE

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Time of treatment (h)</th>
<th>Blood glucose levels (mg/dl ± s.e. mean)</th>
<th>2½h</th>
<th>4½h</th>
<th>6½h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Control</td>
<td>-</td>
<td>121 ± 13</td>
<td>96  ± 4</td>
<td>65  ± 5</td>
<td></td>
</tr>
<tr>
<td>2 Phenylbutazone (56)</td>
<td>0&amp;2</td>
<td>120 ± 4</td>
<td>101 ± 4</td>
<td>68  ± 5</td>
<td></td>
</tr>
<tr>
<td>3 Chlorpropamide (50)</td>
<td>½</td>
<td>83(a) ± 8</td>
<td>80(a) ± 6</td>
<td>54  ± 4</td>
<td></td>
</tr>
<tr>
<td>4 Phenylbutazone (56)</td>
<td>0&amp;2 +</td>
<td>65(bc) + 3</td>
<td>40(bd) + 6</td>
<td>39(a) + 8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chlorpropamide (50)</td>
<td>½</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(a) P < 0.05; (b) P < 0.001, from control; (c) P < 0.05; (d) P < 0.001 from group 3 Mann Whitney U Test. Groups of 8 female BX Wistar rats received 6 m mol /100g oral glucose load at 1 hour. Phenylbutazone was given in a total of twice the molar equivalent of chlorpropamide.
Table 4.8  BLOOD GLUCOSE CONCENTRATION IN RATS RECEIVING
CHLORPROPAMIDE AND CARBENOXOLONE

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Time of treatment (h)</th>
<th>Blood glucose levels (mg/dl ± s.e. mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>21/2h</td>
</tr>
<tr>
<td>1 Control</td>
<td>-</td>
<td>115 ± 7</td>
</tr>
<tr>
<td>2 Carbenoxolone (111)</td>
<td>0.2</td>
<td>121 ± 7</td>
</tr>
<tr>
<td>3 Chlorpropamide (50)</td>
<td>1/2</td>
<td>81(a) ± 7</td>
</tr>
<tr>
<td>4 Carbenoxolone (111) + Chlorpropamide (50)</td>
<td>0.2</td>
<td>81(b) ± 8</td>
</tr>
</tbody>
</table>

(a) P < 0.01; (b) P < 0.001, from controls Mann Whitney U test. Groups of 8 BX Wistar rats were given an oral glucose load of 6 m mol /100g at 1 hour. Carbenoxolone was given in a total of twice the molar equivalent of chlorpropamide.
<table>
<thead>
<tr>
<th>Phenytoin (mg/kg)</th>
<th>Tonic seizure</th>
<th>Righting reflex</th>
<th>Protection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>number of rats</td>
<td>duration (s ± s.e. mean)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>10/10</td>
<td>12.5 ± 0.2</td>
<td>0/10</td>
</tr>
<tr>
<td>30</td>
<td>9/10</td>
<td>8.7 ± 1.0</td>
<td>2/10</td>
</tr>
<tr>
<td>34</td>
<td>8/11</td>
<td>6.6 ± 0.4</td>
<td>11/11</td>
</tr>
<tr>
<td>39</td>
<td>5/10</td>
<td>5.7 ± 1.1</td>
<td>10/10</td>
</tr>
<tr>
<td>44</td>
<td>3/10</td>
<td>5.9</td>
<td>10/10</td>
</tr>
<tr>
<td>80</td>
<td>1/10</td>
<td>6.0</td>
<td>10/10</td>
</tr>
<tr>
<td>100</td>
<td>2/12</td>
<td>3.8</td>
<td>11/12</td>
</tr>
</tbody>
</table>

Results are mean ± s.e. mean where sufficient numbers allow. Phenytoin was administered in 1% sodium carboxymethylcellulose 2 hours prior to shock. Electroshocks were administered for 1 sec by 2 subcutaneous electrodes adjacent to the ears at a rate of 100/sec. Pulse width was 5msec, with a potential difference of 80v and a current of 200mA. Righting reflex was assessed 2 minutes after tonic seizure.
Table 4.10 THE EFFECT OF PHENYL BUTAZONE AND CARBENOXOLONE ON PHENYTOIN INHIBITION OF ELECTROSHOCK SEIZURE IN RATS

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Tonic seizure number of rats</th>
<th>duration (s ± s.e. mean)</th>
<th>Righting reflex</th>
<th>Protection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10/10</td>
<td>12.3 ± 0.5</td>
<td>0/10</td>
<td>0</td>
</tr>
<tr>
<td>Phenylbutazone (200)</td>
<td>10/10</td>
<td>14.4 ± 0.5</td>
<td>0/10</td>
<td>0</td>
</tr>
<tr>
<td>Carbenoxolone (400)</td>
<td>10/10</td>
<td>11.3 ± 0.4</td>
<td>0/10</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>10/10</td>
<td>11.8 ± 0.7</td>
<td>0/10</td>
<td>0</td>
</tr>
<tr>
<td>Phenytoin (39)</td>
<td>7/11</td>
<td>6.7 ± 0.8</td>
<td>11/11</td>
<td>36</td>
</tr>
<tr>
<td>Phenytoin (39) +</td>
<td>6/10</td>
<td>5.7 ± 1.1</td>
<td>10/10</td>
<td>40</td>
</tr>
<tr>
<td>Phenylbutazone (100)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenytoin (39) +</td>
<td>7/10</td>
<td>8.1 ± 1.0</td>
<td>10/10</td>
<td>30</td>
</tr>
<tr>
<td>Carbenoxolone (200)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Phenytoin was administered 2 hours before electroshock and the remaining drugs in divided doses at 1 and 2 hours before shock. Righting reflex was assessed 2 minutes after electroshock. Phenytoin results were significantly different from controls (P < 0.001) but did not differ significantly in the presence of a second drug. Significance tests were performed by the Fisher exact test and the Student t test.
the pharmacological potency of phenytoin by this mechanism has not been demonstrated \textit{in vivo}. However, an increase in the clinical side-effects of phenytoin by concomitant therapy with chlorpheniramine has been attributed to displacement from plasma binding sites (Pugh, \textit{et al}, 1975). Similarly, carbenoxolone did not alter the effect of phenytoin.
4.4 CONCLUSIONS

The potentiation of the pharmacological activities of warfarin, tolbutamide and chlorpropamide by concomitant administration of phenylbutazone was clearly demonstrated, confirming the various animal and clinical findings and the predictions from the previous studies \textit{in vitro}. The mode of action of the potentiation has been elucidated as displacement from plasma binding sites for warfarin but has only been suggested for the sulphonylureas (O'Reilly & Levy, 1970; Slade & Iosefa, 1967; Tannenbaum, \textit{et al}, 1974).

