THE INTERACTIONS OF DRUGS AND PLASMA PROTEINS*

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INTRODUCTION. The capacity of drug molecules to enter into specific combinations with proteins poses for pharmacology its most fundamental task—to comprehend the intimate nature of drug action in terms of these molecular interactions. The great contributions of Clark and of Ehrlich, in their time, shifted the focus from organism to cell. But modern advances in enzymology, protein chemistry, microbiology and electron microscopy have made the cell appear gross and complex. Modern pharmacology, of necessity, has also come to reckon in Ångstrom units and to approach its problems at the level where drug molecules exert their peculiar effects upon the molecular structure and function of living protoplasm.

Most drugs have been established, or can be surmised, to act by virtue of combination with specialized functional proteins.[1] To these proteins the vague term "receptors" has been assigned in the past, but their precise natures and roles are finally being clarified. Ehrlich (79, 80), on the basis of his observations of dye affinity for tissue proteins and the remarkable specificities of antibody-antigen reactions, proposed that chemical combination of drugs with tissue constituents was the *sine qua non* of pharmacological action. "Corpora non agunt nisi fixata." As he had hoped, the field of chemotherapy now provides most eloquent confirmation of this concept. The "chemoreceptors" of parasitic organisms prove to be protein enzymes, vital links in a metabolic chain, exquisitely sensitive to chemotherapeutic agents of precise specificity. Thus the mode of action of all chemotherapeutic agents is being investigated currently at the molecular level. In the field of general pharmacology the cholinesterase inhibitors are outstanding examples of drugs whose actions are now understood in terms of specific interaction with a protein enzyme playing a special role in certain highly localized regions. The vitamins, on the other hand, function as prosthetic groups to widely distributed enzymes. Heparin owes its characteristic effect to interference with a complex system of interacting proteins in the plasma and the evidence points toward a specific interaction with one or more of these proteins. Can it be seriously doubted that the barbiturates, the salicylates, morphine and its congeners, the cardiac glycosides, the curare compounds, the local anesthetics, the autonomic drugs will also prove to enter into definite specialized relationships with particular tissue proteins?

Curiously, the specificity of drug-protein interactions is often more apparent than real. Drugs are in fact capable of forming a multitude of alliances with

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[1] There are certain obvious, if trivial, exceptions, *e.g.*, saline cathartics and diuretics.

the proteins of the body, some reversible and transient, others firm and persistent. The key fits many locks but there is only one door! That single combination which is responsible for the "primary drug action" forces itself upon our attention. If certain other interactions also lead to obvious physiological changes we note that a "side effect" has occurred, and if such changes are detrimental we apply the term "toxic effect." But the great bulk of drug molecules may enter into "silent" combinations which, from the standpoint of therapeutic effect, seem irrelevant. An example is the fixation of quinacrine to the liver, where its concentration may be a thousand times that in the parasitized erythrocytes which are the sites of therapeutic action (163).

Among these numerous secondary interactions are those in which the plasma proteins participate. Are these in any sense unique? Bennhold (11), whose many accomplishments in this field will be reviewed in later sections, was led by his observations to the view that the plasma proteins constitute a specially designed transport mechanism for the regulated distribution of naturally occurring and medicinal substances throughout the body. "Blut ist die flüssige Transportform des Gewebes im tierischen Organismus." Whether one accepts the teleological overtones of Bennhold's formulations one must recognize that the plasma proteins are indeed endowed with remarkable reactivity, that associations of the most varied kind occur with the most diverse of chemical substances, and that the sojourn of such molecules in the body may be profoundly influenced by these interactions. Whether the proteins of other tissues are equally versatile in their reactivities cannot be established at present. Indeed, the difficulties inherent in studying interactions with the proteins of fixed tissues present a sharp contrast to the readiness with which those of the plasma lend themselves to investigation. Furthermore, the remarkable progress of recent years in the isolation and characterization of plasma proteins has provided considerable impetus to the study of these rather than other interactions. A major influence in this direction has been the large scale program of plasma fractionation through which investigators have been able to obtain standardized human plasma protein preparations of known homogeneity (47, 48).

Consequently a resurgence of interest in drug-protein interactions is apparent among pharmacologists today, further stimulated perhaps by the demonstration that in the clinical use of the sulfonamides and penicillins these phenomena are of some practical importance. Interactions have, in fact, been the subject of perennial investigation, for over half a century, by pharmacologists, physiologists and chemists alike. But much of this work, in its application to the living organism, was purely empirical in concept and execution and inconclusive in result. Lacking a clearly formulated theoretical orientation, it led as often to greater confusion as to greater clarity.

It will be the aim of this review not only to present a factual summary of some of the most significant studies on interactions but also to set forth the author's concept of a rational approach to the problem from the standpoint of pharmacology. A systematic collection of the pertinent data obtained in numerous interaction studies will be presented in tabular form, but the work of various

investigators will be cited in the text only when it bears directly upon the topic under discussion. There will also be no attempt to exclude interacting compounds other than drugs when their consideration seems appropriate.

METHODS OF DEMONSTRATING INTERACTION. At least sixteen different methods have been employed to demonstrate the presence of an interaction between drug and protein molecules but these rest upon only three basic principles:

(1) The concentration of free drug and its thermodynamic activity are reduced, and its biological action may also be diminished. Whether such effects are measurable depend upon their magnitude, and upon the sensitivity of the methods employed.

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

(3) The protein component may be measurably altered with respect to its properties or behavior.

The several methods based upon each of these principles will be discussed, showing for each the kind of results that can be obtained, the conditions which must be observed, and the conclusions that can safely be drawn.

1. *Methods based upon reduction in free drug concentration.*

a. *Biological action.* The observation that serum is capable of interfering with the action of many drugs has often served as initial stimulus to the study of drug-protein interactions. Busck (33), as early as 1906, reported on the inhibitory effect of serum on the photodynamic action and other toxic effects of certain dyes, using paramecia as test organisms. He showed that serum not only interferes with dye action but also alters diffusibility, fluorescence or light absorption, solubility, and other properties. These phenomena he attributed to the formation of dye-albumin complexes.

Storm van Leeuwen and his collaborators (245, 246) discovered that rabbit serum inhibits the action of pilocarpine and atropine on the isolated gut of the cat; serum alone produced no effect. The alkaloids are not destroyed, as evidenced by their complete recovery on alcohol denaturation of the serum proteins.

Reduction of biological activity on the frog heart was used by McLean and Hastings (169) (after the technique introduced by Trendelenburg (263)) as the basis of their classical quantitation of the serum binding of calcium. A similar quantitative analysis is embodied in the studies of Fawaz and Farah (86), and of Farah (85), on cardiac glycosides, using systolic arrest of the frog heart for the assay of free drug concentration in the presence and absence of various serum protein fractions.

More recently the inhibitory effect of albumin on the hemolytic action of fatty acids has been employed as an investigative tool by Boyer, Ballou and Luck (24). Davis and Dubos (56, 58) studied the abolition by albumin of the antibacterial action of oleic acid on the tubercle bacillus, and the binding of diverse chemotherapeutic agents has been examined in similar fashion.

