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REVIEW ARTICLE

Binding of Drugs by Albumin and Plasma Protein

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Many drug–protein binding papers have been published since the often-cited review by Meyer and Guttman (1). This review is intended to be a sequel to that one; published research on drug–albumin binding since 1969 is covered. Only results with human or bovine serum albumin are discussed, and attention is focused on the physical and clinical significance of drug–protein binding and on results obtained with particular drug classes.

Various reviews and articles have appeared since 1969 concerning methods of investigating drug–protein binding phenomena. These methods can be summarized into two broad categories: (*a*) equilibrium methods based upon measuring changes in ligand concentration as a result of the establishment of a binding equilibrium, and (*b*) direct measurement methods in which a property of the drug, protein, or complex may be examined during the binding process. A short but complete coverage of the various specific methods in these two categories was pubished (2). Other articles dealing solely with one method may also be of interest (3–9).

Once binding data are accumulated, the binding parameters must be obtained by employing some data reduction technique. Various articles have appeared dealing with graphical and computer treatment of binding data. It is now well recognized that the Scatchard (10) and Klotz (11) methods of examining binding data are suitable when only one class of sites is displayed by the protein but are at best approximate when more than a single class exists on the protein. The subject of binding data reduction is not dealt with here; however, Refs. 12–22 are either applications of various methods or critical treatises of existing techniques.

Articles dealing with ways to estimate drug–albumin affinity constants rapidly (23) have extremely limited use, since most apply mathematics of the mass action law based upon a single protein site for the drug and require that the free concentration of the drug in the blood be known or determined. Presumably, if one is interested in the binding constant from the clinical standpoint, the affinity is high and measurement of the minute amount free from a therapeutic dose is at best hazardous.

Drug binding to various blood proteins and tissue proteins can influence the therapeutic, pharmacodynamic, and toxicologic actions of drugs. Protein binding may also exert profound effects on drug distribution. The protein–drug complex acts as a transport system to carry drug to the sites of action; this transport is extremely important for drugs that exhibit low solubility in the water portion of the plasma. Protein binding slows the disappearance of free drug from the plasma into tissues by decreasing the concentration gradient. It also provides a source of free drug to replace that removed by various distribution and elimination processes.

In a short overview of the consequences of drug–protein binding, Gillette (24) suggested some important questions to be answered from experiments examining binding phenomena. For example, he pointed out that not only are drugs bound to serum albumin but that other proteins in the blood and tissues may be important in the drug complexation interaction. In addition, the volumes of distribution of the bound forms of the drug usually differ from that of unbound drug. Also lacking in most articles is the consideration that the blood contains other substances that compete with drugs for binding sites. In addition,

correlation of *in vitro* binding results with *in vivo* drug–macromolecule interactions is sorely lacking.

Serum or plasma protein binding, principally to albumin, may have a profound effect on overall drug activity. The following effects have become evident from protein binding investigations: (*a*) only free drug (unbound) is available for activity or tissue distribution; (*b*) tightly bound drugs tend to be distributed in a smaller body space or volume; (*c*) marked decreases in protein binding occur in uremia, hypoalbuminemia, hyperbilirubinemia, and hepatic failure; and (*d*) the delayed elimination of highly bound drugs is a result of glomerular filtration and hepatic uptake being directly proportional to free drug in the serum.

PHYSICAL SIGNIFICANCE OF PROTEIN BINDING

Pharmacokinetics—Until the 1960's, protein binding as a basic principle in drug behavior was neglected in pharmacokinetic models. Drug–albumin interactions may profoundly influence the kinetics of drug elimination, *e.g.*, some sulfa drugs, or the relationship between renal clearance and the apparent biological half-life of the drug (25, 26).

In a short review article, Wagner (27) discussed plasma protein binding effects on nonlinear pharmacokinetic models. He pointed out that drugs showing a high affinity for serum albumin may have their plasma concentration–time profiles convex curvilinear in the terminal phase, and, indeed, this was the case in several reports (25–27). Wagner also pointed out, as did Gillette (24), that tissue binding may be much more important pharmacokinetically than albumin binding.

Gillette (24) added a useful criticism of some pharmacokinetic interpretations of bioavailability data by stating that estimations of volumes of distribution from area under the plasma concentration–time curves and the rate constant of the terminal phase of elimination assume that the drug is not highly bound to plasma proteins and that the ratio of the concentrations of bound and unbound drug in the plasma is constant. The researcher may make erroneous conclusions if it is not determined that the areas under the blood level–time curves are dose dependent.

The binding of chlorpromazine was examined pharmacokinetically (28) in an attempt to explain rapid drug decreases in the plasma. Through theoretical computer simulations, it was determined that a rapid redistribution is possible for such a highly protein-bound drug after small changes in tissue binding. As with many other studies, these results suggest that more experimental work on the actual degree of tissue binding may be important in determining a drug's time course in the plasma.

The pharmacokinetic consequences of plasma protein binding were examined for one- and two-compartment models (29). The computer-simulated data treatment suggested that, if an elimination process operates on bound drug in the plasma for either the one- or two-compartment case, considerable alterations in half-life and area under the free drug concentration–time curve would be seen. However, no *in vivo* data were presented to show the existence of such an elimination mechanism for the bound drug.

Drug Displacement—Concurrent administration of drugs with high albumin binding affinities may produce competition at the binding sites, producing a higher free drug concentration and greater biological activity of each drug than if either is administered alone. Competitive protein binding was studied by using sulfaethidole and bovine serum albumin (30). By using a circular dichroic technique, the drug was found to exhibit optical activity only at its primary binding site, resulting in the unique opportunity to examine the effects of added drugs on the optical activity of sulfaethidole. Drugs that underwent binding at the same site, but were not themselves optically active, reduced the signal due to the sulfa drug. The data for acidic drugs showed that the same primary binding site may be shared by a wide variety of these molecules; basic drugs did not compete for the sulfaethidole site on bovine albumin. However, these same investigators reported that a series of basic alkyldimethylbenzylammonium chlorides did displace sulfaethidole from bovine serum albumin (31). Tryptophan and tyrosine residues of the protein may be involved, and some conformational changes may take place when these surfactant molecules are added to the protein solutions.

In another study employing the circular dichroic technique (32), various drugs were examined with regard to their competition for the two binding sites of dicumarol on human serum albumin. Acidic drugs displaced dicumarol, whereas basic drugs displayed no competition at the binding sites.

The displacing effect of endogenous fatty acids on diazepam bound to human serum albumin was examined (33). At therapeutic levels of diazepam, about 14% of the drug was displaced by laurate anion. The investigators concluded that, if the albumin binding of a drug was extensive, displacement interactions with other endogenous substances should not be ignored. Also, fluctuations of diazepam in blood could result from such a free fatty acid competition phenomenon. Considerable support for such a hypothesis could have been warranted if an attempt had been made to support these *in vitro* results with *in vivo* findings.

Diazepam competed for binding sites on thyroxine binding globulin and thyroxine binding prealbumin in a study using electrophoresis of serum containing thyroid hormone isotopes (34). Since this steroid binding globulin and prealbumin are endogenous macromolecules, the *in vivo* displacement of diazepam may be quite complex. A series of equilibrium–displacement–redistribution may take place *in vivo*. In some individuals having either a diminished concentration or malfunctioning macromolecules, a clinical manifestation of fluctuating drug concentrations and perhaps related toxicities may ensue.

Wardell (35) and McQueen (36), working independently and together (37), examined displacement of protein-bound drug and found that the displacement of initially bound drug upon injection of a more highly protein-bound drug results mainly in redistribution of the initially administered agent. The redistribution is caused by a displacement of drug from plasma protein, not from changes in absorption, metabolism, and excretion.

Dayton *et al.* (38), in a brief discussion of possible clinical effects resulting from plasma binding displacement, noted that drug displacement had not been studied adequately *in vivo*. A clinically oriented study was published concerning the displacement of warfarin from human al-

bumin (39). Drugs used as competitors included diazoxide and ethacrynic, mefenamic, and nalidixic acids. Levy and Oie (40) suggested that displacement can be quantitated for bilirubin. They found a strong correlation between the free fraction of bilirubin in plasma containing no drugs and the free fraction of bilirubin when sulfisoxazole or salicylic acid was present.

The effects of sodium 2-(4-chlorophenoxy)-2-methylpropanoate, the active form of clofibrate, on the possible displacement of phenindione derivatives from human albumin were investigated (41). This work was undertaken because clofibrate presumably had the same binding site on albumin as phenylbutazone. Moreover, phenylbutazone was shown to induce hemorrhagic accidents if patients were concurrently receiving coumarin drugs but not if they were receiving phenindione derivatives. 2-(4-Chlorophenoxy)-2-methylpropanoate inhibited the binding of coumarin agents but did not alter the binding of phenindione derivatives.

The displacement of urate from plasma proteins by anti-inflammatory drugs was examined (42, 43). A relationship was shown between the ability of acidic uricosuric agents to displace albumin-bound urate and to interact at the primary albumin binding site of 5-dimethylaminonaphthalene-1-sulfonamide *in vitro*. Since some of the uricosuric action of these agents was thought to depend on displacement of urate, four of the seemingly best displacing agents were tested clinically. All four drugs tested *in vivo* lowered serum urate concentrations. These two papers demonstrated excellent *in vitro–in vivo* correlations of drug displacement data.

Effect of Fatty Acids—The effects of free fatty acids on the subsequent binding of drugs to human and bovine serum albumins were studied extensively (44). Albumin solutions were prepared with 7, 3.5, or <0.05 mole of palmitate or oleate per mole of protein. Palmitate and oleate are the major free fatty acids of mammalian plasma. By using the equilibrium dialysis technique, studies were conducted on the binding of eight drugs to these fatty acid–albumin solutions. The results indicated that inhibition of drug binding was greater with palmitate than oleate and that inhibition was manifested either as a reduction in the number of drug binding sites or in the association constant for that binding site. The experiments suggested that free fatty acids in serum in the 7–3.5-mole ratio range are inhibitors of serum albumin binding of many drugs. At ratios less than 3.5 (free fatty acid to albumin), the inhibition is essentially negligible.

Spector *et al.* (45) examined the effects of palmitate and oleate at free fatty acid to albumin ratios of 0 to 4 on the ability of previously defatted human serum albumin to bind the antihypertriglyceridemics 2-(4-chlorophenoxy)-2-methylpropanoate and halofenate. The added fatty acid greatly reduced the binding of the 2-(4-chlorophenoxy)-2-methylpropanoate while little effect on halofenate binding was seen. The affinity of halofenate to fatty acid free albumin is much greater than the affinity of the 2-methylpropanoate drug to free albumin. As a result of their data, the investigators suggested that the ability of free fatty acids to regulate drug binding depended upon the affinity with which the drug was bound to albumin. They also suggested a hypothetical mechanism to explain the variation in results for free fatty acid effects on drug binding: that at low mole ratios, allosterism predominates, while both allosterism and a competitive interaction ensue at high mole ratios.