Carbenoxolone did not potentiate the activity of warfarin, which confirms the predictions from the study \textit{in vitro} where different classes of binding site on human albumin were seen for the two drugs. Whereas warfarin was binding at only a single class of site on human albumin, chlorpropamide was bound at two classes of site so that displacement from binding by either phenylbutazone or carbenoxolone was not certain, but possible. It was clear that phenylbutazone potentiated the pharmacological effect of chlorpropamide, whilst carbenoxolone did not. This finding leads to the suggestion that carbenoxolone binding did not prevent that of chlorpropamide, whilst phenylbutazone did prevent chlorpropamide binding to plasma proteins in the rat.

The initial results with tolbutamide and carbenoxolone were not as well defined. It appears that, although there was no potentiation of tolbutamide activity, there was either an inhibition of activity or an elevation of blood glucose concentrations. Carbenoxolone is known to possess mineralocorticoid-like activity (Finney & Tárnok, 1960). Further evidence for the lack of glucocorticoid activity was gained from preliminary studies in alloxan diabetic rats, where a similar rise in blood glucose concentration was obtained.
with combined carbadoxolone and tolbutamide treatment; because it is known that glucocorticoids will cause an increase in gluconeogenesis in the presence of normal insulin levels (Bell, et al., 1956). This finding also eliminates the possibility of interference with the absorption of the glucose load. It is interesting to note that carbadoxolone had no inherent activity on blood glucose levels when administered alone, whereas glycyrrhetinic acid was observed to exhibit glycolytic activity in mice (Finney & Somers, 1958).

However, from the studies with adrenalectomised rats, and the second treatment regimen in whole rats, it is clear that no alteration of the pharmacological activity of tolbutamide was induced by the presence of carbadoxolone and that the effect of elevation of glucose levels by combined drug therapy was probably due to adrenal stimulation.

Neither phenylbutazone nor carbadoxolone induced any change in the pharmacological activity of phenytoin, in contrast to the effect upon chlorpropamide, which had exhibited a similar binding to phenytoin in the study in vitro. It is possible that phenytoin remained bound at an alternative site to the two interacting drugs, or even became bound to other proteins such as globulin. It must also be appreciated that phenytoin is not as highly bound to plasma proteins in the rat as it is in man and thus the pharmacological activity may not be significantly influenced by a slight change in the free drug concentration.

Thus with these findings from experimentation in vivo some of the predictions from the study in vitro are validated. Drugs binding at a single class of site on human serum albumin followed predictions and did or did not interact, depending on the classes of binding site involved. Of the drugs binding at two classes of site, chlorpropamide did interact with phenylbutazone, but not with carbadoxolone, whilst phenytoin did not interact with either of these drugs. It is evident that influences other than plasma binding may be involved and that the plasma pharmacokinetics of these drugs should also be studied. Such studies are presented in Chapter 5.
Chapter 5

INTERACTIONS OF CARBENOXOLONE INVOLVING DRUG KINETICS

IN VIVO
Chapter 5

INTERACTIONS OF CARBENOXOLONE INVOLVING DRUG KINETICS
IN VIVO

5.1 INTRODUCTION

5.2 EXPERIMENTAL

5.2.1 Carbenoxolone-warfarin interaction
5.2.2 Carbenoxolone-tolbutamide interaction
5.2.3 The effect of various drugs on serum carbenoxolone concentration in the rat and in man

5.3 RESULTS

5.4 CONCLUSIONS
5.1 INTRODUCTION

Displacement of a drug from plasma binding sites, with the consequent modification of its pharmacological activity, is one aspect of interaction which was investigated in the previous chapter. An alternative aspect of displacement may result in an increased metabolism of the drug, due to a higher concentration of the free drug being made available for biotransformation. In addition to displacement an interacting xenobiotic may alter the rate of metabolism of the drug by enhancing or inhibiting enzymic activity.

The displacement of warfarin by phenylbutazone results in a reduced plasma half-life of warfarin (O'Reilly & Levy, 1970) whilst the pharmacological activity of warfarin is enhanced, as demonstrated by Aggeler and colleagues (1967) and confirmed in the present study (Chapter 4).

In contrast, barbiturates increase the metabolism of warfarin in rat and man, but reduce its pharmacological activity in man whilst the drug distribution remains unchanged (Ikeda, et al, 1968; Levy, et al, 1970). Warfarin is a good example of the effect of plasma binding of drugs on their excretion, because it is non-polar and highly bound to plasma proteins thus resulting in high plasma levels. The hydroxylated metabolites of warfarin are more polar and only weakly bound to plasma proteins, therefore they occur in the urine in high concentrations (O'Reilly, 1969).

The pharmacological activity of tolbutamide is also potentiated by concomitant administration of phenylbutazone, but in this case the half-life of tolbutamide in blood is increased (Slade & Iosefa, 1967; Tannenbaum, et al, 1974).
In the present study (Chapter 4) concomitant administration of carbenoxolone with either warfarin or tolbutamide did not alter their pharmacological activities. In the present chapter it is intended, therefore, to measure possible alternative effects of carbenoxolone on the blood half-lives of warfarin and tolbutamide. In addition, the effect of phenylbutazone on the half-lives of warfarin and tolbutamide will be monitored, to validate the experimental models.

A suitable model to assess the pharmacological activity of carbenoxolone in vivo does not, unfortunately, exist. Therefore, in order to evaluate possible displacement of carbenoxolone by other drugs, serum levels of carbenoxolone will be measured in the presence of warfarin, tolbutamide, chlorpropamide and phenytoin. These measurements will be carried out in rat and in man.
5.2 EXPERIMENTAL

5.2.1 CARBENOXOLONE-WARFARIN INTERACTION

Warfarin concentration in plasma was measured by the fluorescence method of Corn and Berberich (1967). The drug was extracted from 0.2 ml plasma into 4 ml acetone and the fluorescence measured at 340 nm (activation) and 410 nm (fluorescence), using a Baird Atomic FP100 Fluoripoint spectrofluorimeter. The quenching of fluorescence by the addition of 0.05 ml 0.1 M HCl was proportional to the concentration of warfarin in the samples.

Groups of 8 female Biorex Wistar rats (200 ± 10g) were fasted for 16 hours (overnight) prior to treatment and fed 8 hours later. Warfarin was administered orally as an aqueous solution at a dose level of 10mg/kg (5ml/kg). Carbenoxolone or phenylbutazone was administered at the same time as the warfarin, subcutaneously in 0.9% NaCl (5ml/kg) at a dose level of twice the molar equivalent of warfarin, i.e. 40 and 20 mg/kg respectively. Blood samples were taken under ether anaesthesia from the ventral aorta using citrate/hepes buffer (0.13 M trisodium citrate, 0.05 M N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) as anticoagulant at a level of 0.1 ml per ml of blood collected. Plasma samples were prepared from blood collected at 1, 2, 4, 6, 8 and 24 hours after dosing.