This method is fundamentally sound from the pharmacological standpoint

provided the drug concentrations employed are within the range to be expected *in vivo*, and provided the protein concentration has not been artifically altered (*e.g.*, by dilution). If these provisions have been met one can then expect to detect an interaction *if an appreciable fraction* of the total drug is bound to protein at the concentration investigated. As bioassay methods are crude at best it is probably safe to assume that unless the unbound drug concentration is reduced by one-quarter to one-half, reliable results will not be obtained. Furthermore the conditions must be such that the binding is not disturbed by the determination itself. For example, if a cardiac glycoside were three-quarters bound to plasma protein in a loose, reversible combination, and the affinity of the drug for cardiac muscle were greater than for plasma protein, *all* the drug might be rapidly removed from combination and it would appear that none had been bound. Again, in the bioassay of penicillin, preliminary dilution in a protein-free solution results in rapid dissociation of the bound drug so that the original binding is no longer evident in the assay. Therefore while reduction of biological effect as a criterion of interaction may yield extremely useful information, especially if positive results are obtained, interpretations must be extremely cautious when "no binding" is found. The possibility of direct effect of the protein upon the biological assay material must, of course, be ruled out.

b. *Dialysis.* Confining the protein component within a semi-permeable membrane through which unbound drug molecules can freely diffuse is one of the oldest and most direct methods of demonstrating interaction (184). At equilibrium the unbound drug activities on both sides of the membrane must be equal and any increment in the protein compartment is presumed to represent bound drug. Outstanding recent applications of this technique are the studies of Klotz (138, 140, 141) on the binding of anionic dyes and purine derivatives. Provided the membrane (usually cellophane sausage casing) is proved to be impermeable to protein and permeable to the drug in question, and that equilibrium is actually attained within the period of the experiment, the accuracy of the method is determined wholly by that of the procedure for ascertaining the drug concentration. This need not require simultaneous determinations in the aqueous and protein solutions for a method of differences can be employed, on the assumption that the observed loss of drug from the protein-free dialysate represents drug in combination with protein (138). However, inaccuracies may result from irregular adsorption to the dialysis bag itself. In the author's experience with methylene blue, such loss may not only be large (up to 20% of the total dye at low concentrations) but variable from bag to bag. The Donnan effect must also be considered and appropriate corrections made in the case of ionic drugs, especially when the total ionic strength is low. Indeed the same corrections apply to all methods wherein a portion of the aqueous phase is separated from the protein regardless of the presence of a physical membrane.

Bendien and Snapper (9, 10) employed a technique of differential dialysis, the pores being of such size that albumin molecules passed through but larger proteins did not. This method is unwieldy and unpredictable, and offers no real advantage over other currently available techniques.

Dialysis is eminently suited for quantitative studies, especially when an inter-

action is to be explored through a range of drug concentrations. In this way nearly complete saturation of a protein with a given drug can often be achieved and information of prime physico-chemical importance derived. It is one of the few methods conducive to such quantitative work and it is thermodynamically sound. On the other hand it does not yield accurate information about the status of an interaction in the plasma *in vivo*. Even if whole plasma containing the substance under study is dialyzed, physiological conditions will no longer obtain because of the dilution of the water phase. If the drug-protein combination is reversible, dissociation may occur and neither the amount bound per mole protein nor the fraction of total drug bound will necessarily reflect the state of affairs before the equilibrium was disturbed.

c. *Ultrafiltration.* This method (6, 8) differs from dialysis in that the water phase is expressed through a semipermeable membrane by pressure and there is consequently no dilution of the system. The technique is appealing in its simplicity and various types of apparatus have been described (51, 147, 159, 234). It probably approximates most closely the process of glomerular filtration since the hydrostatic pressures employed are usually of a magnitude comparable to the renal filtration pressure.

Bennhold (11) suggests that the lability of a drug-protein bond is somehow connected with ultrafiltrability, that bonds which are not disrupted under milder conditions (electrophoresis, diffusion) may be broken by the pressure under which ultrafiltration is carried out. In support of this view he cites instances of apparently complete binding in diffusion and electrophoresis experiments of substances which nevertheless appear in an ultrafiltrate. If this view were correct ultrafiltration would yield little valid information about reversible reactions for it presumes that equilibria can be shifted by the conditions of filtration. However, from the thermodynamic standpoint the argument is palpably implausible. An equilibrium can be altered in favor of diminished binding only by reducing the *concentration* of a reactant or increasing the *dissociation constant* of the complex. Expression of the water phase with its dissolved drug does not accomplish the former; and the latter, depending as it does upon the rate at which molecules attach to and detach from the protein surface, could hardly be affected significantly by the filtration pressure.

For precise quantitative studies the method is inferior to dialysis because of the continually changing *protein concentration* and accumulation of protein on the membrane surface. The consequent errors, fully discussed by Grollman (110), are in the opposite direction to those postulated by Bennhold. Despite its limitations, ultrafiltration is a fairly accurate indicator of the extent of interaction. For convenient and rapid approximation of the fraction of drug bound in a serum sample it is invaluable.

d. *Conductivity and E.M.F. measurements.* These methods are obviously only applicable to the study of electrolytes. The E.M.F. method lends itself beautifully to quantitating the interaction of an ion that can be incorporated into the electrode system (*e.g.*, chloride) but is otherwise inapplicable. The studies of Pauli and Schön (187) and of Hayasida (117) on chloride binding by serum

proteins illustrate these methods. The determinations must be preceded by removal, so far as possible, of all extraneous ions, so that the use of buffers is consequently much limited.

e. *Osmotic pressure, vapor pressure, surface tension, freezing point.* Although they have found little use, these methods are mentioned because they offer theoretically valid possibilities for detecting a reduction in the expected thermodynamic activity of a drug consequent to interaction. The method of osmotic pressure was employed by Scatchard and his associates (220) for studying the interaction of chloride with serum albumin. Lowering of vapor pressure was the basis of some early experiments on the binding of chloroform in serum (173).

Lecomte du Noüy (148, 149) observed that bile salts lowered the surface tension of serum only briefly, the original value being restored over a period of several minutes. If the facts are to be interpreted, as he suggests, in terms of bile salt-protein interaction, one must also accept the unusual slowness of the combination. Employing the same methods, Laporta (146) could not repeat these observations. Tayeau and Rolland (255) made the curious discovery that after extraction of lipids, serum is able to antagonize the surface tension lowering action of ten to fifty times as much bile salt as previously.

Except for the observation of Moore and Roaf (174) that the freezing point of serum was not depressed to the calculated degree by chloroform, no studies by this method have been reported.

f. *Differential adsorption.* A substance interacting with plasma protein may fail to be adsorbed in the expected manner to some solid adsorbant like charcoal. Ehrstrom (81) used this fact to characterize the "adsorption power" of the plasma in health and disease. He noted, for example, that while congo red was removed by a given amount of charcoal to an extent not exceeding 8% in normal plasma, the plasma of nephrotics and of cachectic patients with neoplastic disease readily gave up 50% or more of the dye.

The method has not been employed extensively nor is it more than a qualitative indication of the firmness of binding. However, it appears to be, in certain respects at least, a model of what occurs when a reversibly bound substance is fixed by various tissue elements in the body. This analogy was demonstrated by von Jancso (265), and earlier by Schulten (227), who were able to show that the staining of various perfused animal tissues by certain dyes was prevented by the addition of serum to the perfusate.

The process must evidently be viewed as a competition for unbound molecules whose distribution between plasma protein and fixed tissue protein (or other adsorbant) depends upon their relative affinity for the two. With the recent advent of specialized exchange resins and refined techniques of chromatographic adsorption, the method of interaction analysis by differential adsorption may prove very useful.