Effect on Bilirubin—Albumin binds significant amounts of the endogenous substance bilirubin. Numerous clinical studies document displacement of bilirubin by sulfa drugs, particularly sulfisoxazole. Many drugs have the ability to displace bilirubin from albumin and thereby potentiate its cytotoxicity (46). Albumin was shown to have two classes of bilirubin binding sites from *in vitro* studies. Of highest affinity was a single site from which bilirubin was not displaced by drugs; however, the availability of this site to bilirubin may be related to free fatty acid concentrations in the serum. The second class of site was multiple in nature, and it was from this class of site that drugs may have displaced bilirubin.

A model was postulated for bilirubin–albumin binding and the propensity of drugs to compete with this highly bound endogenous material. A rapid methodology for detection of free bilirubin in plasma or serum, especially in the newborn, has become important from the standpoint of concurrent drug administration. Published methods attempt to quantitate bilirubin in the presence of various drugs, and some of these methods are quite complex (47–51).

Although drugs may increase the free concentration of bilirubin, as already pointed out, a recent study (52) suggested that phototherapy in the case of hyperbilirubinemia did not increase the unbound fraction of bilirubin in blood. In fact, the bound fractions of both bilirubin and salicylate to albumin remained unchanged after phototherapy compared to prephototherapy levels.

CLINICAL SIGNIFICANCE OF PROTEIN BINDING

Most clinicians and pharmaceutical scientists believe that a drug's action is dependent on its concentration in plasma water. However, most blood level measurement methods include the amount of drug bound to plasma proteins as well as the free amount. The concept of a plasma therapeutic level is now becoming more important (53). Information on the changes in free and bound levels in the presence of various disease states is in its initial stages.

In Renal Dysfunction—Recent studies indicated that, for many drugs, the percent of therapeutic amounts of drug bound to albumin in the plasma was significantly reduced in patients with uremia (54). Patients with renal dysfunction have a higher incidence of adverse drug reaction. Drug dosage modification to obtain plasma therapeutic levels appears most beneficial in patients with these abnormalities of drug excretion. The dosage modification in uremic patients was quantitated using a one-compartment model to predict changes in the elimination rate constant of drugs (55).

The protein binding of phenytoin (diphenylhydantoin) in the uremic patient has been well studied. Phenytoin binding was reduced in such patients, and this reduction was not strongly dependent on concentration of albumin or total protein (56). It was suggested that there had been a qualitative change in the drug binding proteins in uremic patients. The decrease in drug binding was not due to the presence of dialyzable substances in the plasma.

Blum *et al.* (57) concurred with these results and also showed that the principal metabolite of phenytoin was not responsible for a competition phenomenon and therefore could not be decreasing phenytoin binding. They postulated that the free plasma concentration of phenytoin may have been much lower in the uremic patient than in a normal individual, yet adequate seizure control was maintained. To the contrary, Andreasen (58) found phenytoin to have increased binding after dialysis of the plasma proteins from both normal and uremic individuals. He concluded that: (*a*) a competing substance of molecular weight greater than 700 may have been present (nondialyzable in his experiments), (*b*) some very strongly bound small molecules may have been present, or (*c*) alterations in the macromolecules may have been involved. Shoeman *et al.* (59) also suggested a qualitative difference in the albumins of normal and uremic patients.

Other studies showed decreased phenytoin binding in patients with renal abnormalities (60–62). In an excellent comparative study (62), it was pointed out that both normal and uremic patients should have about equal levels of unbound drug in plasma, provided tissue binding has not changed. The total plasma phenytoin concentration would, however, be decreased since there was a smaller amount of protein-bound drug and therefore a shift to a new equilibrium amount. The investigators stated that decreased plasma protein binding in uremic patients would potentiate pharmacological effects only if the drug was highly protein bound in normal subjects and, thus, minimal free drug levels were required for the principal pharmacological action. They further stated that monitoring of the plasma levels of drugs in uremic patients should be combined with the knowledge of the extent of the drug's binding to plasma protein. A more recent study (63) provided information on phenytoin binding to blood cells and showed that the distribution of drug in whole blood was different in the uremic *versus* the healthy subjects.

The binding of sulfamethazine to albumin was reduced in six nephrectomized patients (64). Dialysis of the patient blood did not increase the *in vivo* binding capacity over nondialyzed blood samples, supporting the belief that a defect in the binding properties of serum albumin existed in uremic patients. Also, a study of furosemide binding in patients with acute renal failure seemed to support the possibility of a defective drug binding mechanism (65, 66). The furosemide binding affinities determined in these two studies were in close agreement.

In the case of diazoxide in uremia (67), it appeared that the main determinant of decreased binding was the reduction in serum albumin concentration. Likewise, morphine binding was reduced in patients with renal failure and was shown to be dependent on the concentration of total serum proteins and albumin but not on the severity of renal dysfunction measured by creatinine clearance (68). Other studies were conducted with phenylbutazone (69) and cardiac glycosides (70, 71) in the plasma of uremic patients, and similar reductions in binding in uremic conditions were found.

The pharmacokinetics of trimethoprim, sulfamethoxazole, and cefazolin were studied in normal and uremic patients (72, 73). The distribution of free sulfamethoxazole in tissues increased during renal insufficiency, and this result was suggested to be due to reduced protein binding. However, no change in tissue distribution was seen for trimethoprim. With cefazolin, higher serum levels were observed in uremic subjects after identical doses to uremic and normal subjects, and serum half-lives increased with decreasing renal function. The apparent volume of distribution of cefazolin was elevated in the uremic state, probably due to decreased protein binding.

Campion (74) and Dromgoole (75) examined the reduction in binding of dyes during renal failure. Both found a decrease in binding; but while Campion determined that the decrease was unrelated to competition with metabolites, Dromgoole suggested either competition or conformational changes in albumin as the reason for the reduction. The binding of drugs, essential amino acids, and waste metabolites was investigated in normal and uremic subjects (76). Binding was reduced in the uremic subjects in all cases.

In Hypoalbuminemia—Generally, the concentration of albumin or total protein is decreased in various chronic diseases. Therefore, the unbound fraction of most drugs could be elevated; that is, the concentration of drug in plasma water could be increased. The intensity of drug effect may become greater than expected and may fall into the toxic range, as was documented in the case of phenytoin (77). Phenytoin was extensively bound to albumin and had increased adverse reactions when decreased serum albumin levels were present. A similar explanation was advanced for toxicity due to prednisolone, the major metabolite of prednisone (78).

Perhaps the best *in vivo* correlation of serum protein concentration and adverse drug reaction was reported by Greenblatt and Koch-Weser (79). In more than 1200 patients monitored while receiving diazepam, adverse reactions ranged from 2.9% in patients with normal serum albumin concentrations ($\geq$4.0 g %) to 9.3% in those with hypoalbuminemia (<3.0 g %). Similarly, methadone has a large variation in its binding fraction as a result of varying albumin levels in patients (80).

Another study (81) examined diluted serum from two subjects with regard to its binding of four drugs. In all cases, the amount of unbound drug increased as the albumin concentration was diminished. It was recommended that the albumin concentration be determined when examining plasma drug levels.

Wosilait (82) computed the theoretical amount of free drug in the plasma at various clinical concentrations of a number of drugs and at levels of albumin ranging from 2 to 5 g %. His results implied that the amount of free drug increased significantly in hypoalbuminemia and that care should be exercised when using highly protein-bound drugs in patients with decreased albumin levels.

In Liver Dysfunction—The influence of liver disease on the binding and kinetics of drugs in humans has received limited attention. Some reported results conflict as to the importance, if any, of drug–protein binding in hepatic dysfunction. The binding of five drugs to plasma proteins from patients with alcoholic liver disease was studied (83). The data for basic drugs indicated that liver disease decreased binding. With quinidine, the unbound fraction increased three times over normal amounts, an elevated level that may be clinically significant. For the organic bases studied, the decreased binding was most sensitive to decreased levels of serum albumin. Since cir-

rhotic patients have lower albumin concentrations, they consequently should have increased fractions of unbound drug. The data presented for acidic drugs lacked correlation with liver disease or albumin levels.

The percentage of unbound phenytoin increased by about 33% in five patients with acute viral hepatitis (84). In patients with hepatic dysfunction, monitoring plasma drug concentrations was the best means for adjusting dosages and schedules. The unbound phenytoin fraction in patients with hepatic disease increased by about 50% over that in normal healthy individuals (61). These changes in protein binding correlated better with changes in albumin and bilirubin levels in plasma than with any of 13 other biochemical parameters monitored. The binding of both morphine and phenytoin was decreased in liver failure (66). The binding depended on albumin concentration, but more data are needed to assess the effect of liver dysfunction on protein binding.

The degree of protein binding of amobarbital was reduced in five chronic liver disease patients who also had abnormally low serum albumin levels but was normal in five chronic liver diseased patients with normal serum albumin concentrations (85). Meperidine disposition and elimination were examined in patients with acute viral hepatitis (86). The significant increase in plasma half-life in patients with hepatitis was tested against protein binding of meperidine in normal *versus* hepatitis patients. The binding of meperidine was about equal in both groups; although elimination was significantly impaired in acute viral hepatitis, the effect seemed independent of protein binding.

Likewise, the effect of cirrhosis on the disposition and elimination of clindamycin was examined (87) and compared to normal individuals. Although the half-life was prolonged in the cirrhotic condition, the effect of clindamycin protein binding was about equal in both groups. The effect of liver disease in patients taking diazepam was studied (88). Plasma protein binding of diazepam in cirrhosis was decreased compared to that in normal subjects.

In Neonates—The neonate may respond quite differently than adults to drug substances (89). Drug binding to plasma proteins is of special interest in the neonate due to its regulatory effect on pharmacodynamic processes. Neonatal distribution, metabolism, and excretion processes differ from adults partly because infants have a greater total body water and lower fat content. This composition difference is larger and of extreme importance in premature infants.

The binding of phenytoin, salicylate, and nafcillin to plasma protein from newborn infants was investigated (90). The unbound fraction of phenytoin was significantly greater in neonates than adults, and the percentage of unbound drug correlated with the serum bilirubin level, suggestive of a possible competition phenomenon. Likewise, salicylate binding differed in the newborn as compared to adults. Nafcillin binding in the neonate exhibited large reductions from amounts bound to adult serum, and the levels of unbound drug were much higher. In a more recent paper (91), the low degree of nafcillin binding suggested that albumin was not the primary binding protein for this drug. Diazepam binding to neonatal and adult serum proteins also was discussed; in this case, the same binding affinity was observed with both the adult and newborn proteins.

Two studies (92, 93) compared the binding of some drugs to adult, fetal, and neonatal plasma. Both studies found reduced binding capacity in the newborn as compared with plasma from adults. Differences in the total protein concentration between these two age groups may have been responsible for the binding disparity. Hyperbilirubinemia further decreased the binding capacity. Data were given for phenytoin, imipramine, diazoxide, and cephalothin (92) at levels comparable to therapeutic concentrations; neonatal binding was reduced by 4–12% compared to adult plasma in all cases.