5.2.2 CARBENOXOLONE-TOLBUTAMIDE INTERACTION

Tolbutamide concentrations in whole blood were measured by the spectrophotometric method of Kern (1963). The drug was extracted from 1 ml whole blood into 8 ml 1,2-dichloroethane in the presence of 1 M citrate buffer at pH 4.6. After back extraction with 1 M ammonia solution the tolbutamide was nitrated with potassium nitrate in sulphuric acid and then reduced with stannous chloride. The resultant product was diazotised and coupled with N(1-naphthyl)ethylene diamine and the absorption at 547 nm measured on a Pye Unicam SP500 spectrophotometer.
Groups of 8 female Biorex Wistar rats (200 ± 10g) were fasted for 16 hours (overnight) prior to treatment. All drugs were administered orally in 0.01% aqueous Tween 80 (5 ml/kg), tolbutamide at a dose level of 50mg/kg, carbenoxolone and phenylbutazone at a dose level of twice the molar equivalent of tolbutamide, i.e. 227 and 114 mg/kg. Blood samples were taken under ether anaesthesia from the ventral aorta, using heparin as anticoagulant. Tolbutamide concentration was measured from 1 ml whole blood samples taken 1, 2, 4, 6 and 8 hours after treatment.

5.2.3 THE EFFECT OF VARIOUS DRUGS ON SERUM CARBENOXOLONE CONCENTRATION IN THE RAT AND IN MAN

Carbenoxolone serum concentration was measured by the gas chromatographic method of Rhodes and Wright (1974). The drug was extracted from acidified serum with ether, the ether evaporated and the residue redissolved in ethanol, followed by 0.5 M Na₂CO₃. The alkaline solution was washed with ethyl acetate, the aqueous layer was then acidified and extracted with ether. The ethereal extract was then methylated by the action of diazomethane prior to injection on to a 1 foot 1% OV-1 glc column at 285°C. The internal standard used was 18-a carbenoxolone.

Rat study. Groups of 6 female Biorex Wistar rats (200 ± 10g) were used for each estimation and fasted for 16 hours (overnight) prior to treatment. Carbenoxolone (50mg/kg) and warfarin (10mg/kg) were administered orally as aqueous solutions whilst phenytoin (40mg/kg), tolbutamide (50mg/kg) and chlorpropamide (50mg/kg) were administered in 0.01% aqueous Tween 80. Blood samples were taken under ether anaesthesia at ½, 1, 2, 4 and 8 hours after treatment.

Human study. Volunteers received a single oral dose of 100mg carbenoxolone (2 Biogastrene tablets) after which blood samples were taken at 1, 2, 4 and 8 hour intervals.
Blood samples were also taken prior to the administration of carbenoxolone. On a subsequent day each subject repeated the carbenoxolone dose and took a concomitant dose of either warfarin (10mg), tolbutamide (500mg), chlorpropamide (250mg) or phenytoin (100mg) after which blood samples were taken as before.
5.3 RESULTS

5.3.1 CARBENOXOLONE-WARFARIN INTERACTION

The results of the plasma assay of warfarin produced a linear fluorescence quenching over the range of warfarin concentrations employed (Fig. 5.1) with a correlation coefficient of 0.999.

Plasma concentrations of warfarin in the rat decreased in a uniform manner over an 8 hour period after dosing, and were just detectable 24 hours after treatment, by the method of assay used. The plasma half-life of the drug was determined from regression analysis of log. plasma concentration versus time and was 8.4 and 8.5 hours in the two control groups (Fig. 5.2).

Concomitant administration of phenylbutazone markedly reduced the plasma half-life of warfarin to 2.3 hours, resulting in a significantly lower plasma concentration at each stage from 2 hours after treatment (Table 5.1). It is interesting to note that the warfarin concentration 8 hours after concomitant phenylbutazone treatment were just measurable and that no detectable warfarin was present 24 hours after concomitant treatment. This effect of phenylbutazone is known and validates the model (O'Reilly & Levy, 1970).

In contrast, the concomitant administration of carbenoxolone did not produce plasma warfarin concentrations which were significantly different from controls at any stage (Table 5.2).

The inference from these results using the rat is that the potentiation of the pharmacological activity of warfarin by phenylbutazone (section 4.3.1) and the increased rate of
The method was based on the assay of Corn and Berberich (1967). Warfarin was extracted from plasma into acetone and the fluorescence intensity measured at 340/410 nm. Acidification of the plasma reduced the fluorescence in direct proportion to the concentration of warfarin.
Figure 5.2: Plasma Warfarin Concentration Following Oral Treatment of Female Rats with Warfarin

The points represent the means of 6 rats per group and are from 2 experiments.

The rats were fasted for 16 hours and treated orally with an aqueous solution of warfarin at a dose level of 10 mg/kg. The rats were fed 8 hours after treatment.
Table 5.1  THE EFFECT OF PHENYL ButAZONE ON PLASMA WARFARIN CONCENTRATION, AND PLASMA HALF-LIFE, IN RAT

<table>
<thead>
<tr>
<th>Time after dosing (h)</th>
<th>Plasma warfarin concentration (µg/ml)</th>
<th>Warfarin</th>
<th>Warfarin + Phenylbutazone</th>
<th>P&lt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>3.2 ± 0.3</td>
<td>2.8 ± 0.2</td>
<td>0.005</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>3.0 ± 0.2</td>
<td>1.8 ± 0.2</td>
<td>0.001</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>2.6 ± 0.2</td>
<td>1.6 ± 0.1</td>
<td>0.005</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>2.0 ± 0.3</td>
<td>0.7 ± 0.1</td>
<td>0.001</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>1.8 ± 0.3</td>
<td>0.3 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td></td>
<td>0.2 ± 0.2</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Plasma half-life (h)</td>
<td></td>
<td>8.4</td>
<td>2.3</td>
<td></td>
</tr>
</tbody>
</table>

Means are of separate groups of 6 rats ± s.e. mean. Statistical differences were derived from a Student t test. All rats received warfarin in an aqueous solution at a dose level of 10 mg/kg orally. The rats which also received phenylbutazone at a dose level of 20 mg/kg (twice the molar equivalent of warfarin) were treated subcutaneously at the same time as the warfarin treatment. The rats were fasted for 16 hours prior to treatment. Warfarin concentration was determined by the method of Corn and Berberich (1967).
Table 5.2 THE EFFECT OF CARBENOXOLONE ON PLASMA WARFARIN CONCENTRATION, AND PLASMA HALF-LIFE, IN RAT

<table>
<thead>
<tr>
<th>Time after dosing (h)</th>
<th>Plasma warfarin concentration (µg/ml)</th>
<th>Warfarin</th>
<th>Warfarin + Carbenoxolone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Warfarin</td>
<td>Warfarin + Carbenoxolone</td>
</tr>
<tr>
<td>1</td>
<td>3.4 ± 0.2</td>
<td>3.4 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3.1 ± 0.4</td>
<td>2.8 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2.7 ± 0.4</td>
<td>2.9 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>2.4 ± 0.3</td>
<td>2.3 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1.9 ± 0.3</td>
<td>1.9 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>0.3 ± 0.3</td>
<td>0.2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Plasma half-life (h)</td>
<td>8.5</td>
<td>9.2</td>
<td></td>
</tr>
</tbody>
</table>

Means are of separate groups of 6 rats ± s.e. mean. There were no statistical differences by Student t test. All rats received warfarin in an aqueous solution, at a dose level of 10 mg/kg, orally. The rats which also received carbenoxolone at a dose level of 40 mg/kg (twice the molar equivalent of warfarin) were treated subcutaneously at the same time as the warfarin treatment. The rats were fasted for 16 hours prior to treatment. Warfarin concentration was determined by the method of Corn and Berberich (1967).
excretion of warfarin are due to displacement of warfarin from plasma binding sites by phenylbutazone. These results are in agreement with those of O'Reilly and Levy (1970) in man.