2. *Methods based upon alteration of drug properties.*

a. *Solubility.* One of the simplest ways to demonstrate a drug-protein interaction is to show that a sparingly-soluble drug dissolves more readily in the presence of protein. Drug is removed from the aqueous phase through combi-

nation so that at equilibrium, when the true solubility in the water has been reached, a considerably greater amount of total drug has *apparently* been dissolved.

As early as 1904, Moore and Roaf (173, 174) demonstrated that chloroform, ether and other volatile compounds were more soluble in serum than in saline solution, a fact they attributed to interaction with plasma proteins. A simliar difference in solubility is to be observed with ethylene and cyclopropane (183) (cf., however, p. 137). Mercury salts were shown to dissolve much more readily in serum than in water (256). Recently it was demonstrated that certain substituted naphthoquinones become more soluble in the presence of various plasma protein fractions. In this case interaction was also demonstrated by a biological technique and results by the two methods were in general agreement (124).

It is now obvious that all the naturally occurring lipid-soluble components of the plasma (cholesterol, fatty acids, steroid hormones, carotenoid pigments, water-insoluble vitamins) are in solution only by virtue of their close association with certain of the plasma globulins (49).

Large solubilizing effects are only to be noted with water-insoluble substances. As most drugs do not fall into this category the method of solubility has not often been applied to the study of drug interactions.

b. *Diffusion.* Bennhold (11), refining the early work of Busck, employed a diffusion technique in the study of dye interactions. If an aqueous solution of a monodisperse dye is layered over gelatin the dye will diffuse at a predictable rate over a period of several days. The slower diffusion of albumin can also be determined. It is found that many dyes in albumin solution do not diffuse at the expected rate but travel entirely with the albumin. Paradoxically, a colloidal dye which fails to diffuse into the gel from aqueous solution also travels with albumin. This result is evidently to be attributed to a dual effect of the protein—dispersion of the colloidal particles on the one hand, and interaction with the dispersed dye on the other. Unfortunately Bennhold did not vary dye concentrations through a wide range to determine the upper concentration limit of the apparently complete binding. One may not conclude from these experiments that the dyes were irreversibly bound. In principle the diffusion method is not very different from ultrafiltration, the gel performing the function of a semipermeable membrane which separates rapidly-diffusing small molecules from the colloidal proteins.

c. *Stabilization.* Molecules which tend to undergo spontaneous breakdown in aqueous solution may be protected in the presence of plasma protein. Such an effect, resulting from interaction, has been demonstrated in the case of bilirubin (3). It must be proved in every case that no specific stabilizing agent is present in the protein solution.

d. *Electrophoresis.* The refinements of the Tiselius apparatus, allowing accurate identification of a number of protein moieties in plasma, with provision for sampling as desired, affords the possibility of determining with which migrating protein component, if any, a given drug is associated, and within what concentration range each association occurs. Precise quantitative relations be-

tween drug concentration and molar binding ratio are not readily obtained. Bennhold employed this method as his principal tool in the study of a great many interactions. When the substance in question is colored or fluorescent its migration with one or another plasma component is particularly easy to follow. This is illustrated by the studies of Schubert (226) on riboflavin, and of Martin (164, 165) on bilirubin.

Limiting interpretations to the particular conditions of the experiment and extending observations, when possible, over a concentration range are as important in electrophoresis as in diffusion studies. At low concentrations an interaction may appear to involve a single protein exclusively and no free drug may be detected. But at higher concentrations other proteins may participate or unbound drug may appear, migrating independently of any protein peak. Such behavior was observed by Rawson (201) in the case of Evans blue which combined with albumin in a ratio of 8 moles dye to 1 mole albumin, and then began to associate with an α-globulin. Schubert (226) found that riboflavin, which combined initially with a euglobulin, also interacted with a pseudoglobulin at higher concentration.

e. *Spectrophotometry.* Some colored compounds undergo pronounced changes in their absorption spectra upon combination with protein. This effect has long been recognized in the case of various heme pigments acting as enzyme prosthetic groups. The so-called "protein error" in the use of certain indicator dyes, first discussed by Sörensen (240), is caused by an alteration in the usual color or intensity as a result of dye-protein combination. The absorption peak is shifted (usually toward the red) or the magnitude of the peak absorption is altered (usually diminished), or both. Robinson and Hogden (208) showed that serum produces both these changes in the spectrum of phenol red but their observations were largely confined to a narrow pH range near the pK of the dye (7.7). They observed that the protein effect is abolished in alkaline solution and this has been confirmed in the course of investigations by the author (103). The interpretation of such influences of pH upon binding will be discussed at length elsewhere (p. 145).

Klotz (136, 139, 140) made the fullest use of this method in studying the binding of azosulfonamides and other anionic dyes. Because spectral shifts were large he was able to quantitate the data to obtain estimates of bound and unbound dye in a single protein solution.

However, the protein interactions of a number of dyes cause no spectral change. An interesting hypothesis intended to explain these differences in dye behavior was advanced by Hartley (116) but numerous unexplained cases remain. Methylene blue was shown by the author (103) (dialysis method) to enter into combination with albumin and other protein fractions but to display no spectral change in the visible region. However, the substance displays two peaks in the ultraviolet, one of which (at 246 mμ) undergoes a sharp increase in magnitude with rising protein concentration. Michaelis and Granick (172) observed similar differences between the behavior of visible and ultraviolet spectra of this and other dyes and proposed an explanation of the phenomenon. Alterations of the

ultraviolet spectra might prove very useful in interaction studies were it not for the limitation imposed on the concentration of protein, whose own absorption in this region soon interferes with the dye spectrum.

3. *Methods based upon alteration of protein properties or behavior.*

a. *Precipitation.* The titration of proteins by dyes has been used to determine the available reactive groups on the protein molecule, in confirmation or extension of the results of acid and base titration (90, 199, 200). Experiments with synthetic detergents have yielded very similar results (41, 194, 196). The method generally entails addition of the reagent to complete precipitation of the protein, or back-titration of excess reagent after precipitation. In either case the use of the method is limited to a particular group of apparently stoichiometric, irreversible interactions that ultimately denature the protein. Useful information may be obtained, particularly with regard to the molar combining ratio of the two components. Hewitt (123) carried out such studies with serum albumin and the dyes eosin and rose bengal, finding ratios of 20 and 34 (moles dye per mole albumin), respectively. The principal difficulty in interpreting such data is that the combining ratios thus obtained do not necessarily represent the maximal binding capacity of the native protein. In all likelihood they only indicate the amount of dye required to neutralize the protein net charge to the point of insolubility.

Bound drug can sometimes be determined simply and directly in a protein precipitate. Protein-bound iodine (4, 204, 205, 217), bilirubin in combination with an α-globulin (49), and the lipids and carbohydrates of plasma lipo- and glyco- proteins (40, 49, 171) appear in precipitates of the respective protein fractions. One investigator (271, 272) has claimed that thrombin-active euglobulin fractions contain firmly bound calcium but this is not generally accepted. Among drugs, suramin (Bayer 205, Germanin) (20, 66, 168, 241), arsphenamine (179), and bismuthyl tartrates (5) have been recovered from protein precipitates.

The objection has been raised that the substance in question may not have been in combination with the native protein before precipitation (59). It is true that more elegant means of demonstrating interaction are often to be preferred but the evidence is clear, at least for naturally-occurring substances, that those found in fractionated protein precipitates are also firmly combined *in vivo*. The tightness of the binding should be stressed here, for substances in easily reversible combination will not be precipitated. On the contrary, coagulating a protein usually abolishes its binding capacity (11, 14, 15, 59, 85, 86, 113, 127), and bound drug is released. This is why most routine chemical methods for determining plasma drug levels give estimates of total (bound and unbound) drug.