It was similarly reported (94) that drugs were less bound in newborn than in adult plasma, but this study also fractionated the plasma from each group into albumin and globulin fractions. With the six drugs studied, no difference was found in binding in the albumin fraction of newborn or adult plasma; however, binding to the globulin fraction was less in the newborn than in adults. Three possible mechanisms were suggested for the observed binding disparities: (*a*) a bilirubin competition phenomenon, (*b*) the decrease in globulin–drug interaction, and (*c*) the possibility for less drug binding due to albumin–globulin interaction in the plasma of the newborn.

A binding disparity between maternal and fetal plasma protein affinities for bupivacaine also was reported (95). Maternal protein bound approximately twice as much drug as did fetal protein on a per gram basis. The drug concentration was in the therapeutic range of 0.05–5.0 μg/ml, and the binding decreased from 92–78% to 35–31% in the maternal and fetal plasma, respectively. However, Gorodischer *et al.* (96), in an attempt to rationalize high digoxin doses in infants, reported that at therapeutic levels the affinity of digoxin for cord serum was low and about equal to that in adult serum. The higher therapeutic concentrations of digoxin in infants probably are a result of a higher dose (per unit body weight or surface area) than in adults. The pharmacological basis for the traditionally used high doses of digoxin in infants cannot be rationalized as due to binding differences, and more work is required.

In a study of hydrocortisone binding to plasma proteins from maternal, fetal, and infant plasma, a large difference was found in the binding capacity of maternal and fetal plasma (97). That this result might be due to a physicochemical difference in the respective proteins was deemed unlikely because the binding or affinity constants (K_a) were similar ($5–8 \times 10^8\ M^{-1}$) in all blood compartments examined. It was also reported (98) that the binding proteins in the maternal compartment may be highly active in the control of steroid metabolism. This report (98) found that, in comparison with nongravid women, fetal and neonatal steroid serum binding protein concentrations were one-thirtieth in the 5th month of gestation, one-twentieth immediately postpartum, and at normal adult nongravid levels 1 month postpartum.

In attempts to understand the binding disparity of phenytoin in neonatal and adult stages, it has been suggested (99) that there are large variations in circulating free fatty acids during the neonatal period and that the differences in both the acids present and their concentrations can influence the degree of drug binding during this period.

In plasma from newborn infants, phenytoin binding correlated with the albumin concentration and with the concentration ratios of bilirubin and free fatty acid to albumin. A reasonable correlation coefficient for phenytoin binding was obtained when compared to the ratio of total plasma bilirubin and albumin concentrations in infants (100).

RESULTS OBTAINED WITH PARTICULAR DRUG CLASSES

Antibacterial Chemotherapeutic Agents—*Penicillins and Cephalosporins*—A paper dealing with the pharmacological effects of the binding of various antibiotics (101) noted that, although most penicillins are bound by serum proteins, in only a few cases does this binding exceed 80%. (This percentage figure is misleading, because it is definitely related to some specific concentration or at least a concentration range.) Consequences of extensive binding of antibiotics by serum proteins may be interference with biological activity, delayed renal excretion, reduced urine concentration, restricted distribution volume, and decreased penetration of drug into intracellular spaces or inflammatory fluids. These effects were examined in relation to the binding of antibiotics by serum proteins and other endogenous substances. It was concluded that serum protein binding exerted an effect on these parameters and that this effect was particularly important for penicillinase-resistant penicillin and newer analogs of tetracyclines. Also included in this paper was a tabulation of binding percentages for various penicillin and tetracycline analogs.

In a study of six antibiotics, binding was well correlated with the ability of antibiotics to diffuse into interstitial fluid (102), as suggested previously. By using an *in vivo* skin chamber device, antibiotics with lower percentages of protein binding were found to diffuse into the interstitial fluid better than those with high binding percentages. Floxacillin (flucloxacillin), 95% of which is bound to protein, exhibited no detectable interstitial fluid levels. Also, increased protein binding influenced the renal clearance of four penicillins inversely (103).

Using a diafiltration technique to examine the association–dissociation rate of drug–protein interactions, a question about which information is still scarce, Barza *et al.* (104) presented data for oxacillin, nafcillin, and cephalothin. The binding of all three antibiotics was rapidly reversible and complete. The results of their experiments, in terms of percentages bound, are included in Table I, along with the results of some other papers having protein-binding data on penicillin and cephalosporin drugs.

In another study designed to demonstrate the reversible-ion binding between a series of penicillins and bovine serum albumin (105), the penicillin side chain had considerable influence over the binding process. According to the reactions of hydrated electrons produced by pulse radiolysis, the binding of the first molecule of penicillin G (benzylpenicillin) or penicillin V (phenoxymethylpenicillin) resulted in a greater decrease in protein reactivity towards e_{aq}^- than succeeding molecules. The results were interpreted in terms of the carboxyl group of penicillins interacting with cationic sites on the protein; however, other short-range weak forces may be significant.

In a study of the pharmacokinetics of various cephalosporins compared to cefazolin (106), extensive binding of a few congeners to serum protein took place. This degree of binding (Table I) apparently affected the serum half-life, the renal clearance, and the apparent volume of distribution of these drugs. Cefazolin, the most highly bound cephalosporin, had the lowest distribution volume, a result also obtained by other workers (110). In a study of the levels of ampicillin and cloxacillin in synovial fluid of arthritic patients, the non-highly-bound ampicillin reached about the same concentration in synovial fluid as in serum; the highly bound cloxacillin had synovial fluid levels much lower than serum levels (111). A rapid equilibrium apparently was established between the free concentration of cloxacillin in serum and synovial fluid. The primary effect of binding was to confine the drug to the vascular fluid. Distribution to other fluids was governed by levels of free drug in serum and not total levels. Along these same lines, a wide variation was found in the clearance of seven antibiotics by an artificial kidney (112). These differences were attributed to the variation in protein binding among the antibiotics.

Table I—Penicillin and Cephalosporin Antibiotic Binding to Human Serum

Antibiotic	Antibiotic Concentration, μg/ml[a]	Percent of Antibiotic Bound[b]	Reference
Oxacillin	20 (99)	85.2 (83.5)	103
Nafcillin	20 (100)	87.2 (80.5)	103
Cephalothin	10 (40)	57.0 (53.9)	103
Carbenicillin	80	50	107, 109
Ticarcillin	124	65	107
Penicillin V	30 (60)	52.7 (45.2)	108
Cefazolin	30	86	106
Cephanone	30	88	106
Cephaloridine	30	20	106
Cephalexin	30	15	106
Cephalothin	30	65	106
Cephradine	86.9	12.5	110
Ampicillin	—	13.5	111

[a] Number in parenthesis denotes second concentration of drug used in the literature report; results for this concentration are given in parenthesis in percent bound column. [b] Results are given in terms of percent of drug concentration bound. The serum consisted of 100% human serum or 100% pooled human serum.

Tetracyclines—A quantitative study of the interaction of therapeutic concentrations of tetracycline with human serum proteins was reported (113). In serum, 53% of tetracycline was complexed with various proteins and just over half of that amount was with albumin (most of the rest being associated with lipoprotein fractions). The results showed that tetracycline bound to two sites on albumin. The high affinity site had an association constant of $4.38 \times 10^4\ M^{-1}$ but very limited capacity (0.032 site/mole of albumin); the low affinity site exhibited a binding constant of $1.01 \times 10^2\ M^{-1}$ but had 39.50 sites associated with it. The physical meaning of having less than one-tenth of a binding site per mole of albumin was not discussed and was quite confusing to the reader. Such a minute number of sites on a large globular protein is difficult to envision.

The binding of tetracycline and various analogs to human serum albumin was examined using a probe displacement technique (114). Methacycline displaced the greatest amount of probe followed, in order, by doxycycline, chlortetracycline, oxytetracycline, and tetracycline. The association constants are included in Table II. As with

Table II—Literature Values for the Binding of Tetracycline Antibiotics to Serum Albumin

Tetracycline Analog	Primary Binding Constant or Percentage Bound	Number of Binding Sites or Concentration of Tetracycline	Reference
Tetracycline[a]	$4.38 \times 10^4\ M^{-1}$	0.032	113
Tetracycline[a]	$3.15 \times 10^4\ M^{-1}$	—	114
Oxytetracycline[a]	$3.53 \times 10^4\ M^{-1}$	—	114
Chlortetracycline[a]	$4.03 \times 10^4\ M^{-1}$	—	114
Doxycycline[a]	$5.32 \times 10^4\ M^{-1}$	—	114
Methacycline[a]	$6.28 \times 10^4\ M^{-1}$	—	114
Oxytetracycline[a,b]	$4.44 \times 10^3\ M^{-1}$	0.27	115
Demeclocycline	38%	10 μg/ml	116
Oxytetracycline	31%	10 μg/ml	116
Doxycycline	52%	10 μg/ml	116

[a] The tetracycline concentration was varied over a wide range, and more than one protein concentration was used in most cases. [b] Study used bovine serum albumin; 50% of the drug was bound at therapeutic concentrations.

other probe studies, the number of binding sites associated with these binding constants cannot be calculated. However, the technique was confined solely to the number of sites upon which the probe itself was bound. A drug binding at a site different than the probe may not have been detected. In addition, the possibility of probe-caused conformational changes in the protein structure was not, or cannot be, accounted for with such a technique.

Aminoglycosides—Conflicting reports concerning aminoglycoside antibiotic binding exist. The problem seemingly arose due to the ion content of the buffers used in various studies. For example, human serum binding of gentamicin, tobramycin, and kanamycin was about zero whereas streptomycin was about 35% bound (117). The system was not described except that a physiological pH was used with an ultrafiltration method.

In contrast, at gentamicin concentrations between 1.7 and 8.9 μg/ml (therapeutic levels of this antibiotic), 70% was bound to human serum albumin (118, 119). When the concentration was increased to 10–20 μg/ml, the binding of gentamicin was decreased to about 50% (118). Similar results were obtained for tobramycin, sisomicin, kanamycin, and amikacin; they were bound to the extent of 75, 85, 54, and 69%, respectively, at therapeutic levels. However, under *in vivo* conditions, these binding levels would not be expected to be reached; these values were obtained in 0.05 *M* tromethamine–hydrochloric acid buffer, which was void of any divalent cations. As levels of Ca^{2+} and Mg^{2+} increased, the binding of these agents decreased (118). The variations reported for *in vitro* experiments might carry over to the *in vivo* situation only under certain pathological conditions.

Another conflict arose from the results of a report comparing the pharmacokinetics of kanamycin and amikacin (120). Only 3.6% binding of amikacin could be detected when 15 μg of drug/ml was present in an *in vitro* system. This figure was about 200% less than the results reported previously (118).