5.3.2 CARBENOXOLONE-TOLBUTAMIDE INTERACTION

The spectrophotometric absorption from the assay of tolbutamide in whole blood was linear over the range of concentrations observed in the rat (Fig. 5.3).

There was an exponential fall of tolbutamide blood concentration over an 8-hour period following an oral dose of tolbutamide (Fig. 5.4). From regression analysis of log. blood concentration versus time (Table 5.3), the half-life in blood was found to be about 3 hours. Concomitant administration of phenylbutazone increased the half-life to 6 hours, resulting in blood concentrations which were significantly higher than controls at each measurement from 2 hours after administration. In contrast, concomitant treatment with carbenoxolone did not produce blood concentrations which were significantly different from controls at any stage (Table 5.4).

From the present results it may be inferred that the potentiation of the pharmacological activity of tolbutamide by phenylbutazone (section 4.3.2) may be partially due to the decrease in the rate of excretion of tolbutamide. However, it is clear that carbenoxolone does not alter the half-life of tolbutamide in blood in the rat.

5.3.3. THE EFFECT OF VARIOUS DRUGS ON SERUM CARBENOXOLONE CONCENTRATION IN THE RAT AND IN MAN

None of the drugs administered with carbenoxolone caused any interference with the assay of carbenoxolone itself.

Rat study. In the fasted rat, carbenoxolone was rapidly absorbed following an oral dose, a second peak
Fig 5.3 STANDARD CURVE FOR THE DETERMINATION OF TOLBUTAMIDE

The absorption of the tolbutamide- N(1-naphthyl) ethylenediamine complex was measured at 547 nm, from the assay of Kern (1963). The points represent the means of duplicate estimations from whole rat blood, using heparin as anticoagulant.
Fig 5.4 BLOOD TOLBUTAMIDE CONCENTRATION FOLLOWING ORAL TREATMENT OF FEMALE RATS WITH TOLBUTAMIDE

The points represent the means of 6 different rats per group and are from 2 experiments.

The rats were fasted for 16 hours prior to treatment, then dosed orally with tolbutamide at a dose level of 50 mg/kg.
Table 5.3. THE EFFECT OF PHENYLButAZONE ON THE BLOOD CONCENTRATION OF TOLButAMIDE AND THE BLOOD HALF-LIFE, IN RAT

<table>
<thead>
<tr>
<th>Time after treatment (h)</th>
<th>Tolbutamide concentration (µg/ml)</th>
<th>Tolbutamide + Phenylbutazone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tolbutamide</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>148 ± 4</td>
<td>163 ± 6 (p &lt; 0.01)</td>
</tr>
<tr>
<td></td>
<td>116 ± 4</td>
<td>144 ± 4 (p &lt; 0.001)</td>
</tr>
<tr>
<td></td>
<td>81 ± 2</td>
<td>113 ± 3 (p &lt; 0.001)</td>
</tr>
<tr>
<td></td>
<td>43 ± 3</td>
<td>93 ± 4 (p &lt; 0.001)</td>
</tr>
<tr>
<td></td>
<td>20 ± 2</td>
<td>72 ± 3 (p &lt; 0.001)</td>
</tr>
<tr>
<td>Half-life (h)</td>
<td>2.5</td>
<td>6.0</td>
</tr>
</tbody>
</table>

Means are of separate groups of 6 rats ± s.e. mean. Statistical differences between the groups were assessed by Student t test. The rats were fasted 16 hours (overnight) prior to treatment. Blood samples were taken into heparin under ether anaesthesia. Phenylbutazone was administered at a dose level twice the molar equivalent of tolbutamide. All rats received tolbutamide, in 0.1% aqueous Tween 80, at an oral dose level of 50 mg/kg. The rats which also received phenylbutazone at a dose level of 114 mg/kg were treated orally at the same time as the tolbutamide treatment. Tolbutamide was assayed by the method of Kern (1963).
Table 5.4 THE EFFECT OF CARBENOXOLONE ON THE BLOOD CONCENTRATION OF TOLBUTAMIDE AND THE BLOOD HALF-LIFE, IN RAT

<table>
<thead>
<tr>
<th>Time after treatment (h)</th>
<th>Tolbutamide concentration (µg/ml)</th>
<th>Tolbutamide + Carbenoxolone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tolbutamide</td>
<td>Tolbutamide + Carbenoxolone</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>142 ± 7</td>
<td>135 ± 6</td>
</tr>
<tr>
<td>2</td>
<td>115 ± 6</td>
<td>120 ± 4</td>
</tr>
<tr>
<td>4</td>
<td>77 ± 4</td>
<td>75 ± 2</td>
</tr>
<tr>
<td>6</td>
<td>46 ± 3</td>
<td>40 ± 2</td>
</tr>
<tr>
<td>8</td>
<td>28 ± 1</td>
<td>24 ± 1 (P&lt; 0.05)</td>
</tr>
<tr>
<td>Half-life (h)</td>
<td>3.0</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Means are separate groups of 6 rats ± s.e. mean. Statistical difference was assessed by Student t test. The rats were fasted 16 hours prior to treatment. Blood samples were taken into heparin under ether anaesthesia. Carbenoxolone was administered at a dose level twice the molar equivalent of tolbutamide. All rats received tolbutamide, in 0.1% aqueous Tween 80, at an oral dose level of 50 mg/kg. The rats which also received carbenoxolone at a dose level of 220 mg/kg were treated orally at the same time as the tolbutamide treatment. Tolbutamide was assayed by the method of Kern (1963).
of carbenoxolone serum concentration was seen 2 hours after treatment, which was subsequently reduced with a half-life of 9.5 hours (Fig. 5.5). The two peaks of carbenoxolone serum concentrations may represent gastric and intestinal absorptions. The serum concentrations measured by this selective method are of carbenoxolone per se and not a metabolite, enoxolone, in contrast to earlier studies using radiochemically-labelled materials (Parke, et al., 1963 & 1972; Iveson, et al., 1971).

None of the drugs administered concomitantly with carbenoxolone caused any significant changes in the pattern of serum concentrations in the rat (Table 5.5).

*Human study.* The rate of absorption of carbenoxolone was not as rapid in man as in the rat, although the volunteers were not fasted as were the rats (Table 5.6). The serum half-life of carbenoxolone in the volunteers varied between 5.6 and 10 hours. Unfortunately, not all the volunteers adhered to the protocol, in that blood samples were taken at 6 hours after treatment (instead of 1 hour) in two cases, and 5 hours after treatment (instead of 4 hours) in one case. The average serum half-life of carbenoxolone as Biogastrone has been previously found to be 13 hours (Baron, et al., 1975).