An interesting illustration of these generalizations is to be found in the recent studies of Halpern, Dolkart and coworkers (115). Plasma containing benzylpenicillin (G) was fractionated by the method of Cohn *et al.* (48) and the fractions assayed for penicillin. The drug was almost quantitatively recovered in the final supernatant despite its well-known binding to albumin *in vivo*. Dissociation of reversible complexes therefore occurs during the preparation and washing of precipitates, when the latter are undenatured.

It may be concluded that recovery of a drug from a protein precipitate suggests, but does not establish, its firm combination with native protein. Failure to discover a drug in a protein precipitate is not evidence against interaction.

b. *Viscosity, electrophoretic mobility, sedimentation rate, and other properties.* Certain interactions which ultimately lead to coagulation may first produce more delicate alterations in protein properties. Increasing viscosity appears to be an early stage in the denaturation process. The interaction of urea with serum albumin has been studied (22, 24, 70, 194) by following such a viscosity change, which occurs well before any turbidity can be observed. The process evidently takes place in two steps, only the first of which is readily reversible by a number of stabilizing agents including fatty acid anions, and sulfate at high concentration.

Anionic detergents have been found to interact with albumin, producing complexes with distinctive mobility in an electric field (156, 194–196), and similarly produced complexes were observed to sediment at an altered rate in the ultracentrifuge (196). The interaction complex containing one atom of mercury and two molecules of albumin, reported by Hughes (131), sediments more rapidly than normal albumin. The formation of this complex has been followed by changes in light scattering and the kinetics of the reaction thereby analyzed (154).

Although limited to a special group of proteins, the measurement of enzyme activity or inhibition provides an extraordinarily sensitive method for the study of interaction. Furthermore, changes produced by denaturants are usually detectable by loss of enzyme activity long before the appearance of altered physical properties.

c. *Stabilization of protein.* The capacity of a molecular species to prevent denaturation can be accepted as a criterion of interaction provided direct combination with the denaturant can be excluded. The method has been profitably explored in several investigations from the laboratory of J. M. Luck (2, 22–25, 70, 259). Fatty acids of varying chain length were employed as stabilizing agents for plasma albumin. The progress of thermal as well as urea or guanidine denaturation was followed viscosimetrically, nephelometrically, and by ascertaining the temperature at which gross coagulation occurred in a fixed time. The stabilizing effects of the interacting compounds were so marked as to lead to important new concepts bearing on the molecular requirements for interaction with plasma albumin. Information of considerable practical value in the preservation of human albumin for clinical use also emerged from these studies.

Another interesting example of stabilization through interaction is provided from the field of enzymology, in the work of Koelle (144) who showed that human serum cholinesterase is protected against the denaturant action of di-isopropyl fluorophosphate by physostigmine, which is itself a reversible inhibitor of the enzyme.

Expression and Interpretation of Quantitative Interaction Data.

1. *Binding capacity of the protein and tightness of the binding.*

Considerable interest has centered in the finding that many drug-protein interactions can be shown to follow an adsorption isotherm. But much con-

fusion and not a few irrelevant conclusions have been based upon this simple fact.

As a result of his studies with charcoal Freundlich (91) formulated the relationship:

$$\frac{x}{m} = kc^{1/n}$$

This is known as the Freundlich isotherm. It states that at a given temperature the amount of a substance adsorbed (x) per weight of adsorbant (m) is proportional to some power (1/n) of the concentration of unadsorbed material (c). A number of investigators have expressed their interaction data in terms of this equation, usually plotting the logarithm of x/m against the logarithm of c to obtain a straight line of slope 1/n, whose position on the axes gives k.

It is important to note that the Freundlich isotherm is a purely empirical function. It has no thermodynamic validity and the constants are without special physical significance. The simplest proof of its incorrectness is the fact that it has no end-point. It implies infinite adsorption with increasing concentration of solute, an absurdity since the finite adsorbant surface must ultimately become saturated.

The Langmuir isotherm (145, 145a), on the other hand, is a theoretically valid expression of the facts of adsorption. The equation is:

$$y = \frac{x}{m} = \frac{bc}{\frac{1}{a} + c}$$

where x/m and c have the same significance as above, while a and b are constants whose values can be rationally interpreted. The equation correctly expresses the fact that, at very high values of c, the adsorbant becomes saturated, the whole term on the right approaching a limiting value b. The Langmuir isotherm is entirely equivalent to the mass law equilibrium (cf. p. 113), the more usual form for characterizing the reversible association of two components. A more involved treatment of multi-layer adsorption was proposed by Brunauer, Emmett and Teller (29), and has been applied to the binding of water vapor by dry plasma (and other) proteins (32).

It seems appropriate to restate an important point about adsorption previously emphasized with considerable passion in an entertaining review by Mathews (166) about twenty-five years ago. If a substance is concentrated reversibly at a surface we say it is adsorbed. The term is simply descriptive of this fact and does not imply anything about the nature of the responsible forces. Therefore the mere finding that an adsorption isotherm is followed *does not mean* that the forces are physical rather than chemical in nature. "There is no present justification for dividing interatomic (or intermolecular) forces into *physical* and *chemical* forces. It is much more profitable to consider all such forces as strictly chemical in nature." This statement, made over thirty years ago by Langmuir (145) , has its counterpart in the identity of his adsorption isotherm with the law of mass action.

If it be assumed that all groups on a protein molecule capable of interacting have identical affinities for the drug molecules and that the affinity of any group is unaffected by the combination of drug molecules with other groups, several related equations can be derived from the mass law (137):

$$P_f + X \rightleftharpoons P_f X$$

$$\frac{(P_f)(X)}{(P_f X)} = K$$

where (P_f) is the total concentration of *free receptors* (regardless of how many are carried by each protein molecule), (X) is the concentration of unbound drug, $(P_f X)$ is the concentration of *combined* receptors, and K is the dissociation constant. Now let n be the number of receptor groups carried by each protein molecule, and P be the molar concentration of total protein. Then nP represents the total concentration of receptors, $(P_f X) + (P_f)$. Substituting $(P_f) = nP - (P_f X)$, we find

$$nP(X) - (P_f X)(X) = K(P_f X)$$

$$[K + (X)](P_f X) = nP(X)$$

Now let r be the moles drug bound per mole total protein, or $(P_f X)/P$. Then

$$r = \frac{(P_f X)}{P} = \frac{n(X)}{K + (X)} \tag{1}$$

This is the expression that is identical in form with the Langmuir isotherm.

One important type of interaction experiment is designed to reveal the number of interacting groups on the protein, and the tightness of the binding, *i.e.*, n and K in Equation (1). Such experiments are based upon varying drug concentration and measuring the amounts bound at each concentration by one of the methods previously outlined. To illustrate clearly the results that can be expected when actual data are plotted in various ways, and the interpretation of such data, a hypothetical case will be analyzed. Suppose an interaction experiment in 0.69% albumin solution (1×10^{-4} M/L), in which 10 binding centers per mole albumin are available to the drug under study. Let the dissociation constant of the drug-albumin complex be 1×10^{-3}. Assume we are able to vary the unbound drug concentration a thousand-fold.

Figure 1 shows the variation in r as a function of unbound drug concentration, a typical "adsorption-type" curve, asymptotic to 10, the maximal value of r. The points are chosen at drug concentrations 0.01, 0.1, 1.0 and 10.0×10^{-3} molar and the same symbols are used in this and subsequent figures so that points for high and low drug concentration can be readily identified.