Erythromycin, Lincomycin, and Clindamycin—Few papers have been published since 1969 concerning the binding of these drugs to human serum albumin. Prior to 1969, various reports stated that these molecules bound with high affinity while other papers indicated that only a low binding of these drugs occurred.

The binding of these three related antibiotics to serum albumin was studied using an ultrafiltration technique (121) (Table III). Also included in this work was a discussion of erroneous techniques and conclusions that resulted in some previously reported low levels of binding of these drugs. In a report examining the protein binding influence on the relative merits of the blood levels produced by erythromycin stearate and erythromycin estolate (erythromycin propionate lauryl sulfate) (122), doubts were raised as to the ability of blood levels to predict the effectiveness of these two forms of the same drug. Of utmost importance, in the author's opinion, was that the unbound levels of the estolate form were significantly lower than the unbound parent drug levels, even though total levels were higher. Thus, the difference in serum levels produced by the two forms of erythromycin reflected the difference in protein binding (Table III). It was suggested that unbound drug was a better measure of activity in the case of erythromycin congeners (122).

Table III—*In Vitro* Human Serum Binding of Erythromycin, Lincomycin, and Clindamycin[a]

Antibiotic	Percentage Bound	Antibiotic Concentration, μg/ml	Reference
Erythromycin base	73.4	5	121
	81.4	0.59–2.87	122
Erythromycin propionate	92.6	5	121
Erythromycin estolate	95.7	0.94–4.05	122
Lincomycin	71.9	5	121
	57	1	123
Clindamycin	93.6	5	121
	93	1	123

[a] Protein consisted of human serum (in a few cases pooled). The results reported are with 100% human serum, although some studies also used 25, 50, and 75% serum solutions.

Sulfas—In a series of articles dealing with drug–macromolecule complex formation, sulfa drug–albumin interactions were studied (124–127). For the many sulfas studied, the binding was considerably greater for the ionic form of the drugs than for the nonionized form. Also, two sets of binding sites apparently existed on albumin for most of these agents. Various drugs, such as phenobarbital and penicillin V (phenoxymethylpenicillin), were able to compete for these sulfa binding sites. Some of the binding data results are given in Table IV.

Table IV—Literature Parameters for Sulfa Drug Binding by Human Serum Albumin

Agent[a]	Primary Binding Affinity	Number of Binding Sites	Reference
Sulfadiazine	$3.6 \times 10^3\ M^{-1}$	1	125
Sulfamerazine	$2.9 \times 10^4\ M^{-1}$	1	125
Sulfamethazine	$4.4 \times 10^4\ M^{-1}$	1	125
Sulfameter	$5.9 \times 10^4\ M^{-1}$	1	125
Sulfadimethoxine	$1.8 \times 10^5\ M^{-1}$	1	125
Sulfisomidine	$5 \times 10^3\ M^{-1}$	1.1	128
Sulfamethomidine	$1 \times 10^4\ M^{-1}$	2.1	128
Sulfaortho-dimethoxine	$1.25 \times 10^4\ M^{-1}$	2.1	128
Sulfadimethoxy-pyrimidine	$4 \times 10^3\ M^{-1}$	2.2	128
Sulfadimethoxine	$1 \times 10^4\ M^{-1}$	1.9	128
Sulfaethidole[b]	1.2×10^5 liters/mole	1 primary (3 secondary)	129
Sulfathiazole[b]	2.96×10^3 liters/mole	1.98 primary (9.77 secondary)	130

[a] The concentration of drug used for these data varied over a wide range; the albumin concentration was also varied in most cases. [b] Study used bovine serum albumin.

The influence of sulfa drug binding on the excretion of two very long-acting, two long-acting, and one short-acting sulfa drugs was reported (128). The short-acting agent was bound to a lesser extent at clinical concentrations, and only a single binding site was evident for this drug in contrast to the two sites for the other sulfas. Small changes in sulfa drug structure may have altered plasma protein binding. At the plasma concentration of 0.4 mM, which is within the therapeutic range of these drugs, the short-acting agent was 67% bound whereas the longer acting drugs were 90–96% bound.

Perrin and Nelson (129), using the highly specific direct technique of induced optical activity, especially appropriate at low levels of added drug, found that with sulfaethidole only the single high affinity site was being examined by their method. Dialysis investigations had shown two classes of binding sites and thus made drug competition examination difficult. A primary binding affinity of 1.2×10^5 was obtained for sulfaethidole binding to bovine serum albumin. Binding constants of various drugs competing for the sulfaethidole binding site were reported.

Recently, the binding of sulfathiazole to bovine serum albumin and other blood constituents was examined (130). The results for serum albumin are included in Table IV. Also, some interesting possibilities were offered to explain unusual binding data. Data treatment of binding may be altered in whole blood systems, so normal methods to characterize the data may be inappropriate in these instances.

Rifampin—The binding of rifampin (rifampicin), a semisynthetic antibiotic with a broad antibacterial spectrum, often used as an antituberculous agent, has been examined (131–135). Rifampin concentrations ranging from 0.8 to 1000 μg/ml were used, and all studies except one (134) showed the drug to be 70–91% bound. This extent of binding resulted from using human and bovine serum as well as the albumin fractions of each. The report with percentage binding outside this range used human serum and a rifampin concentration of 10 μg/ml in an equilibrium dialysis investigation (134). The binding was 8–41%; however, these results are confusingly reported as rates of binding.

In one study (135), binding was examined in healthy volunteers (mean 88.9% bound) as well as in tuberculous patients (mean 86.1% bound). The difference between these two groups was significant, but the binding of rifampin to plasma proteins was of relatively minor importance clinically.

Anticoagulants—The interaction of warfarin and dicumarol with human albumin has been the subject of many investigations. This is to be expected since the relatively low therapeutic index of these agents and the propensity for other concurrently administered drugs to displace small amounts of the anticoagulants from their binding sites raise free drug levels and possibly increase prothrombin time.

O'Reilly (136–138) found that warfarin was bound to albumin with an affinity constant of $2.17 \times 10^5\ M^{-1}$ at 37° (137) and that the 6-, 7-, and 8-hydroxy metabolites showed a 7–23-fold reduction in binding (136). These data (136) suggested that the interaction was exothermic and had a positive entropy and that the albumin–drug interaction was probably the result of cooperative hydrogen bonding–hydrophobic forces. Recent studies (139, 140) discussed the effect of protein binding of warfarin in rats related to its pharmacological action, distribution, and elimination. A substantial variation was found in the free fraction of drug in the serum, and strong correlations were observed between the free serum fraction and the elimination rate constant, the volume of distribution, and the total plasma clearance of the drug. The amount of total warfarin required to elicit a specific anticoagulant response varied widely between animals, and this response was a function of free warfarin levels in plasma (140).

An investigation of warfarin and dicumarol interaction with human serum albumin was reported using a circular dichroic technique (141). Warfarin, in contrast to dicumarol, gave no induced Cotton effect, suggesting a different mode of binding for the two anticoagulants. On the other hand, another study (142) found that warfarin was capable of generating extrinsic Cotton effects and that the binding constant could be calculated from such data. Based on equilibrium dialysis data, Chignell (141) also reported that dicumarol (bishydroxycoumarin) was bound to human serum albumin at three primary homogeneous sites and exhibited a binding constant of $2 \times 10^5\ M^{-1}$. However, he also studied the interaction by monitoring the quenching of the single tryptophan residue of human serum albumin and concluded that one primary binding site existed with a binding affinity of $5.2 \times 10^5\ M^{-1}$. In addition, he showed that the dicumarol–albumin interaction gave rise to large negative extrinsic Cotton effects. Thus, it was proven that the interaction could be studied by the circular dichroic technique, but no quantitative attempt was made.

Recently, a report (32) appeared using this sensitive circular dichroic method for low dicumarol to protein ratios (*i.e.*, those of clinical significance), and a binding constant of $2.9 \pm 10\% \times 10^6\ M^{-1}$ at the single high affinity site was calculated. Results from dicumarol displacement studies using other acidic drug molecules are contained in the same report (32). A competition between warfarin and dicumarol for a human albumin binding site also was shown.

A computer program was used to determine the binding capacities, association constants, and amounts of unbound and bound dicumarol at different drug levels as well as at various levels of protein concentrations (143–145). An association constant of 2.2×10^6 liters/mole containing two homogeneous sites was reported. A second class of seven sites with a binding constant of 1.3×10^4 liters/mole also was determined (144), and for warfarin a primary binding affinity of 8.9×10^4 liters/mole at two equivalent binding sites was found.

Warfarin, dicumarol, sulfonamides, and other acidic drugs may share the same binding site on albumin (146). However, such acidic drug competition may not always be the case. A recent report dealt with the lack of interaction between warfarin and ibuprofen (147). Ibuprofen, an acidic drug bound to plasma protein when administered alone, failed to inhibit competitively or to interfere with warfarin binding, thus producing no alteration in the degree of hypoprothrombinemia.

Antiepileptics—Phenytoin has been well studied with respect to its protein binding and pharmacokinetic characteristics. In normal human plasma, 92.6% of the drug was bound at a therapeutic concentration of 16 μg/ml using an

ultrafiltration technique (148). No binding differences were found with respect to sex, and there was a 64% increase in the unbound fraction of drug at 37°. Thirteen drugs were also examined as to their propensity to affect phenytoin binding. Only salicylic acid, sulfisoxazole (sulfafurazole), and phenylbutazone were able to increase the unbound fraction of phenytoin. In another study (149), phenytoin was extensively bound to both human and rat albumin and the major metabolite (the 5-*p*-hydroxy species) was also strongly plasma protein bound. The binding levels of both the parent compound and the metabolite were suggested as possible explanations of the species selectivity in the drug-induced toxicity observed.

A study was designed to examine the protein binding of phenytoin in eight patients with epilepsy and in drug-free volunteers as well as to determine total drug in plasma and cerebrospinal fluid (150). Only minor variations were seen between individuals in each group. The mean unbound fraction of phenytoin in patient plasma was 6.3% at room temperature and 10.3% at body temperature at a mean drug level of 11.2 μg/ml. The ratio of total phenytoin in cerebrospinal fluid to that in plasma in the eight patients was unusually constant with a mean of 9.6%. A comparison between the unbound fraction of phenytoin in plasma and the same cerebrospinal fluid to plasma ratio exhibited a good correlation, a result not unexpected since only the unbound fraction of drug was available for equilibration.

Phenytoin binding was examined to determine if individual differences in the degree of protein binding might explain the variance between total serum levels and clinical intoxication (151). Adverse clinical symptoms were better correlated to free levels of the drug than to total serum concentration. When free phenytoin levels were above 5 μg/ml, signs of intoxication would probably be present no matter what the total serum level. These individual variations in ability to bind this drug may have been due to a qualitative change in the drug binding proteins (152). In a study of plasma from 11 different species of mammal, such a change in binding proteins was observed.