*Phenytoin (Mr. W.).* The concomitant dose of phenytoin caused a minimal change in the serum half-life of carbenoxolone from 7.5 to 8.0 hours. This cannot be considered a significant difference.

*Warfarin (Dr. B.).* The volunteer who took warfarin with BiogastroneR had a higher carbenoxolone serum concentration (up to 26 µg/ml) than the other volunteers with a short serum half-life of 5.6 hours. The concomitant administration of warfarin produced no change in the serum half-life of carbenoxolone.
Fig 5.5 SERUM CARBENOXOLONE CONCENTRATION FOLLOWING ORAL TREATMENT OF FEMALE RATS WITH CARBENOXOLONE

Each point represents the mean of 6 different rats per group. The rats were fasted for 16 hours prior to treatment, then dosed orally with carbenoxolone at a dose level of 50 mg/kg in aqueous solution. Serum concentrations were estimated by the method of Rhodes and Wright (1974).
Table 5.5 THE EFFECT OF CONCOMITANT ADMINISTRATION OF OTHER DRUGS ON THE SERUM CONCENTRATION OF CARBENOXOLONE IN RAT

<table>
<thead>
<tr>
<th>Time after treatment (min)</th>
<th>Second drug treatment (mg/kg)</th>
<th>None</th>
<th>Warfarin (10)</th>
<th>Tolbutamide (50)</th>
<th>Chlorpropamide (50)</th>
<th>Phenytoin (40)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>6.7 ± 1.1</td>
<td>6.3 ± 0.9</td>
<td>6.0 ± 0.8</td>
<td>7.9 ± 1.2</td>
<td>7.1 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>3.8 ± 0.9</td>
<td>5.2 ± 0.8</td>
<td>4.4 ± 0.7</td>
<td>5.9 ± 0.8</td>
<td>3.4 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>3.6 ± 0.6</td>
<td>3.9 ± 0.5</td>
<td>3.2 ± 0.4</td>
<td>3.8 ± 0.5</td>
<td>2.9 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>4.6 ± 0.7</td>
<td>4.2 ± 0.6</td>
<td>3.8 ± 0.7</td>
<td>3.4 ± 0.9</td>
<td>5.1 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>240</td>
<td>4.4 ± 1.5</td>
<td>4.9 ± 0.7</td>
<td>5.6 ± 0.8</td>
<td>5.1 ± 1.2</td>
<td>4.3 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>480</td>
<td>3.2 ± 1.0</td>
<td>3.8 ± 0.8</td>
<td>4.2 ± 0.7</td>
<td>2.5 ± 0.6</td>
<td>2.5 ± 0.5</td>
<td></td>
</tr>
</tbody>
</table>

Results are carbenoxolone serum levels (µg/ml). Means are ± s.e. mean from groups of 6 female Biorex Wistar rats (200 g ± 10g). The rats were fasted overnight and all received carbenoxolone at a dose level of 50 mg/kg in aqueous solution at the same time as the second drug in the following vehicles: warfarin, water; phenytoin, 0.5% sodium carboxymethylcellulose; tolbutamide & chlorpropamide, 0.01% aqueous Tween 80. Serum samples were stored at -14°C until assayed by the method of Rhodes and Wright (1974).
Table 5.6 THE EFFECT OF CONCOMITANT ADMINISTRATION OF OTHER DRUGS ON THE SERUM CONCENTRATION OF CARBENOXOLONE IN MAN

<table>
<thead>
<tr>
<th>Volunteer</th>
<th>Mr. W.</th>
<th>Dr. P.</th>
<th>Dr. B.</th>
<th>Mr. Y.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Second drug</td>
<td>Phenytoin</td>
<td>Chlorpropamide</td>
<td>Warfarin</td>
<td>Tolbutamide</td>
</tr>
<tr>
<td>Time of serum sample (h)</td>
<td>alone + drug</td>
<td>alone + drug</td>
<td>alone + drug</td>
<td>alone + drug</td>
</tr>
<tr>
<td>0</td>
<td>31.5</td>
<td>0.5</td>
<td>0</td>
<td>1.7</td>
</tr>
<tr>
<td>1</td>
<td>7.9</td>
<td>13.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>11.1</td>
<td>12.7</td>
<td>9.7</td>
<td>3.8</td>
</tr>
<tr>
<td>4</td>
<td>10.8</td>
<td>13.7</td>
<td>8.9</td>
<td>3.9</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
<td>8.0</td>
<td>10.7</td>
</tr>
<tr>
<td>8</td>
<td>6.0</td>
<td>6.4</td>
<td>6.9</td>
<td>13.3</td>
</tr>
</tbody>
</table>

Approximate $t_2$ (h) | 7.5 | 8.0 | 10.0 | - | 5.6 | 6.5 | 5.7 | 6.5 |

Results are the means of duplicate assays, µg/ml carbenoxolone. Each volunteer (male) received a single oral dose of 100mg carbenoxolone (2 BiogastroneR tablets) on day 1 and the same on day 2 together with a single oral dose of the second drug: phenytoin 100mg, chlorpropamide 500mg, warfarin 10mg and tolbutamide 250mg. Serum samples were stored at -14°C until assayed by the method of Rhodes and Wright (1974).
**Chlorpropamide (Dr. P.).** The volunteer who took Biogastrone\(^R\) and chlorpropamide had the lowest overall serum concentration of carbenoxolone but this decayed with the longest half-life, namely 10 hours.

The concomitant dose of chlorpropamide inhibited the serum concentration of carbenoxolone from rising above 4 µg/ml until 6 hours after the dose when the serum concentration continued to rise, until the final measurement 8 hours after treatment. It was not possible to estimate a plasma half-life in this case.

**Tolbutamide (Mr. Y.).** The volunteer who took Biogastrone\(^R\) and tolbutamide displayed a very slow absorption pattern for carbenoxolone when taken alone, peak serum concentration was not achieved until 4 hours after ingestion of the dose. When the second Biogastrone dose was taken with tolbutamide, peak serum concentration was achieved more rapidly, i.e. by 2 hours, after which the serum concentration declined similarly, giving a half-life of approximately 6 hours.
5.4 CONCLUSIONS

The potentiation of the pharmacological effects of warfarin by phenylbutazone is well known and was demonstrated, in the rat (Chapter 4). The mechanism of potentiation is known to be mediated via the displacement of warfarin from plasma binding sites, resulting in a simultaneous reduction in the plasma half-life of warfarin (Aggeler, et al, 1967; O'Reilly & Aggeler, 1968a). In the present studies the plasma concentration of warfarin was greatly reduced in the presence of phenylbutazone, validating the model and confirming earlier predictions from studies in vitro (Chapter 3). Carbenoxolone showed no influence on warfarin plasma concentration. This finding is in accord with the previous demonstration of the failure of carbenoxolone to modify the pharmacological activity of warfarin and with the predictions from studies in vitro, that displacement interaction would not occur.