In *Figure 2* the same data are plotted according to the method commonly employed for showing conformity to the Freundlich isotherm. The expected linearity is obtained only at low concentrations, when the binding capacity of the protein is but little saturated. The limit approached by the slope ($\lim_{1/n}$) at very low concentration is *always* unity, but with increasing concentration it falls off. Furthermore, the curve over *any* small concentration range would be very nearly linear. These matters are well illustrated in the studies of Grollman (109) on the binding of phenol red by various sera. Varying concentration no more than twenty-fold, this investigator obtained linear log-log plots of different slopes with sera of various species. Obviously his data for each serum fall on a different portion

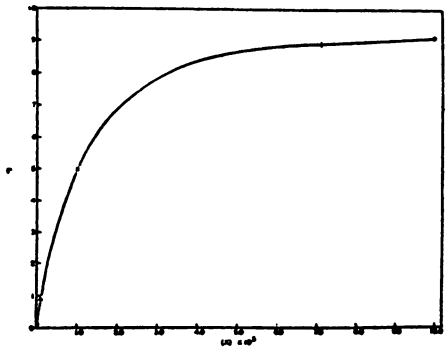

FIG. 1

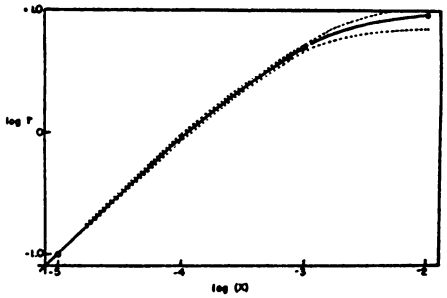

FIG. 2

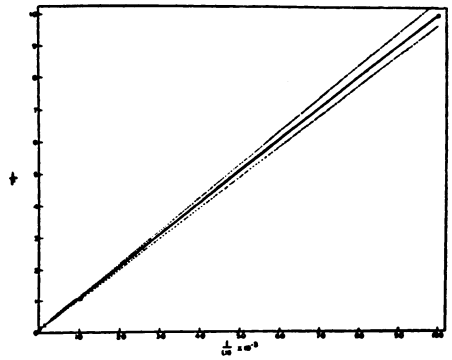

FIG. 3

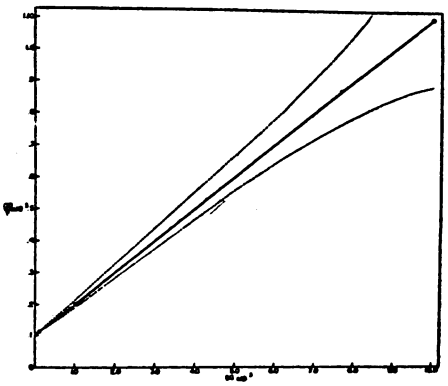

FIG. 4

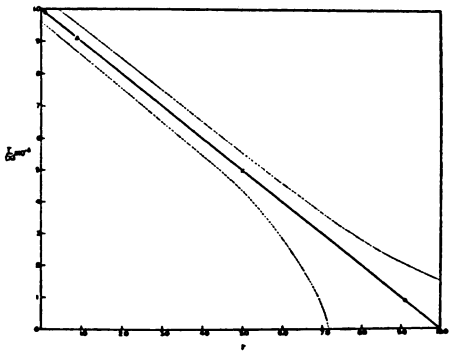

FIG. 5

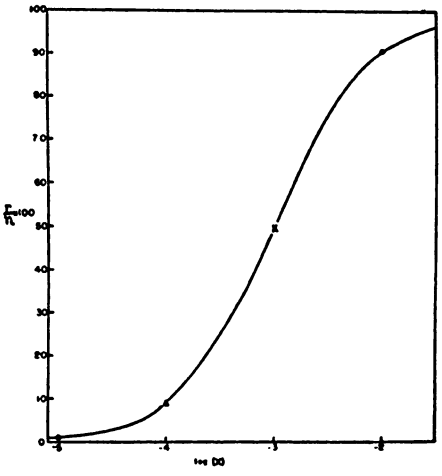

FIG. 6

FIGS. 1–6. For explanation see text

of the true curve and apparent conformity to the Freundlich isotherm can only be attributed to the narrow concentration range he employed.

Equation (1) can be rearranged in a number of ways for convenient plotting to yield values of K and n by extrapolation. If reciprocals of r and (X) are plotted, as in *Figure 3*, one obtains a straight line with slope K/n and intercept 1/n. This form will be familiar to enzymologists as the Lineweaver-Burk plot (152) and its use in interaction studies is well illustrated in Greenberg's (106) review of cation-protein interactions. In this and the other figures the disproportionate weighting of points representing high and low drug concentration should be noted. Here a few *low* drug concentrations will outweigh many high ones. The broken lines indicate the possible variation resulting from a maximum error of ±1% in the determination of drug concentration. The differing appearances of this variation on the several plots should be especially observed.

In *Figure 4*, (X)/r is plotted against (X), as in the investigations of Klotz (137, 138). Here the slope is 1/n and the intercept K/n. In contrast to *Figure 3*, *high* drug concentrations here weigh especially heavily.

Figure 5 represents the type of plot advocated by Scatchard (221a), in which the ordinal and abscissal intercepts give, respectively, n/K and n.

In *Figure 6*, 100 × r/n is plotted against log (X). When a negligible *fraction* of the total drug is bound, the abscissae can be expressed as "log *total* drug concentration." This is then the familiar dose-response curve or, alternatively, the course of saturation of an enzyme by its substrate or inhibitor, the ordinates representing per cent saturation of available groups.

Regardless of the manner of rearranging the equation it will not accurately describe a situation which fails to conform to the principal underlying asumptions.

(1) The interaction must be reversible and the data must be obtained at complete equilibrium.

(2) All groups must have the same affinity for the drug. If certain groups react more avidly than others, *sharp* breaks in the various linear curves will be obvious only if the affinities of the several species of receptor differ widely. Although this question has been scantily explored, it seems very likely that affinity of drugs to various binding centers does vary. The data of Thimann and Rothschild (260) on the interaction of indole-acetate with plasma albumin suggest that the first interacting groups on each albumin molecule bind more firmly than subsequent ones. Similar complex behavior might be expected in the case of chloride and thiocyanate, under study at present by Scatchard and coworkers (221, 222). At equivalent concentrations 24 moles of thiocyanate but only 6 moles of chloride are bound to albumin. If, as seems probable, the groups binding chloride are included among those binding thiocyanate, it might follow that thiocyanate binding involves at least two species of receptors of different affinity. This, however, has not yet been demonstrated.

(3) Systematic deviation of the data in the direction of reduced binding may result from electrostatic repulsion, by initially bound drug, of the approach of subsequent ions of the same charge. Such effects, which may occur with multivalent ions (and which can be corrected for), were observed by Klotz (138, 140) in the case of di- and tri-sulfonated azo dyes.

(4) If drug concentration is not varied through a sufficient range the extrapolations will not even approximate the true maximum number of available groups

but will only show the number of groups readily available, *i.e.*, those with highest affinity for the drug molecules. Such information is valuable but should not be misinterpreted.

(5) The quantitative methods described here are obviously only applicable to interactions with purified and homogeneous proteins of known molecular weight. In view of the widely differing combining power of diverse proteins it is extremely hazardous to attempt an estimate of molar binding capacity in the presence of even the smallest quantities of contaminant protein.