For highly bound phenytoin, the unbound plasma level can be readily obtained in the clinical laboratory by measuring the red blood cell to plasma concentration ratio of an unknown sample (153). The red blood cell binding to plasma binding ratio was constant throughout therapeutic plasma phenytoin levels. As a result of this finding, one could readily individualize drug dosage and minimize adverse effects. A recent paper (154) concerned with phenytoin effects and plasma protein binding reported that, in rats, prior treatment with phenylbutazone had no effect on the potency of unbound phenytoin. Thus, the action of this antiepileptic depends upon the unbound concentration in plasma and not upon dose or total plasma concentration. These results agree well with those previously described (151–153).

A recent abstract (155) further supported the idea of binding when more than one highly bound drug was present. Diazoxide lowered the phenytoin binding levels, requiring considerable dosage adjustment. Concurrent drug administration may also have changed the rate of drug metabolism, further necessitating dosage adjustment.

The protein binding of the antiepileptic carbamazepine was the topic of two recent investigations (156, 157). Carbamazepine had a low association constant for albumin (1.35×10^3 liters/mole) at approximately one site per mole (156). For therapeutic concentrations of drug (5–30 μg/ml), the unbound fraction was 24% following ultrafiltration of plasma samples from six volunteers. The results from an excellent study (157) confirmed this observation of the unbound fraction. A linear relationship was observed between unbound and total drug throughout the therapeutic region of 5–50 μg/ml. Carbamazepine binding in 54 plasma samples from treated patients was examined with the result that 26.9% of the drug was found to be unbound. Erythrocyte uptake, measured in 23 of these patients, was 38.3% of the plasma levels. Six other anticonvulsants were examined at high therapeutic concentrations for effects on carbamazepine binding and were shown not to alter carbamazepine free drug levels significantly. Also, no difference was seen when renal disease was present and only a slightly lower percentage of carbamazepine was bound if the patient had hepatic dysfunction.

Antihypertensives—The binding of diazoxide to human albumin was investigated, and the binding accounted for its long half-life. At 37°, the dissociation constant for the diazoxide–albumin interaction was $5.2 \times 10^5\ M^{-1}$, with 1.2 sites exhibiting this constant. At pH 7.4, the free energy change upon binding was −5.9 kcal/mole and was accounted for from hydrogen and ionic bonds (158). Protein binding may be implicated in the kinetics of the diazoxide effect (159). A rapid intravenous injection produced an effective hypotensive action, whereas injections lasting longer than 1 min greatly reduced the intensity. At therapeutic concentrations, 97.6% of the drug was in the protein-bound form, so free diazoxide reached effective levels at the arteriolar wall only during rapid injection. These results duplicated those of Andreasen (160).

Propranolol binding was studied and related to its disposition in humans (161–163). At therapeutic concentrations, 93.2% of the drug was bound to human plasma (163); the volume of distribution increased as the free propranolol fraction in the blood increased (162). However, the ratio of the volume of distribution to the free drug was relatively constant. After examination of propranolol binding in four species (humans, monkeys, dogs, and rats) as well as the clearance, half-life, and volume of distribution, it was concluded that increased drug binding was associated with a decrease in drug half-life when the influence of variable drug clearance was accounted for. Differences in binding were responsible for interindividual variations in the half-life of the drug in humans after intravenous administration. This was the result of an increase in the rate of drug delivery to the elimination sites (163). Human albumin (5%) alone accounted for only about 50–60% of the binding to plasma over the drug concentration of 1–10 μg/ml. Similarly, serum albumin alone accounted for only 42% of propranolol binding at drug concentrations less than $1 \times 10^{-4}\ M$ (164). From these data, an intrinsic binding constant of very low magnitude was calculated for propranolol (about 120 liters/mole). The binding of the β-adrenergic receptor antagonists alprenolol, its 4-hydroxy derivative, and a chemically related compound metoprolol[1]

[1] H 93/26.

Table V—Summary of Results Obtained from Investigations of Analgesic–Anti-Inflammatory Agent Binding to Plasma or Serum Albumin

Drug	Drug Concentration	Protein	Protein Concentration	Affinity Constant or Percent Bound	Number of Sites	Reference
Salicylate	50 μg/ml	Human plasma	5.3%	72%	—	166
Salicylate	50 μg/ml	Human albumin	3%	73%	—	166
Salicylate	Wide range	Bovine albumin	4%	5×10^4 liters/mole	1.04	130
Salicylate	Wide range	Human albumin	0.7%	$2.19 \times 10^5\ M^{-1}$	4	169
Salicylic acid	$0.002–5 \times 10^{-3}\ M$	Human albumin	0.3%	$7.07 \times 10^4\ M^{-1}$	1.28	168
Indomethacin	$0.002–2 \times 10^{-3}\ M$	Human albumin	$1 \times 10^{-4}\ M$	$8.4 \times 10^5\ M^{-1}$	4–5	169
Indomethacin	57–756 μg/ml	Human plasma	2.35%	$3 \times 10^5\ M^{-1}$	1	174
Phenylbutazone	Wide range	Human albumin	$1 \times 10^{-5}\ M$	$1 \times 10^5\ M^{-1}$	1	177
Fenoprofen	40 μg/ml	Human albumin	$7.1 \times 10^{-5}\ M$	$3 \times 10^4\ M^{-1}$ (>99% bound)	4–5	180
Fenoprofen	$0.075–37.9 \times 10^{-5}\ M$	Human albumin	$7.25 \times 10^{-5}\ M$	$1.86 \times 10^5\ M^{-1}$	1	181
Pentazocine	—	Human plasma	—	61.1%	—	182
Diftalone	Wide range	Human albumin	$2.5 \times 10^{-5}\ M$	3.85×10^4 liters/mole	1.38	183
Methadone	$0.006–2.87 \times 10^{-5}\ M$	Human plasma	—	83–87%	—	185
Morphine	$1.3–1.7 \times 10^{-7}\ M$	Human albumin	4%	31.4 ± 1.4%	—	187

also was studied. Alprenolol had about the same percentage binding and intrinsic constant for human serum albumin as propranolol; however, alprenolol was bound to a much higher extent in human serum *in vitro* (165).

Analgesic–Anti-Inflammatory Agents—Many of these agents have been examined for the intensity of their interaction with albumin or plasma. Some of these results are given in Table V.

Salicylic acid and its salts and aspirin have been the subject of many investigations, often with wide variations in results. Salicylate binding in the plasma obtained from six mammalian species was compared (166). After equalizing the protein concentration from each species, the binding was greatest in humans (72% at a drug concentration of 50 μg/ml). Salicylate bound to albumin was closely correlated with that bound to plasma in four species, including humans, but was not correlated in rats and dogs. Similar results were obtained for salicylate binding to adult plasma, but binding was progressively and perhaps significantly less in children (5–8 years) and newborns (to 5 days old) (167).

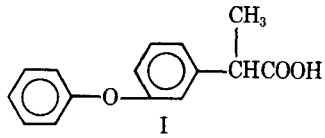
Cruze and Meyer (130), studying salicylate binding to bovine serum albumin and bovine plasma, found close agreement in the extent of binding for these two systems. Keresztes-Nagy *et al.* (168) examined the salicylate–albumin interaction by two methods and found results compatible with those of Cruze and Meyer (130). Another investigation of the salicylic acid–human albumin interaction found four strong primary binding sites exhibiting an affinity constant of about 2.19×10^5 (Table V) (169). The culmination of such *in vitro* binding experiments can be seen in a report by Levy and Yaffe (170), which pointed out the protein binding and dose-dependent change in the volume of distribution of salicylate. These data were used to explain the clinical severity of salicylate poisoning in cases of single-dose ingestion (171): once the binding capacity of available serum proteins was surpassed, the apparent volume of distribution increased and a given serum salicylate level reflected larger amounts of total salicylate in the body than at low doses. The larger volume of distribution possibly includes the central nervous system, which then may manifest toxicity.

Aspirin and salicylates also were examined from the standpoint of their effects on endogenous substances. The ingestion of aspirin caused a release of tryptophan from its serum albumin binding site (172). Subsequently, there was a decrease in bound and total serum amino acid levels, a rise in free levels of tryptophan, and an increased effect on tryptophan metabolism. Aspirin produced an increase in free 11-hydroxysteroids in an *in vitro* system employing human plasma (173). In summary, salicylate may not be highly protein bound, but the levels ingested may have some deleterious effects on the binding of concomitantly administered agents or on endogenous substances.

The interaction of indomethacin with human plasma protein was investigated at 37° using an ultrafiltration technique (174). A surprisingly high affinity constant was obtained for indomethacin (Table V), and plasma protein displayed one primary site and at least seven secondary sites for the drug. Displacement of albumin-bound indomethacin was also investigated. Hvidberg *et al.* (175) found about 90% indomethacin binding to human plasma over the therapeutic concentration range of 0.5–10 μg/ml. From these data, they calculated an association constant of $8.6 \times 10^2\ M^{-1}$ with about 15 sites present on the albumin; however, this binding constant may be somewhat suspect in relation to other indomethacin binding studies due to the fact that only 4 hr was used for the equilibrium dialysis procedure and more time to equilibrate may be required. The indomethacin–human plasma interaction also was studied using an equilibrium dialysis procedure (176). The results showed plasma binding to an extent of about 75% at a drug concentration of $2 \times 10^{-4}\ M$.

The binding of phenylbutazone, flufenamic acid, and some of their analogs to human serum albumin was investigated using a circular dichroic method (177, 178). Based upon the induced Cotton effects and solvent perturbation studies, it appears that hydrophobic interactions, along with polar binding, were important for the phenylbutazone–protein complex (177). Likewise, these forces may play a major role in the flufenamic acid–albumin interaction. The primary association constant for flufenamic acid bound to albumin appears to be very large; when less than 2 moles of drug were bound per mole of protein, no free drug apparently existed (178).

The binding of the nonsteroidal anti-inflammatory drug fenoprofen (I) and some of its analogs to human serum



I

albumin was investigated using the circular dichroic technique (179). Fenoprofen was bound nonstereospecifically to albumin as a result of hydrophobic bonding of the aromatic rings and hydrogen bonding of the carbonyl and ether oxygen. Another study (180) found an association constant of about 3×10^4 for the fenoprofen–human albumin interaction. In an investigation of competition phenomena, only the highly bound phenylbutazone, in concentrations 10 times that of fenoprofen, was able to displace fenoprofen from human serum albumin. These data supported the binding constant data of Vallner (181) for the fenoprofen–human albumin interaction. He found a primary association constant of 1.86×10^5 liters/mole at a single site on albumin, indicating that displacement by added drugs would probably require a strongly bound agent.

In a study of the relationship of the nonsteroidal anti-inflammatory agent diftalone to its interaction with human serum proteins (183), the largest association took place with the albumin fraction, and an association constant of 3.85×10^4 liters/mole was calculated (Table V). The diftalone–albumin interaction was strongly temperature dependent. It was concluded that the binding was primarily due to either van der Waals or hydrophobic forces.