The pharmacological activity of tolbutamide was also potentiated by phenylbutazone, in the rat, but not by carbenoxolone. The former interaction is known in man (Tannenbaum, et al, 1974) and is mediated via an increase in the serum half-life of tolbutamide (Slade & Iosefa, 1967) which is confirmed in the present model in the rat. Although it is known that phenylbutazone will displace tolbutamide from plasma binding sites, it is probable that the main mode of potentiation is exerted via the inhibition of the metabolism of tolbutamide because of the ability of phenylbutazone, in an acute dose, to inhibit the microsomal oxygenation system (Cho, et al, 1970; Griffin & D'Arcy, 1975).

The fact that carbenoxolone binds to human serum albumin in vitro at differing sites to warfarin and tolbutamide led to the suggestion that carbenoxolone would not enter into displacement interactions with the two other drugs and this has been confirmed here. In addition, the serum half-life of carbenoxolone was
unaffected by concomitant administration of warfarin or tolbutamide both in rat and man, indicating that a reverse interaction has not occurred.

In contrast, carbenoxolone shared one of the two binding sites on human serum albumin for both phenytoin and chlorpropamide, therefore the possibility of interaction with these drugs involving displacement from binding sites exists. However, phenytoin caused no alteration of serum levels of carbenoxolone either in man or rat.

Conversely, chlorpropamide showed no effect upon the serum levels of carbenoxolone in the rat but appeared to reduce the absorption of carbenoxolone in man. However, the human results are based upon paired administrations in only one patient per interacting drug. The absorption of carbenoxolone in the presence of chlorpropamide was similar to that seen for carbenoxolone alone in the fourth volunteer (Mr. Y.) and may, in fact, be more closely linked with the times and contents of meals rather than the presence of a second drug. Carbenoxolone, as Biogastrone, is known to be absorbed more slowly when taken after meals (Baron, et al., 1975) and serum levels are lower in fed rats than in fasted rats used in the present experiments (Gilbert, personal communication). It is interesting to note that tolbutamide did not cause a similar inhibition of carbenoxolone absorption, suggesting that the delay of absorption is not likely to be due to an increased rate of insulin secretion.

The inferences from these results must be that carbenoxolone does not interact with warfarin, phenytoin, chlorpropamide or tolbutamide in the rat, nor do these drugs alter the serum half-life of carbenoxolone in rat or man. However, it is possible that chlorpropamide prevented the absorption of carbenoxolone in man.
Chapter 6

FINAL DISCUSSION
Chapter 6

FINAL DISCUSSION

6.1 INTRODUCTION

6.2 EXPERIMENTAL METHODS IN VITRO

6.3 COMPARATIVE PROTEIN BINDING OF CARBENOXOLONE AND OTHER DRUGS IN VITRO

6.4 CARBENOXOLONE INTERACTIONS IN VIVO

6.5 PROPOSALS FOR FUTURE RESEARCH
Chapter 6
FINAL DISCUSSION

This chapter presents an overall view of the experimental work completed, the conclusions drawn and consequent suggestions for further research.

6.1 INTRODUCTION

Interaction of a drug with a xenobiotic, or a second drug, is a problem when changes in the bioavailability of the drug lead to an increased activity or toxicity. These possible influences are summarised in Fig. 6.1.

For a drug such as carbenoxolone, which is highly bound to plasma proteins, the available form may not necessarily be the 'active' form, because no correlation between blood levels and efficacy has been established. Thus high blood levels of carbenoxolone are a hazard and large free concentrations may be toxic, since the free drug has been shown to be a potent uncoupling agent of oxidative phosphorylation (Whitehouse, et al, 1967). The possible effects of other drugs on carbenoxolone, as a model of potentiation drug interaction, are summarised in Fig. 6.2. In order to investigate possible interactions of this drug, its binding to plasma proteins was first investigated.

Experiments to evaluate protein binding-drug interactions per se are generally performed in vitro and often with isolated protein fractions, such as bovine serum albumin. In the present study it was decided to carry out a correlation between such methods of study in vitro and to compare human and bovine serum albumins, with a view to extrapolation from studies in vitro with bovine albumin to the human clinical situation.

Before studying interactions in vivo it was considered necessary to carry out some form of preselection of drug candidates, and this was based on the results of a single
Fig 6.1 SCHEME OF POSSIBLE MODES OF DRUG INTERACTIONS

- Green arrows represent effects resulting in inhibition and
- Red arrows represent potentiation of drug activity.

Bioavailability

DRUG FORMULATION

Absorption

Food (Fat etc.)

Antispasmodics

Excess fatty acid

Induction

Inhibition

METABOLIC ACTIVATION

Antagonism

Synergism

Pharmacological

METABOLIC INACTIVATION

Induction

Inhibition

Transport

Site of Action

Increased concentration of unbound drug

Decreased plasma half-life

Displacement

Plasma Binding

Excretion

Prevention of tubular secretion eg. Probenecid

Diuretics

Laxatives
Fig 6.2 SCHEME OF POSSIBLE INTERACTIONS OF DRUGS WITH CARBENOXOLONE IN CLINICAL USE

- Represents increased blood levels/toxicity
- Represents decreased blood levels/toxicity
study in vitro. The drugs used in the experiments in vivo were all subject to 'known' interactions with phenylbutazone, therefore this drug was used to validate the animal models prior to the investigation of the effects of carbenoxolone. Measurements of the pharmacological activities and blood concentrations of the drugs under study were made following administration of the drug alone and with carbenoxolone. Reciprocal measurements of the serum concentrations of carbenoxolone were made in the presence of these drugs in rat and man.

6.2 EXPERIMENTAL METHODS IN VITRO

'Classical' methods of quantitative measurement of protein binding use a visking dialysis membrane, but this membrane binds carbenoxolone to a significant degree. The binding of macromolecules to visking membranes has not generally been acknowledged as a problem by other workers. Carbenoxolone was bound to visking membranes perhaps because it is relatively larger (mol.wt. 615) than most other drugs, or because it is more lipophilic and hence more extensively bound to protein than other drugs. However, this binding of carbenoxolone was reduced by various washing techniques and was found to be a major problem only with dilute protein solutions.

Comparison of the binding characteristics of carbenoxolone in vitro did not result in a good correlation between the different methods. However, it is known that comparison of the results of binding studies, not only between different methods but also between laboratories, is not uniform (Swillens & Dumont, 1975; Vallner, et al, 1976) and that minor variations in conditions such as pH or osmolarity may cause major differences in binding. Furthermore, the mathematical analyses employed may also introduce a known bias (Madsen & Robertson, 1974; Vallner, et al, 1976).
A comparison of the results from different methods of measuring drug binding to both human and bovine serum albumins is presented in Table 6.1. From this survey it becomes clear that it is unwise to consider comparing the results of drug binding performed under differing conditions. Using a standard technique, namely equilibrium dialysis, under identical conditions, Chignell and Starkweather (1971b) have compared the binding of phenylbutazone to human, rat, dog and rabbit serum albumins. The drug was bound most strongly to human albumin but significant differences in binding characteristics were observed for all four species studied. In the present study, even though the results obtained by differing techniques in vitro did not show good agreement, it was clear that human and bovine serum albumins presented differing binding characteristics for each drug studied.