2. *The ratio of bound to total drug.*

In the foregoing discussion prime attention was focussed upon the protein molecule and the course of its saturation by an interacting drug. There is good reason to examine the problem also from the standpoint of the drug molecules and their partition between bound and unbound forms.[2] This partition largely determines the degree to which pharmacological properties of a drug can be modified by interaction with plasma proteins. A further interest in this approach arises from the frequent inapplicability of the techniques at hand to the kind of saturation experiments already described, either because of impurity of the proteins or because drug concentration cannot be suitably varied.

It is therefore desirable to know how maximal binding capacity and tightness of binding determine the ratio of bound to total drug. Bechhold (7) long ago stated the basic principle that large effects upon this ratio are to be observed, as a rule, only when *few* of the available protein groups have been saturated, *i.e.*, at low drug concentration.

Equation (1) can be rearranged to yield an expression for the fraction $\frac{\text{bound drug}}{\text{total drug}}$, which we shall designate by the symbol β.

$$\frac{(X)}{(P_tX)} = \frac{K + (X)}{nP}$$

$$\beta = \frac{(P_tX)}{(P_tX) + (X)} = \frac{1}{1 + \frac{K}{nP} + \frac{(X)}{nP}} \qquad (2)$$

This equation states that the *fraction bound* depends upon the protein concentration, the drug concentration, the dissociation constant of the drug-protein complex, and the number of groups available for drug binding on each protein molecule. Either K or n, or both, may be affected by pH, ionic strength and temperature. Consequently the values of these variables should always be reported.

[2] The term "free drug" is employed in two utterly unrelated contexts. It is meant to distinguish a drug in its original state from the same drug after conjugation (*e.g.*, "free sulfonamide" as opposed to conjugated sulfonamide). It is also used to connote unbound as opposed to protein-bound drug. While the latter sense would conform to general usage in physical chemistry, the former is well established in pharmacology. It will probably lead to a minimum of confusion if the term "unbound" be uniformly adopted. This convention will be followed throughout this paper.

Figure 7 is the graphical representation of Equation (2), where β is plotted as a function of log (X)/nP for discrete values of K/nP. For each particular drug-protein system a single one of these curves will apply, the abscissae being then proportional to the logarithms of unbound drug concentrations. From the equation and figure, the following behavior can be predicted:

(1) As the protein concentration increases, β also increases toward unity. Regardless of any other factors, all interacting drugs should be *completely bound* at high enough protein concentration, but in real instances, when affinity is low, such concentrations might be out of the question. Conversely, dilution of a system containing bound drug results in dissociation of the complex.

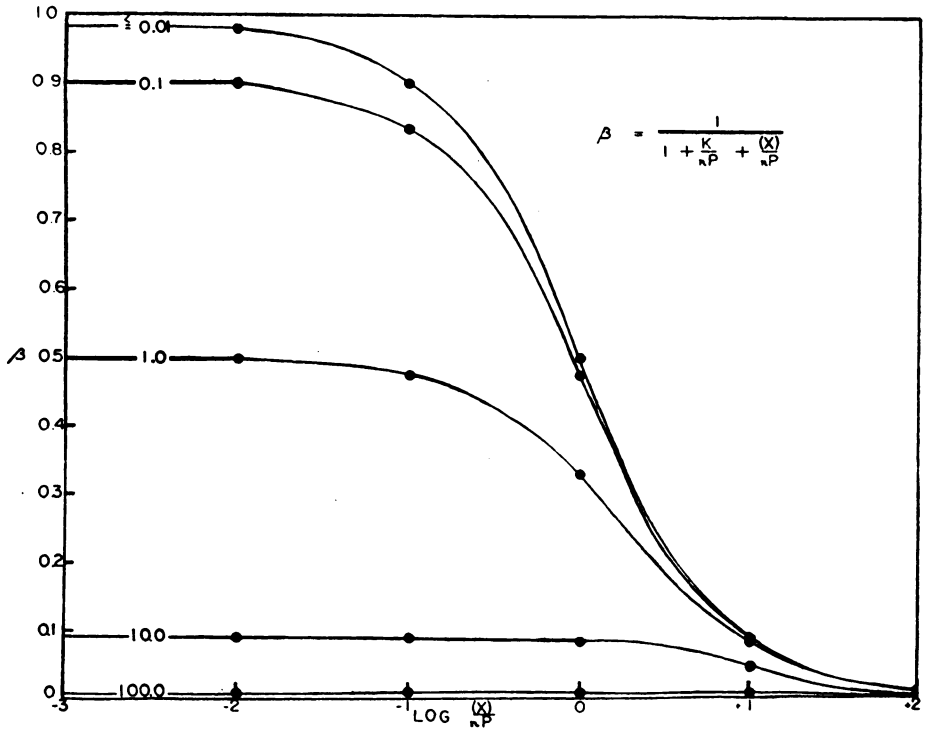

FIG. 7. FRACTION BOUND (β) AS A FUNCTION OF LOG (X)/nP FOR DISCRETE VALUES OF K/nP

(2) Drug concentration is crucial. As it is increased more drug is bound per protein molecule, but at the same time the *fraction bound decreases.* Inspection of the equation and figure shows that if the drug concentration is high enough essentially all the drug is unbound in a system held constant in other respects. Conversely, as drug concentration falls, the fraction bound approaches a maximum. *This maximum is not necessarily unity* but is represented by the term $\frac{1}{1 + K/nP}$. Consequently for certain interactions the maximal fraction bound, at very low drug concentration, may be extremely small while for others (e.g., if K/nP < .01) the binding may be practically complete. *The statement that a*

given fraction of drug is bound in the plasma is meaningless unless qualified by an indication of the unbound drug concentration at equilibrium. If conclusions bearing upon pharmacological action are to be drawn, interaction studies must be carried out at therapeutic drug levels, for experiments at arbitrary higher levels will usually (but not necessarily) give underestimates of β.

(3) The smaller the dissociation constant the tighter will be the binding and the greater the fraction bound. On the other hand the equation shows that a small K can be offset by a large (X), so that at high enough concentration essentially all of a drug will be present in unbound form. As noted above, if K is considerably smaller than nP, the whole term containing K becomes negligible. This means that all drugs with very high affinity should behave identically. Affinity *differences* will be obscured and fractional binding will depend upon the drug concentration alone. All such drugs will be completely bound at comparably low concentrations.

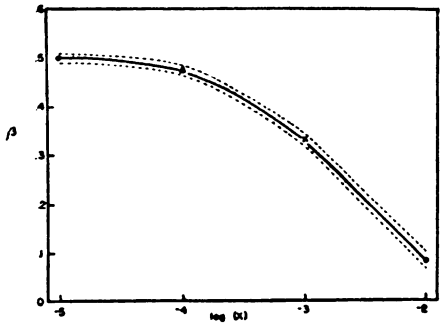

FIG. 8. For explanation see text

(4) Other things being equal, the greater the total number of receptor groups per molecule, the larger will be the fractional binding of interacting molecules.

In *Figure 8* the hypothetical interaction analyzed in the previous section is plotted in terms of β. The curve, as was to be expected, is identical with that of Figure 7 for which K/nP = 1, and the *maximal* binding is 0.5. Frequently it is only possible to investigate an interaction at low drug concentrations. If, in such a case, two measurements at concentrations differing by a factor of ten give the same fractional binding, it can safely be assumed that the maximal value of β has been nearly reached. The system can therefore be characterized in certain important respects, and the ratio K/n obtained, even though distinct values for each constant cannot be determined. It is to be recognized that *every* interaction will show an unchanging, maximal value of β below a certain drug concentration. For example, the finding that chloromycetin is 45% bound to crystalline bovine albumin through a considerable range of concentration, reported by Smith and his associates (238) as an anomalous finding, should in fact have been expected, and could have been used to derive information of some quantitative value about the interaction.