Recently, morphine, heroin, and methadone have been examined with regard to their plasma and albumin binding capacity. Initially, Olsen (184, 185) found that the percent of methadone bound to albumin was independent of drug concentration but dependent on albumin concentration. He obtained a range of 8.0–43.8% bound as the albumin increased from 0.4 to 5.0 g % (184). Subsequently, Olsen (185) examined the contribution of γ-globulin to the total amount of methadone binding and found a small addition of 13–17% bound to this macromolecule fraction. At therapeutic levels in human plasma, methadone was about 85% bound (185).

Morphine and heroin binding to human serum proteins and red blood cells was examined and found to be minimal (186). The morphine–plasma protein interaction also was investigated; morphine bound to albumin and γ-globulin (187). In the therapeutic range, 34.0–37.5% of morphine was bound to human plasma, with albumin accounting for the largest binding fraction. A recent report concerned the possible production of a morphine-binding globulin in subcutaneous pellet-implanted rabbits receiving narcotic drugs (188). Opiates could form specific protein conjugates, so binding to albumin may be minimal. The possibility that immunogenic drug–protein complexes were formed deserves further study.

Antitumor Agents—A paucity of information is available concerning the plasma protein binding of antineoplastic agents. Binding information coupled with pharmacokinetic data could be exploited advantageously in the clinical cancer chemotherapy setting. In a report (189) on protein binding and renal clearance data for methotrexate, the mean protein-bound methotrexate was 70% of the serum levels (in 15 patients) over the 90–200-ng/ml range. Alterations in renal clearance of the antimetabolite were affected by concomitant administration of organic acid drugs. These alterations may have been due to altered protein binding or renal mechanism and may be useful in the clinical situation. These results (189) were confirmed in a recent study (190) employing a different technique.

Camptothecin binding to various plasma proteins as well as to whole plasma has been investigated in various species including humans (191). This antitumor agent was extensively bound in human plasma: 98.3% at a drug concentration of 30 μg/ml. It also showed a high association constant, $7.9 \times 10^6\ M^{-1}$, for human plasma albumin at a single site on the macromolecule. Albumin, however, was not the only plasma protein with significant affinity for this agent. High binding levels in the human may be one reason that significant antineoplastic effects have not been seen, whereas this agent showed marked potential in the mouse but had a low binding affinity.

The binding of the antineoplastic agents vinblastine, vincristine, and colchicine to plasma and serum proteins was examined (192). These agents adsorbed to serum proteins, with the result that vinblastine and vincristine were about 75% bound while colchicine was 50% bound. Saturation of binding could not be shown with these drugs; however, it was evident that α- and β-globulins were the more important macromolecular serum species involved in the adsorption.

A subsequent study (193) showed that, although vinblastine binding in two patients was highest in their platelet fractions, red blood cells and white blood cells also accounted for significant binding. The extent of protein binding of this agent was not so great as to prevent rapid distribution into other body organs. A study (194) of the binding of a number of antineoplastic dinitrophenylaziridines found that the five congeners studied were weakly and reversibly bound to bovine serum albumin.

Barbiturates—Most protein binding work concerning these agents was done prior to 1969. However, the influence of pH on the bovine albumin binding of barbituric acid and amobarbital was evaluated (195). At pH 4, barbituric acid had an association constant of $1.6 \times 10^3\ M^{-1}$ for albumin; at pH 2, no binding occurred. According to the report, such behavior was consistent with an ionic mechanism of binding for this agent. Similarly, with amobarbital, no binding was evident at pH 5.8 but was detected at a higher pH.

In a subsequent study (196), the binding of amobarbital, pentobarbital, and phenobarbital to human albumin was examined over the pH range of 5.7–8.4; the ionized forms of these drugs were preferentially bound, with the primary binding constant ranging from 2.0 to 6.2×10^3. In a pharmacokinetic study of pentobarbital at therapeutic levels in seven human volunteers, little of this drug was associated with plasma protein whereas tissue binding was extensive (197). The large extent of tissue binding elevated the distribution volume such that it exceeded total body water. In another patient study (198), thiopental binding was better correlated with hemoglobin than with plasma protein levels. That such a conclusion was reached was not surprising since therapeutic levels of barbiturates do not seem to have a high affinity for albumin or other plasma proteins.

Cardiovascular Agents—An excellent study concerned the binding of digitoxin and digoxin and related cardiac glycosides and genins (199). Digitoxin in the plasma was bound almost exclusively by a single site on albumin and to the extent of 97% of concentrations ranging

Table VI—Literature Results of *In Vitro* Binding of Diuretics to Human Serum Albumin

Drug	Drug Concentration, μg/ml	Albumin Concentration, %	Binding Constant or Percent Bound	Number of Sites	Reference
Chlorothiazide	5–100	0.1–4	3–3.4 × 10^4 M^{-1}	1 or 2	208
Hydroflumethiazide	—	5	74%	—	209
Bendroflumethiazide	—	5	94%	—	209
Furosemide	3.4	5	97.2%	—	212

as high as 12 μg/ml. An association constant of 9.62 × 10^4 liters/mole at 37° was calculated. The digitoxin–albumin interaction was endothermic with a gain in enthalpy of 3.5 kcal/mole, a free energy change of −7.06 kcal/mole, and an entropy change of 33.8 cal/mole/°K. These results were suggestive of a hydrophobic mechanism between digitoxin and albumin. In contrast to digitoxin, only 23% of digoxin bound to albumin at concentrations up to 2 μg/ml. The binding differences for these two closely related drugs are reflected by higher plasma levels, lower urinary excretion rates, and a longer half-life of digitoxin compared to digoxin when these agents are administered to humans.

In other studies of digitoxin (200) and digoxin (201) binding to human serum proteins, digitoxin had a large association constant for albumin (K = 1–5 × 10^5 liters/mole), but the number of sites calculated was 0.5 in contrast to the 1.0 site seen previously. The binding was observed to be pH dependent; maximum binding occurred at pH 4.8. This finding might explain the somewhat lower binding constant observed by Lukas and DeMartino (199) who carried out their work at pH 7.4. For the digoxin–albumin interaction, Brock (201) found that increases in pH from 5 to 9 decreased the association constant from 8 × 10^2 to 1 × 10^2 liters/mole at 37°. The binding of these same two drugs was examined in a wide variety of mammalian species, and some substantial binding differences were noted, especially in the less tightly bound digoxin (202).

Two other studies (203, 204) on digoxin binding reported results contradictory to those given above. One study (203) found a total lack of protein binding of digoxin in human serum, and the other (204) showed that digoxin was about 30% bound in human serum using a wide range of digoxin concentrations. The calculated association constant was 6.8 × 10^4 liters/mole at an infinite number of digoxin sites on the albumin molecule (204). Seemingly, the binding constant calculated was larger than expected based on results of other studies and the low percentage binding in the plasma.

The antiarrhythmic drug quinidine was bound to albumin at two different classes of sites, and the primary binding affinity was 2.06 × 10^4 M^{-1} (205). Another study also examined the binding of quinidine to human plasma and human albumin (206). At a concentration of 1.6 × 10^{-5} M quinidine, 74% of the drug was bound to plasma and 71% to albumin. Like the previous study, two classes of sites on albumin were found, and the primary binding affinity was calculated to be 1.28 × 10^4. In the same study, binding of the new antiarrhythmic disopyramide had a lower binding affinity (4.6 × 10^3). A computer program was used to predict free and bound levels of quinidine, based on binding to albumin at two sets of sites (209). The analysis was limited to drug concentrations that may be obtained clinically (1–10 μg/ml). Over this concentration range, 74–88% of the drug would be predicted to be bound to serum albumin.

Diuretics—Relatively little work has been done concerning the binding of diuretic drugs to serum albumin since the Meyer and Guttman (1) review. A study of the binding of chlorothiazide to plasma proteins found that plasma binding was due almost entirely to the albumin fraction (208). At 37° with a 0.4% albumin solution and a drug concentration of 5–100 μg/ml, a maximum of 68% of chlorothiazide was bound; larger percentages of binding occurred at lower temperatures. Other binding data for diuretic agents are given in Table VI. In addition, it was found that the chlorothiazide–albumin interaction was accompanied by a negative entropy change and a relatively large change in enthalpy (208). It was suggested that the interactive force was due to nonionic bond formation.

Agren and Back (209), examining the binding of hydroflumethiazide and bendroflumethiazide to human serum albumin, found the primary binding affinity for hydroflumethiazide to be 1.8 × 10^3. For bendroflumethiazide, the affinity constant was 2.6 × 10^5 when the drug was ionized to 6.5% (at pH 7.35) and about 50 times less if 0.7% ionized. It was concluded that bendroflumethiazide was bound primarily by ionic bond formation. Another study (210) mentioned that bumetamide was 95–97% bound to human plasma protein *in vitro*.

Furosemide binding has been studied both *in vitro* and *in vivo* in cardiac patients and normal volunteers. In these individuals, the diuretic was bound exclusively to plasma albumin; at concentrations of furosemide from 10 to 400 μg/ml, 99.1–95.8% of the drug was bound (211). Similar results were observed using therapeutic concentrations of furosemide (212); phenytoin, phenobarbital, diazoxide, trifluoperazine, or chloramphenicol did not displace any furosemide.

Hypoglycemics—The natural hypoglycemic agent insulin has been studied in the serum of normal and diabetic patients. With a gel permeation method, addition of iodine-labeled insulin to normal human serum showed a binding of 4.3% if analyzed immediately and 5.4% if incubated for 24 hr (213). In contrast, these values were 37.7 and 66.9%, respectively, in insulin-treated diabetics. Another study (214) found that the binding of insulin to macromolecular serum proteins was about 10 times higher in the diabetic than the normal individual.