However, in spite of the use of the same technique to measure drug binding, it is not clear whether the binding sites for different drugs on plasma proteins are, in fact, identical. Chignell and Starkweather (1971a) have demonstrated changes in drug binding to human serum albumin; following acetylation of the albumin by aspirin. The binding of phenylbutazone to acetylated albumin was increased, whereas that of flufenamic acid was decreased and that of dicoumarol was unchanged. These findings lead to the suggestion that apparently identical binding sites are, in fact, quite different. It is also appreciated that albumin itself may be sufficiently modified, by fractionation to the crystalline form, to cause a change in drug binding characteristics (Olsen, 1972; Kostenbauder, et al, 1970).

Therefore, if measurements of protein binding in vitro are to have relevance to the human clinical situation, then human albumin should be used in preference to that of non-human species.
Table 6.1 COMPARISON OF DATA FROM DRUGS BINDING TO HUMAN AND BOVINE SERUM ALBUMINS IN VITRO

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Source of albumin</th>
<th>Method of measurement</th>
<th>No. of classes of binding sites</th>
<th>No. of binding sites per class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Warfarin</td>
<td>Human</td>
<td>Dynamic dialysis, Ultrafiltration</td>
<td>2</td>
<td>( n_1=1, n_2=6 )</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>Equilibrium dialysis</td>
<td>1</td>
<td>( n=2.3 )</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>Equilibrium dialysis</td>
<td>2</td>
<td>( n_1=1, n_2=3.7 )</td>
</tr>
<tr>
<td></td>
<td>Bovine</td>
<td>Fluorescence</td>
<td>1</td>
<td>( n=2.9 )</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>Human</td>
<td>Equilibrium dialysis</td>
<td>1</td>
<td>( n=6 )</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>Equilibrium dialysis</td>
<td>2</td>
<td>( n_1=0.04, n_2=7.9 )</td>
</tr>
<tr>
<td>Phenylbutazone</td>
<td>Human</td>
<td>Equilibrium dialysis, Ultrafiltration</td>
<td>2</td>
<td>( n_1=1, n_2=2 )</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>Equilibrium dialysis, Ultrafiltration</td>
<td>2</td>
<td>( n_1=1.5, n_2=3.7 )</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>Circular dichroism</td>
<td>2</td>
<td>( n_1=1.1, n_2=1.9 )</td>
</tr>
<tr>
<td></td>
<td>Bovine</td>
<td>Ultrafiltration</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Bovine</td>
<td>Fluorescence</td>
<td>1</td>
<td>( n=2.9 )</td>
</tr>
<tr>
<td></td>
<td>Bovine</td>
<td>Equilibrium dialysis</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Methyl orange</td>
<td>Human</td>
<td>Ultrafiltration</td>
<td>2</td>
<td>( n_1=3.5, n_2=13.5 )</td>
</tr>
<tr>
<td></td>
<td>Bovine</td>
<td>Dynamic dialysis</td>
<td>1</td>
<td>( n=22 )</td>
</tr>
</tbody>
</table>

From the review of Jusko and Gretch (1976)
Measurement of binding characteristics of a drug by the use of a fluorescent probe was confirmed as a suitable and relevant technique by comparison with other established methods for the known binding of phenylbutazone and warfarin. The fluorescent probe was the method chosen to investigate the comparative binding of carbenoxolone and several other drugs.

6.3 COMPARISON OF THE PROTEIN-BINDING OF CARBENOXOLONE WITH THAT OF OTHER DRUGS IN VITRO

From a study of the binding of some 14 different drugs to human serum albumin, using a fluorescent probe, it is evident that the drugs can be classified into 3 groups. There are 2 distinct drug binding sites present in human albumin; 2 of the groups of drugs bind at only one site and the third group binds at both sites.

The group bound only at the first class of site (n = 1) includes carbenoxolone, which was the most strongly bound member of its group. This finding suggests that carbenoxolone would displace the other members of its group, namely, cicloxdolone, indomethacin, enoxolone and cholesterol from albumin binding sites. In contrast, carbenoxolone would not displace drugs of the second group, flufenamic acid, phenylbutazone, warfarin, tolbutamide and imipramine, which are bound at the alternative binding site (n = 3). The other drugs which carbenoxolone might displace were the third group, that is prednisolone, aspirin, ibuprofen, chlorpropamide and phenytoin, all of which bind at both sites and hence share the carbenoxolone binding site.

Evidence for the validation of the fluorescent probe method was obtained by comparison of the orders of displacement of drugs with those obtained by established methods. These are: phenylbutazone > warfarin > tolbutamide, and between groups;
indomethacin) > aspirin and warfarin > phenytoin (Stockley, 1971; O'Reilly & Levy, 1970; Assandri & Perazzi, 1976). It is also suggested that within each group those drugs with the highest binding affinities would displace the members with lower affinities.

The clinical consequences of carbenoxolone displacing the triterpenoids, cicloxolone and enoxolone, are minimal because these drugs are never likely to be administered concomitantly. However, the pharmacology and toxicity of cicloxolone are very similar to those of carbenoxolone, thus the toxic effects may be increased by an elevated concentration of free cicloxolone. Displacement of indomethacin, from plasma binding sites, by carbenoxolone may result in an enhanced central toxicity such as dizziness (Martindale, 1977). However, the gastro-intestinal irritation and ulcerogenensis of indomethacin may not be potentiated because of the protective effects of carbenoxolone. Because both indomethacin and carbenoxolone are excreted in the bile, there may be competition for the mechanisms of biliary excretion. It is possible that the anti-inflammatory actions of indomethacin may be increased by concomitant administration of carbenoxolone, not only from a possible increased concentration of free indomethacin, but from simple addition with the anti-inflammatory activity of carbenoxolone itself (Thornton, MacDonald, Sacra & Gottfried, unpublished observations). However, it is interesting to note that such synergism is not observed between carbenoxolone and phenylbutazone, which bind at different sites on human serum albumin (Thornton & Sacra, unpublished observations). Displacement of cholesterol by carbenoxolone may result in an increase in the rate of cholesterol metabolism or an increase in the deposition of cholesterol in atherosclerotic plaques. However, long-term toxicity studies in rats and dogs, and indeed a long clinical history, have produced no evidence to suggest that either of these interactions with cholesterol is of clinical significance.
Should carbenoxolone displace prednisolone, aspirin, ibuprofen, chlorpropamide or phenytoin from their common binding site on human albumin, then these drugs may remain bound at alternative sites and thus not result in an increased free concentration of displaced drug. However, indomethacin is known to displace aspirin from plasma binding sites \textit{in vitro}, but when the displaced aspirin binds at its alternative site, a possible conformational change results in aspirin displacing indomethacin \textit{in vivo} (Mason & McQueen, 1974). Therefore it is possible that this group of drugs may displace carbenoxolone from plasma binding sites \textit{in vivo}.