Frequently the concentrations of protein and of total drug are varied simultaneously to determine what molar ratio gives a certain constant effect. Davis

and Dubos (56, 58) observed that expected antibacterial action could be prevented at several different concentrations of oleic acid and albumin provided the molar ratio oleic acid/albumin = 3 was not exceeded, while the ratio could be 9 when hemolysis was taken as an end-point. The underlying principle in such experiments is that at the point of protection the *unbound drug* is at that concentration just insufficient to produce a particular effect. Reference to Equation (1) shows that the only way (X) can remain constant as P varies is for ($P_f X$) to vary also by exactly the same factor. This requirement can be approximately satisfied only when practically all of a drug is in the form ($P_f X$). The case becomes one of apparent stoichiometry, falling into the category represented by the uppermost curve of Figure 7. It is difficult to see why the molar combining ratios thus obtained should have any special significance. They are certainly not to be confused with the maximal binding capacity, n. Nor is it likely that they represent a distinct class of binding centers of high affinity. As the experiments cited above show, the molar combining ratio varies with the concentration of unbound drug taken as end-point, which in turn depends upon the sensitivity of the biological indicator sytem. If 50 or 100 groups on an albumin molecule were capable of interacting with oleic acid and all were of identical affinity, the same molar binding ratios might be obtained under these conditions.

It should perhaps be emphasized again, in concluding this discussion on the quantitative analysis of interaction data, that all such attempts, including that presented above, are oversimplifications. The doubtful validity of some of the assumptions has been pointed out. Nevertheless these first approximations are better than none and often describe interaction phenomena accurately enough to be of real value to the investigator.

SPECIFICITY OF INTERACTIONS. The chemical reactivities of the many plasma proteins differ as widely as their amino-acid compositions and physical properties. Drug molecules, in their interactions with these proteins, obey the same laws as do other interacting substances devoid of pharmacological activity. Any attempt to discover the nature of these laws of specificity must therefore embrace a wide variety of such substances, many of which do not fall into the drug category. Indeed, some of the most revealing investigations have dealt with constituents of normal plasma, or with organic dyes of little pharmacological interest.

Table 1 presents, in summary, the results of numerous interaction studies. In many cases the protein component was simply whole serum or plasma and no attempt was made to discover which proteins were responsible for the interaction. Many investigators, however, have sought this additional information by separation of the protein into two or more "fractions" and observing with which the substance under study specifically combined. Such data cannot be intelligently interpreted except with reference to the fractionation scheme employed. As different techniques may yield variant results a brief digression into some technical aspects of protein fractionation will be necessary.

TABLE 1

Results of some representative interaction studies, classified according to interacting substance.

Concordant results of several investigators are often grouped together. Discrepant results are listed separately. Meanings of the symbols are given at end of table.

SUBSTANCE	PROTEINS INVESTIGATED	METHOD	PRIMARY INTERACTION WITH	SECONDARY INTERACTIONS WITH	NO INTERACTION WITH	EASE OF REVERSIBILITY	COMPLEX DISSOCIATED BY	REMARKS	REFERENCES
I. INORGANIC SUBSTANCES									
Water	Various soluble proteins		A	All other soluble proteins					(32, 45, 224)
ANIONS									
Chloride	A (ho) S (ho) A* (b, h) S (d) S (h)	C E.M.F. OP, D U U	A S A* 0 0			+ + +	High pH	$r > 26$ $\beta < .06$ (0.1 M) $r = 2$–6 (.02–.12 M) (0.375%)	(187) (117) (220, 221) (104) (11)
Iodide	S (b)	U	0					(0.1%) Not done at lower concentration	(61)
(Protein - bound iodine)	F (h)	P	G (α, β) in IV-6	A and other fractions		−		Increased binding if iodide added	(4, 217)
Phosphate	S (ho) A* (h)	U EM	S A*			+		$\beta = .05$ (3.1 mg. %) (.01–.08 M)	(189) (2)
Sulfate	F (b, h) S (h) S (d) S (h)	SP Renal clearance U U	A* S(?) 0 0		G (γ)			 (12 mg. %) (> 4.5 mg. %)	(22, 24) (18) (104) (118)
Thiocyanate	A* (h)	D	A*			+		$r = 11$–24 (.02–.12 M)	(221)

	Cations								
Calcium	S (h)	U	S			(+)			(105)
	S (ho)	U	S			+		β = .40 (7.7 mg. %)	(189)
	S, A, G, (h)	Various	A	G		+	Low pH	Reviews on specificity of interaction	(9, 106, 111, 169)
	G (h)	B (f.h.)	G (eu-)	G (pseudo-) Other G (eu-)		(+)		Data on K	(69)
	F (ho)	P	G (thrombin)		See remarks	−		Tightly bound *only* in fraction containing thrombin	(271, 272)
	Casein	SR	Casein			+	Salt	n about 13-16	(37, 155)
Copper	F (h)	Color reaction	G (β_1-pseudo) in IV-7		All other fractions	(−)		Crystalline metal-combining protein	(49, 252)
	S (h)	P	S			−	Low pH prevents interaction	4N: 2Na: 1 Cu in complex	(158)
	S (h)	E	A						(11)
Gold (colloidal)	S (h)	DA	S			(+)		Perfused liver experiment	(265)
Iron	F (h), S (h)	B (bac.),	G (β_1-pseudo) in IV-7					Crystalline metal-combining protein	(36, 49, 223)
	F (h)	Color reaction	G (β_1-pseudo) in IV-7		All other fractions	(−)	pH < 5	Crystalline metal-combining protein	(49, 252)
Magnesium	S (h)	U	S			(+)		β = .30–.60 (2 mg. %)	(11)
Mercury	A* (h)	Various	A*			+	BAL, cysteine, by removing Hg	Crystallizes with 2 molecules A	(131, 154, 219)
	S (ho)	So	S						(256)
Potassium	S (h)	E	0						(11)
Silver	S (h)	E	A						(11)
Silver (colloidal)	S (h)	DA	S			(+)		Perfused liver experiment	(265)
Sodium	S (h)	E	0						(11)
Thorotrast (colloidal thorium dioxide)	S (h)	E	G		A				(11)
Zinc	S, A (ho)	SP, EM	A (see remarks)			(+)		Probably to metal-combining globulin (49)	(187)