An investigation of the binding of acetohexamide, chlorpropamide, and tolbutamide to human serum proteins showed that the albumin fraction was responsible for the greatest binding (215). At pH 7.4, the primary binding affinities were 3.4 × 10^4, 1.1 × 10^4, and 4.1 × 10^4 for acetohexamide, chlorpropamide, and tolbutamide, respectively. The interaction of a number of drugs with the sulfonylurea–albumin complex was examined for each of these agents. Sulfaphenazole appeared to be a potent in-

Table VII—Reported Sulfonylurea–Albumin Binding Parameters

Drug	Drug Concentration, M	Albumin Source	Albumin Concentration	Primary Binding Constant	Number of Sites	Reference
Tolbutamide	$2–84 \times 10^{-5}$	Human serum	2.9×10^{-4} M	4.06×10^{4} M^{-1}	1.36	215
Tolbutamide	—	Human serum	1%	2.18×10^{5} liters/mole	2.27	216
Tolbutamide	$10^{-6}–10^{-3}$	Human serum	1×10^{-4} M	1.35×10^{5} M^{-1}	—	217
Tolbutamide	—	Human serum	$0.25–1.0 \times 10^{-4}$ M	1.65×10^{5} M^{-1}	—	221
Chlorpropamide	$6–84 \times 10^{-5}$	Human serum	2.9×10^{-4} M	1.09×10^{4} M^{-1}	1.64	215
Chlorpropamide	—	Human serum	1%	4.51×10^{4} liters/mole	2.19	216
Chlorpropamide	$10^{-6}–10^{-3}$	Human serum	1×10^{-4} M	6.80×10^{4} M^{-1}	—	217
Acetohexamide	$2–81 \times 10^{-5}$	Human serum	2.9×10^{-4} M	3.39×10^{4} M^{-1}	1.39	215
Acetohexamide	—	Bovine serum	$0.25–1.0 \times 10^{-4}$ M	1.60×10^{4} M^{-1}	—	221
Carbutamide	$10^{-6}–10^{-3}$	Human serum	1×10^{-4} M	3.75×10^{4} M^{-1}	—	217
Glipizide	—	Bovine serum	1.38×10^{-6} M	$2.45–6.88 \times 10^{4}$ M^{-1}	—	220
Glipizide	—	Bovine serum	$0.25–1.0 \times 10^{-4}$ M	4.90×10^{4} M^{-1}	—	221
Glipizide	—	—[a]	—	92–99% bound	—	219
Glyburide	—	Bovine serum	1.38×10^{-6} M	3.26×10^{5} M^{-1}	—	220
Glyburide	—	Bovine serum	$0.25–1.0 \times 10^{-4}$ M	6.13×10^{4} M^{-1}	—	221

[a] In this report, human plasma was used as the biomacromolecule.

hibitor of the sulfonylurea–albumin binding while aspirin had little displacing ability. In contrast to these data (215), substantial differences were reported in the binding of tolbutamide and chlorpropamide (216). This study demonstrated that an interaction between buffer contents and serum albumin may have had significant effects upon the binding parameters calculated by Judis (215).

In 1% human serum albumin using phosphate buffer and tromethamine buffer, the numbers of primary binding sites were 2.27 and 1.42 and the primary affinity constants were 21.86×10^4 and 0.97×10^4 liters/mole, respectively (216). It appears important to characterize binding at several buffer or protein concentrations and perhaps employ more than a single buffer system to calculate more meaningful constants. Various other studies using the same sulfonylureas, tolbutamide, chlorpropamide, and acetohexamide, and newer agents, such as glyburide and glipizide, have appeared (217–222). These results are given in Table VII.

Hsu *et al.* (220) reported that the binding of glipizide and glyburide to human and bovine serum albumin was fundamentally different than the other sulfonylureas. Using a fluorescent probe study, they found a difference in competition for the probe binding site between glipizide and glyburide, which interacted with one of their probes, and the other sulfonylureas, which interacted with a probe with which glipizide and glyburide did not. In their opinion, this probe-specific interaction differentiated the binding sites. Zia and Price (221), on the other hand, showed that acetohexamide, glipizide, glyburide, and tolbutamide bound at the same or closely located sites on the protein.

An evaluation was made recently of the nature of the molecular interaction between sulfonylureas and serum albumin using a circular dichroic technique (222). The sulfonylurea–albumin complex failed to generate a specific conformation among these various agents. This finding could be interpreted in terms of a dissimilar mode of binding for these agents. The binding of the biguanides metformin, buformin, and phenformin to human and dog whole blood and plasma was studied (223). Relatively small percentages (7.4–12%) of these agents were bound in human plasma at drug concentrations from 0 to 3×10^{-4} M.

Local Anesthetics—The binding of the anilide-type local anesthetic agents lidocaine, mepivacaine, and bupivacaine was reported (224). The binding of these agents was evaluated in two patients at plasma drug concentrations of 0.4–23.3 μg/ml. Binding of bupivacaine was greatest (96.5–61.5%), mepivacaine was less (84.3–31.5%), and lidocaine was least (75.0–28.2%). At levels greater than 5.0–10.0 μg/ml of any of these agents, there was a marked reduction in binding, presumably due to binding site saturation. Identification of the main plasma binding macromolecule for these basic drugs was not successful; however, albumin was probably not the protein of greatest importance. A subsequent paper reported that differences between the plasma binding of these three agents may partly explain observed differences between their umbilical–maternal concentrations (225).

The results of another study (226) examining the interaction of bupivacaine with human plasma proteins are in close agreement with those of Tucker *et al.* (224). It was found (226) that at bupivacaine levels of 1 μg/ml, the mean unbound fraction of drug in 10 healthy volunteers was 6.3%. In a study of lidocaine disposition kinetics (227), 60.8% of the drug was bound over the plasma concentration range of 0.8–4.1 μg/ml. A rapid bolus injection of lidocaine and its seemingly rapid saturation of binding sites may be important in determining the immediate toxicity of this drug (227). The suggestion of administering this drug over 1–2 min may be important in decreasing toxic symptoms.

Psychopharmacological Agents—Many reports concerning the binding of tricyclic and benzodiazepine derivatives to human plasma and albumin have been published since 1969. An ultrafiltration technique was used to examine the binding of various tricyclic antidepressants to human plasma proteins (228). At a total concentration of 0.29 μg/ml, the percentage of unbound desipramine (desmethylimipramine) was 9.5 ± 1.4 in 41 individuals; this degree of binding was relatively constant over a wide drug concentration range. Other tricyclics studied at 1.1 μM concentrations had the following percentages of unbound drug: nortriptyline, 5.5 ± 0.6; amitriptyline, 3.6 ± 0.8; and protriptyline, 8.0 ± 0.6. The *in vitro* percentage of unbound nortriptyline correlated well with *in vivo* measurements of free drug concentration in cerebrospinal fluid. The protein responsible for the greatest amount of binding in the plasma was not determined.

Imipramine interacted with bovine serum albumin at six equivalent binding sites, possibly involving tyrosyl

Table VIII—Reported Tricyclic Antidepressant–Protein Binding Parameters

Drug	Drug Concentration, M	Protein	Protein Concentration, M	Primary Binding Constant	Number of Sites	Reference
Chlorpromazine	—	Human albumin	1.8×10^{-5}	4.2×10^{4} liters/mole	4	7
Trifluopromazine	$0.365–14.6 \times 10^{-5}$	Human albumin	1.8×10^{5}	5.5×10^{4} liters/mole	4	7
Chlorpromazine	$40–1600 \times 10^{-6}$	Human plasma	—	$1.5 \times 10^{4}\ M^{-1}$	—	236
Chlorpromazine	$40–1600 \times 10^{-6}$	Human albumin	—	$1.3 \times 10^{3}\ M^{-1}$	—	236
Imipramine	$40–1600 \times 10^{-6}$	Human plasma	—	$1 \times 10^{4}\ M^{-1}$	—	236
Imipramine	$40–1600 \times 10^{-6}$	Human albumin	—	$4.9 \times 10^{2}\ M^{-1}$	—	236
Chlorpromazine	$1–20 \times 10^{-6}$	Human albumin	6.8×10^{6}	1.9×10^{5} liters/mole	1.94	237
Promazine	$1–20 \times 10^{-6}$	Human albumin	5.8×10^{-6}	8.5×10^{4} liters/mole	1.31	237
Promethazine	$1–20 \times 10^{-6}$	Human albumin	5.8×10^{-6}	7.9×10^{4} liters/mole	1.64	237
Trifluoperazine	$1–20 \times 10^{-6}$	Human albumin	5.8×10^{-6}	2.86×10^{5} liters/mole	1.18	237
Imipramine	$1–20 \times 10^{-6}$	Human albumin	5.8×10^{-6}	2.39×10^{4} liters/mole	1.31	237
Trimipramine	$1–20 \times 10^{-6}$	Human albumin	5.8×10^{-6}	2.38×10^{4} liters/mole	1.24	237
Clomipramine	$1–20 \times 10^{-6}$	Human albumin	5.8×10^{-6}	7.35×10^{4} liters/mole	1.03	237
Desipramine	$1–20 \times 10^{-6}$	Human albumin	5.8×10^{-6}	7.02×10^{4} liters/mole	1.53	237
Chlorpromazine	4.6×10^{-5}, 15×10^{-4}	Bovine albumin	6.9×10^{-5}	$2.57 \times 10^{4}\ M^{-1}$	—	221
Trifluoperazine	4.6×10^{-5}, 15×10^{-4}	Bovine albumin	6.9×10^{-5}	$2.32 \times 10^{4}\ M^{-1}$	—	221
Perphenazine	4.6×10^{-5}, 15×10^{-4}	Bovine albumin	6.9×10^{-5}	$1.96 \times 10^{4}\ M^{-1}$	—	221
Fluphenazine	4.6×10^{-5}, 15×10^{-4}	Bovine albumin	6.9×10^{-5}	$1.82 \times 10^{4}\ M^{-1}$	—	221
Promazine	4.6×10^{-5}, 15×10^{-4}	Bovine albumin	6.9×10^{-5}	$1.04 \times 10^{4}\ M^{-1}$	—	221

residues of the protein, with an intrinsic association constant of $5 \times 10^{3}\ M^{-1}$ (229). The related tricyclic desipramine exhibited a more complicated binding mechanism, perhaps involving a drug-induced conformational change. From a study of nortriptyline binding in seven identical and 10 fraternal sets of twins, Alexanderson and Borga (230) concluded that drug binding was influenced by environmental, that is, statistically significant differences in the binding ratio within monozygotic twin pairs, as well as genetic factors. They found no significant correlation between the bound to free nortriptyline ratio and the steady-state drug concentration.

Another study (231) showed that there was far less variation in plasma protein binding of imipramine among children (bound fraction 77–94.6%) (92) than among adults and that children bound substantially less of this drug. Adult-like binding values may be reached at about age 13, and the relatively high doses of imipramine administered at bedtime to children with behavior disorders may be unsound from a binding–pharmacokinetic standpoint.

In a study relating the plasma protein binding of chlorpromazine to the apparent volume of distribution and the rate constant of elimination, chlorpromazine plasma binding influenced tissue localization; a high degree of tissue localization occurred in the species in which plasma protein binding was low and vice versa (232). It was also shown that over 90% of chlorpromazine in human plasma was present in bound form (233). The binding was reversible, and very large amounts of chlorpromazine, 15 μg/ml of plasma or more, were required to saturate the plasma binding sites.

One study (234) found that the apparent binding constant for chlorpromazine was 1355 with human albumin and 1545 with a human α-globulin fraction; although each of these proteins demonstrated a large number of binding sites for the drug, the results indicated that a different plasma protein fraction may display a larger affinity. Mao and Noval (235) suggested that the serum protein binding of the phenothiazines might explain the low *in vivo* hemolytic activity found for these agents. Administration of large amounts of tricyclics over extended times showed that erythrocytes were protected from hemolysis due to the plasma protein binding of these agents.