However, the binding capacity of human albumin for carbenoxolone must be considered in relation to possible displacement interactions. The present studies with diluted human serum albumin have demonstrated that the binding capacity for carbenoxolone may be in excess of 28 days accumulated dose at a regimen of 300 mg/day. Therefore there is a considerable reserve of plasma albumin, and other plasma proteins, which will bind therapeutic concentrations of carbenoxolone.

From the present results it is evident that the fluorescent probe technique is a rapid, sensitive method for measuring drug-albumin binding. The complications of radio-labelled drugs or of membrane binding are not encountered with this method. Therefore, it is ideally suited to the evaluation of the binding of new drugs to serum albumin prior to study \textit{in vivo}.

### 6.4 Carbenoxolone Interactions \textit{In Vivo}

It is not always possible to predict interactions \textit{in vivo} from studies \textit{in vitro}, due to such factors as drug metabolism and protein dilution (Olsen, 1972). Phenylbutazone displaces prednisolone from plasma binding sites but does not alter its plasma half-life, therefore the
elimination of prednisolone is not dependant upon its degree of protein binding (Maickel, et al., 1966).

Animal models of interaction in vivo are useful for comparison with studies in vitro but contain known hazards in the extrapolation of results to the human clinical situation, due to species differences (Brodie, 1962; Williams, 1974, 1978). Therefore, in the present studies of drug interactions in rat, 'classical' interactions known to occur in man were included to evaluate the animal model.

Phenylbutazone is a drug which is highly bound to plasma proteins and known to potentiate the pharmacological actions of warfarin, tolbutamide and chlorpropamide (see as example Stockley, 1974). These interactions were readily demonstrated in the rat in vivo. Under the same experimental conditions carbenoxolone did not alter the pharmacological activity of these drugs providing confirmation of the results from studies in vitro.

Warfarin is a classical example of the effects of displacement interaction on its rate of metabolism. The plasma half-life of warfarin is decreased following displacement from plasma binding sites by phenylbutazone. This increased free concentration of warfarin results in an enhanced anticoagulant action (O'Reilly & Levy, 1970). Phenylbutazone is also known to displace tolbutamide from plasma binding sites and to potentiate its glucose-lowering action. In this case the plasma half-life of tolbutamide is increased, probably due to inhibition of the metabolism of tolbutamide by phenylbutazone (Slade & Iosefa, 1967; Cho, et al., 1970). These changes in the plasma pharmacokinetics of warfarin and tolbutamide were also easily demonstrated in the rat in the present study. Furthermore, carbenoxolone did not interact to produce alterations in the plasma levels of these two drugs. This confirms the predictions from the results.
in vitro, in that no displacement from plasma binding was observed. In addition, it is clear that carbenoxolone exerted no effect upon the elimination of tolbutamide from blood, following an acute dose.

Phenytoin and chlorpropamide differed from warfarin and tolbutamide in the results of the binding study in vitro. The former two drugs shared only one of their two binding sites with phenylbutazone. Potentiation of the glucose-lowering action of chlorpropamide was demonstrated in vivo by phenylbutazone but not by carbenoxolone, suggesting that carbenoxolone and chlorpropamide may be satisfactorily bound to plasma albumin at separate sites at the same time. In contrast, the pharmacological action of phenytoin in vivo was not affected by either concomitant treatment with phenylbutazone or with carbenoxolone. It is known that phenytoin is bound less strongly to plasma proteins in rat than in man and this may account for the lack of significant interaction (Conard, et al, 1971). Therefore this finding leads to the suggestion that, for phenytoin, the rat is not a suitable model for interaction studies.

Carbenoxolone failed to alter the pharmacological effects of any of the drugs with which it was administered concomitantly. However, these experiments did not provide any information as to whether carbenoxolone itself may have been displaced from plasma binding sites. Because of the difficulty in assessing the pharmacological activity of carbenoxolone, serum concentrations of carbenoxolone, in the presence of other drugs, were measured in rat and in man.

None of the drugs administered concomitantly with carbenoxolone, namely warfarin, tolbutamide, phenytoin and chlorpropamide, altered the serum concentrations of carbenoxolone in the rat. It was anticipated, from studies in vitro, that neither warfarin nor tolbutamide would affect
serum concentrations of carbenoxolone but chlorpropamide and phenytoin both share a common binding site with carbenoxolone and thus may have displaced carbenoxolone from its binding site. However, an almost identical pattern of carbenoxolone concentrations were seen in man and rat, with the exception that chlorpropamide may have initially inhibited the absorption of carbenoxolone in man.

In conclusion, it may be inferred that carbenoxolone is free from many of the untoward interactions associated with other drugs which are highly bound to plasma proteins. However, only a small number of drugs were evaluated \textit{in vivo} and caution must always be exercised when introducing a new drug combination in man.

6.5 PROPOSALS FOR FUTURE RESEARCH

Whatever the answers to a research question are, they always stimulate further questions.

For binding studies \textit{in vitro} it would be of value to use fluorescent probes with differing structures to those used in the present study. A basic fluorescent probe would be novel. In order to study the species differences of plasma-drug binding, purified albumin fractions from various species would be extremely valuable in the use of fluorescent probe techniques.

Whilst a fairly comprehensive group of drugs was screened in the fluorescent probe study, the addition of other classes such as the benzodiazepines, and new drugs such as diflunisal and cimetidine, would be of interest.

Carbenoxolone is excreted in the bile and competition with other compounds for this route of excretion, whilst not directly related to plasma binding, may be of interest.
Phenytoin undergoes extensive biliary excretion and enterohepatic circulation in the rat and is therefore a suitable candidate for this study (Noach, et al, 1958).

**Evaluation of potential interactions of drug candidates**

It is impossible to evaluate all new drugs or, for that matter, established drugs, for interactions with all other clinically used agents.

One of the early investigations which may be made is to evaluate whether a drug candidate is bound to plasma proteins. For this the fluorescent probe method is eminently suitable. If the drug is bound to plasma proteins then the relative binding between laboratory species and man should be assessed, preferably *in vitro* and by the fluorescent probe method, using albumin fractions. These results may assist in the choice of an appropriate laboratory species for toxicological tests.

Studies *in vivo* may follow two lines of approach, the effect upon 'classical' drugs, such as warfarin and tolbutamide, and the effect of enzyme-inducing agents, such as barbiturate and phenylbutazone, upon the plasma levels of the drug. It is preferable to use a laboratory species for toxicity studies where plasma binding and drug metabolism is similar to that in man. The effect of the new drug upon drug-metabolising enzyme systems is also important and may be assessed on such models as barbiturate-induced sleeping times and the plasma half-life of a suitable drug, such as warfarin.

Although much caution should accompany new drug combinations there is a danger of inducing, in the words of Azarnoff (1974), 'a drug-interaction-anxiety syndrome'.


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