Another study determined the groups or positions on the phenothiazines responsible for drug–albumin complexation (7). The whole phenothiazine nucleus takes part in the binding process; predominantly hydrophobic forces are involved, but ionic interactions must not be completely ignored. The binding parameters are included in Table VIII.

Chlorpromazine and imipramine were bound to three major blood components: erythrocyte membranes, albumin, and lipoproteins (236). The affinity displayed by lipoprotein for these drugs was as high as that of albumin. The binding of various tranquilizer–antidepressant drugs to human albumin was examined (237) (Table VIII). A drug displacement study of these drugs found that phenothiazines displace iminodibenzyl compounds but not vice versa, since the affinities of the phenothiazines for the albumin are greater than the affinities of the iminodibenzyl agents.

In a subsequent study (238), the binding of tricyclic compounds to human albumin was correlated with linear free energy models. The major force in the binding of these molecules was electronic, and only a minor contribution of hydrophobic forces was involved. To the contrary, a previous report (221) stated that the interaction of phenothiazine drugs with bovine serum albumin was of a predominantly hydrophobic nature. Another study (239) also showed that the binding of phenothiazines to bovine serum albumin was of a hydrophobic nature. Recently, excellent evidence was presented that hydrophobic forces are more responsible for the phenothiazine–albumin interaction than are charge transfer or electronic forces (240).

Dialysis of plasma from 14 patients receiving diazepam (0.1 mg/kg) showed that plasma bound 97.7% of this benzodiazepine (241). Similarly, in seven patients the plasma proteins bound 98% of the diazepam in the serum from a 10-mg im dose (242). Diazepam was among 11 benzodiazepine derivatives quantitatively studied for binding to bovine serum albumin (243). Diazepam had a binding constant of 8.23×10^{3} liters/mole with 5.56 binding sites on bovine albumin. Binding constants calculated for the 11 benzodiazepines demonstrated that their binding affinities for bovine albumin were smaller than for human

albumin. Furthermore, these agents have more than the characteristic one or two binding sites on bovine albumin than would be expected on human albumin.

Some benzodiazepines were shown to be competitors for the binding of L-tryptophan to human serum albumin (244). There was a single highly stereospecific site on human albumin which bound L-tryptophan and with which the benzodiazepines competed. Lucek and Coutinho (245) presented results showing that the protein binding of benzodiazepines increased with increases in the lipophilic character of substituents in the 1-, 2-, 3-, 4-, 7-, and 4′-positions. The electronic character of 1-, 3-, and 7-substituents also correlated with protein binding but to a lesser degree than the lipophilic parameter.

Amphetamine binding was studied *in vitro* in different species, including humans (246), and was less than 45% in all species in the drug concentration range of 2.5×10^{-7}–4×10^{-6} *M*. Drug diffusion to cerebrospinal and ocular fluids was not retarded by the binding. An *in vitro* investigation of bovine serum albumin and amphetamine and one of its derivatives (247) arrived at similar results (246). Amphetamines appeared to be bound primarily to the albumin fraction of plasma. However, Δ^1-tetrahydrocannabinol was 80–95% bound to lipoprotein fractions *in vitro,* and no binding to albumin could be demonstrated (248).

Steroids and Catecholamines—Most steroid hormones are bound to hormone-specific macromolecules such as various binding globulins. However, some literature reports have concerned steroid–albumin binding. The protein binding of 10 steroids was studied in women with benign breast disease, early breast cancer, and advanced breast cancer and was compared to the binding in normal women (249). The data showed that the percentage binding in the plasma varied considerably between steroids. However, for a given steroid, there was no significant difference in the mean amount of steroid bound among these groups of women.

An investigation of progesterone binding to different polymeric fractions of human serum albumin found similar parameters in the mono- and dimeric species; two independent sets of binding sites were determined (250). In the first class, there was one site with an affinity of 3.6×10^5 M^{-1}; in the second class, there were eight sites with an affinity of 6×10^3 M^{-1}. The trimeric and higher forms had significantly lower binding affinities. Clark and Bird (251) examined the binding of 5α-androstane-3α,17β-diol, an important biologically active metabolite of testosterone, to human plasma proteins. They found that 96.6% of the drug was bound in male plasma and that the albumin fraction contained the greatest amount of the steroid. Albumin bound more of this steroid than the parent testosterone, and the authors concluded that this result was due to a higher association constant for the metabolic dihydroxysteroid.

Estriol and some of its conjugates were studied for their binding to plasma proteins during pregnancy (252). These compounds were bound principally in the albumin fraction, and such binding was stated to play an important role in the transport, metabolism, and excretion of these steroidal agents. Although albumin has been reported to be the main binding protein for some steroid hormones and related molecules, other results suggest that the globulin fraction may be principally responsible (253, 254). Ryan and Gibbs (255) found that Ca^{2+} was bound to human serum albumin in direct competition with testosterone and that such ion binding was associated with inhibition of testosterone binding to hydrophobic albumin sites but not with inhibition to sites involving hydrogen bonding.

Recently, several studies were published concerning the binding of catecholamine hormones to plasma proteins. Two distinct plasma binding sites were found for epinephrine; the binding was highly specific, similar to that reported for steroids (256). The first site was present in small numbers and had a large binding constant (6.9×10^5 liters/mole). The second site was more plentiful and had a smaller binding affinity. The enthalpy associated with the epinephrine–plasma protein interaction was small, because the activation energies for association and dissociation were essentially equal.

Both epinephrine and norepinephrine binding to human serum albumin was studied, and a similar binding affinity of approximately 10^7 was observed (257). Other catechols were studied, and neither the nature of the alkyl side chain nor it presence affected the binding affinity. It was also determined that 50% of a physiologic concentration of norepinephrine circulates bound to plasma protein. In apparent contrast to these results, Zia *et al.* (258) reported that the most probable binding site on epinephrine, when bound to bovine albumin, was the alkyl side chain. A possible reason for such a conclusion was that the concentrations of epinephrine used were high. Therefore, Zia *et al.* (258) were examining nonspecific binding in contrast to Danon and Sapira (257). Another report (259) implicated the phenolic hydroxyl groups as well as the alkyl side chain of catecholamines as being responsible for the binding interaction. In a recent study (260), an excellent discussion of the binding observed (257–259) in human serum was given. Several serum macromolecules were implicated as being responsible for catecholamine binding, and such binding may be responsible for the stability of these agents in blood.

Miscellaneous—The interaction of the antimalarial agent pamaquine with bovine albumin was examined (261), and three pamaquine molecules were bound per molecule of protein; the primary binding affinity was very strong, 6.4×10^7. Because the drug was positively charged and the binding constant was large, the binding site on the protein probably involved negatively charged groups. The binding of dapsone, a drug important in the treatment of leprosy, and its principal metabolite, monoacetyldapsone, was studied *in vitro* and *in vivo* in patients known to be rapid acetylators of the drug and in patients metabolizing the agent normally (262). At therapeutic concentrations of dapsone, 70–80% was bound to plasma protein while 90–100% of the monoacetyldapsone was bound. No difference in binding was seen in the plasma of rapid and normal acetylators. The results suggested that protein binding of monoacetyldapsone may account for limited excretion of this compound in the urine and the long plasma half-lives of these agents.

The binding of the antiprotozoal agent metronidazole and four derivatives to plasma proteins was assessed (263). The nature of the alkyl side chain had a significant effect on binding; however, the lipophilicity of these agents was not correlated with plasma protein binding. The parent compound showed good lipophilic character, as did one of

the metabolites, yet these compounds were bound to very small extents (less than 5% over a wide concentration range). A metabolite with poor ability to partition exhibited extensive binding (65–70%), and the binding was principally related to the frontier electron density on the terminal portion of the alkyl side chain. Recently, a good correlation was observed between lipophilicity in a series of related drugs, measured by a reversed-phase thin-layer technique, and the drug binding constants (264). Seemingly, if hydrophobic forces are responsible for the binding interaction, good correlation between partition coefficient and protein binding should be observed. The binding of therapeutic levels of ornidazole and metronidazole to human plasma proteins was less than 15% (265), and this binding was not correlated with the lipophilicity of these agents.

The binding of dantrolene sodium to human serum albumin recently was reported (266). The binding constant, calculated over a wide range of drug concentrations by two different techniques, was 4×10^4–$4 \times 10^5\ M^{-1}$. Ionic as well as hydrophobic forces were responsible for the interaction. Dantrolene added to a cationic surfactant produced perturbations similar to the drug added to human albumin solutions. Albumin also was shown to be the plasma protein most responsible for the binding of prostaglandins (267, 268). The binding of prostaglandins A_2, E_2 (dinoprostone), and F_2 to human plasma was reported to be 88, 73, and 58%, respectively, over the 10–120-ng/ml concentration range (267). The apparent association constants were $1–4 \times 10^4$ liters/mole. Another report (268) also indicated that albumin was the plasma protein principally responsible for binding prostaglandin E_1 and that no E_1 interaction with blood cells or γ-globulin occurred.

Conclusions—The binding of drugs by plasma proteins in general and isolated specific proteins, such as serum albumin and various lipoproteins and globulins, has continued to interest many researchers. Binding parameters (n's and K's), percentages bound, and possible displacement effects may be the clinically important contributions of this research and may be beneficial to dosage adjustment and prediction of the duration of drug action. From a physical standpoint, drug–protein interactions are studied to gain information about denaturation or unfolding of the protein, to determine cooperative interactions and mechanisms, to examine saturation and the total number of binding sites on the protein, and to examine molecular phenomena such as the active site, molecular conformation, or molecular distance involved in the drug–protein complex.

The vast majority of articles reviewed here were *in vitro* investigations and provided quantitative binding data. However, much of the work was of a more qualitative nature, since data were presented in terms of percent binding without specification of the drug concentration to which the binding data pertain; at times, drug concentrations were higher than therapeutic or toxic concentrations expected in the *in vivo* situation. Often plasma was considered by itself as the binding biomacromolecule without an attempt to define the primary protein component responsible for the binding interaction. Seldom were *in vitro–in vivo* correlations of binding attempted. Many results failed to furnish the reader with the temperature of the binding study, and it is well known that less binding is found at 37° than at ambient temperatures. Much of the binding literature could serve as a useful source of information to the clinician, pharmacokineticist, and other interested pharmaceutical researchers if the data presentation, data reduction, and use of multiple methods to support conclusions could be uniformly imposed on new articles pertaining to drug binding results.

Considerable research attention in the area of drug–protein binding could be directed at one or more of the following points, as well as inclusion of the calculated binding parameters: tissue binding, complexation of drugs by red cells and blood vessels, binding effects on pharmacokinetic models and parameters, protein active sites, and other molecular events associated with binding phenomena, displacement interactions, especially in the *in vivo* situation, and binding changes that may alter the course or effects of therapy associated with specific disease states. It is quite evident that strongly bound drugs influence drug effects and drug dynamics, and new drugs ought to be assessed as to whether or not they fit in the category of strongly bound agents.

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