TABLE 1—*Continued*

SUBSTANCE	PROTEINS INVESTIGATED	METHOD	PRIMARY INTERACTION WITH	SECONDARY INTERACTIONS WITH	NO INTERACTION WITH	EASE OF REVERSIBILITY	COMPLEX DISSOCIATED BY	REMARKS	REFERENCES
II. ORGANIC COMPOUNDS PRESENT IN NORMAL OR PATHOLOGICAL PLASMA									
Glucose	S (b) S (h)	U E	S 0					β = .25–.33 normally Normal and diabetic sera	(60) (11)
Other sugars	F (h)	P	G (α_2) in IV-6			(−)		Firmly combined glyco- and muco-proteins	(49, 78)
Indican	S (h)	E	A	G				With A (< 1 mg. %). Some with G (> 1 mg. %)	(12)
Lipids, various	F (h)	P	G (α_1, β_1) in III-0, IV-0			−		Firmly combined lipoproteins	(49, 78)
Melanogen	S (h)	E	A		G			Patient with melanosarcoma	(11)
Urate	S (h, g)	E, U	A, 0					By electrophoresis partially bound (4.6–12 mg. %); but all ultrafilters (at what concentration?)	(11)
	S (h, ch)	U	S			(+)	Uricosuric agents do not reduce protein binding	β = .20 normally. Suggests polymeric urate in plasma	(151, 276–278)
Urea (or "NPN")	S (d)	U	S			(+)		β = .37 normally	(159)
	S (h)	P	S			+	$MgSO_4$	No evidence for claim here that polypeptides are bound. NPN was measured	(153)
	Blood (d)	P	Blood			+	See remarks	Oleate (25 mg./cc.) releases NPN normally bound to plasma protein precipitate	(214)
	F (b, h)	V	A*	G(γ)		(+)	Various interacting anions	Denaturant (6 M)	(22, 24)
	S (h)	E	0					(148 mg. %)	(11)

PORPHYRINS									
Bilirubin	S (h)	E	A		G	(+)	0.35% desoxycholate	Some unbound (> 24.5 mg. %)	(11)
	S (h)	Sy	S			(+)	pH > 13	r = 46 if all assumed with A	(273)
	F (h, b)	Sb	A°	Bovine G fraction?	I, II, III, IV Gum arabic Glycogen			Protection of bilirubin against oxidation	(3)
	S (h)	D, E, U, Sb	Fraction of "A" or "G"	A			50% acid methanol (indirect van den Bergh)		(10, 50, 160)
	F (h)	D, E, Sy	G (α_1) in V-1	A°, G (α_2)	G (β, γ)	α_1:−, A:+		r = 3.3, β = .86 when A° in equilibrium with solid bilirubin	(164, 165)
Biliverdin	S (h)	P	G (pseudo-)						(50)
Coproporphyrins	S (h)	E	A		G				(11, 97)
Hematoporphyrin	S (h)	E	A		G, ϕ	+		Some unbound (> 3 mg. %)	(97)
	A° (h)	Sy	A°			+			(210)
Urobilin	S (h)	E	A		G			(0.44 mg. %)	(11)
Uroporphyrins	S (h)	E	0		A, G				(11, 97)
STEROIDS									
Bile salts	S (h)	E, Di, B (hem.)	A			+		Displace other compounds from A in diffusion experiments	(11)
	S (ho)	ST	S			(+)		Reduced binding in certain immune sera	(149)
	S (ho)	ST, U	0					Cannot confirm Lecomte du Noüy (149)	(146)
	S (h, ho)	ST	S					Binding *increases* after extracting lipids	(255)
	F (h)	B (hem.)	A°			(+)			(49)
Cholesterol (and esters)	S (h)	E	G		A				(9-11)
	S (h)	U	S			?	Adsorbed by Kieselguhr	Complete binding. (Cholesterol 3 mg. %; ester 10 mg. %) (Protein 0.9%)	(28)
	S (b, ho)	E, D	"Part of A or G"					$\beta \leqq .99$ (0.7-1.4%)	(10, 170)
	F (h)	P	G (eu-)	A, G (pseudo-)		−		16% total sterol in precipitate of which 90% in euglobulin, 10% in "albumin"	(94)
	F (h)	P	G (α_1, β_1)			(−)		With α_1 and β_1 lipoproteins	(49)

TABLE 1—*Continued*

SUBSTANCE	PROTEINS INVESTIGATED	METHOD	PRIMARY INTERACTION WITH	SECONDARY INTERACTIONS WITH	NO INTERACTION WITH	EASE OF REVERSIBILITY	COMPLEX DISSOCIATED BY	REMARKS	REFERENCES
				STEROIDS—*Continued*					
Estrogens	S (b, r, h)	P, D	S			+		(0.1–0.5 γ %)	(253)
	S (h)	U, P, B (m.a.)	S			−	Vigorous acid hydrolysis	All precipitated with proteins, from pregnancy serum	(198)
	S (h)	U	S			(+)		β = .90 (1 mg. %)	(19)
	F (h)	P	G (β_1 - lipoprotein) in III-0			+		β = .67 normally. Mainly estriol; esters unbound	(207)
				VITAMINS					
Ascorbic acid	S (h)	E	A		G	(+)		Complete binding (< 0.75 mg. %)	(226)
Carotenoids	F (h)	P	G (β_1 - lipoprotein) in III-0			?			(49)
Vitamin K	S (h)	E	A					Complete binding (< 400 mg. %)	(12)
Nicotinamide	S (h)	E	G	A				All with G (1–2 mg. %). Also with A (> 3.5 mg. %). Some unbound (> 35 mg. %)	(12)
Vitamin P	S (h)	E	A		G			Complete binding (17–125 mg. %)	(226)
Riboflavin	S (h)	E	G (eu-)		A			Complete binding (< 0.75 mg. %)	(226)
Riboflavin Phosphate	S (h)	E	A	G (eu-)				Complete binding (< 0.15 mg. %)	(226)
Thiamine	S (h)	E	0					(0.15 %)	(226)
Thiochrome	S (h)	E	G (eu-)		A				(226)

III. ALIPHATIC AND AROMATIC ANIONS. DETERGENTS. ALIPHATIC ALCOHOLS

Acetate	A* (h)	EM	A*					(0.1 M)	(2)
Trichloracetate	A* (h, b)	SP	A*			Apparent stoichiometric		Mole ratios for stabilization	(70)
Medium chain-length fatty acids	F (h, b)	SP	A*		G(γ). Egg A	Apparent stoichiom.	High pH	Specificity studies. Quantitative data	(23)
	A* (h, b)	U, B (hem.)	A*			+	High pH	Specificity studies. Quantitative data.	(25)
Oleate	Blood (d)	P	Blood			Apparent stoichiom.		Complete binding (2.5 %)	(214)
	F (b)	B (bac., hem.), So	A*	G (β-lacto)	II, III-1, IV-1, Gelatin	Apparent stoichiom.		Mole ratios for protection	(56, 58)
Aromatic carboxy acids	A* (b)	D	A*			+	High pH	Quantitative data on K, n, ΔF. Specificity study	(259)
Cationic detergents	A (h)	P	A			−		Essentially a precipitation titration	(41)
Dodecyl sulfate	A* (ho)	EM, V	A* (2 complexes)			Complex 1: apparent stoichiom.	High pH Freezing reverses complex 2	Complex 1 = half of acid-binding capacity. Complex 2 = full acid-binding capacity	(194–196)
	A* (h, b)	SP	A*			Apparent stoichiom.		Mole ratios for stabilization	(70)
Aliphatic alcohols	A* (h)	P	A*			?		Decanol crystallises with A*	(49)
	S (p)	So	S			+		Ethyl, amyl alcohol and acetates	(174)
	S (b)	D	S			+		Aliphatic alcohols and esters	(16)

TABLE 1—*Continued*

SUBSTANCE	PROTEINS INVESTIGATED	METHOD	PRIMARY INTERACTION WITH	SECONDARY INTERACTIONS WITH	NO INTERACTION WITH	EASE OF REVERSIBILITY	COMPLEX DISSOCIATED BY	REMARKS	REFERENCES
III. ALIPHATIC AND AROMATIC ANIONS. DETERGENTS. ALIPHATIC ALCOHOLS—*Continued*									
Substituted phenols	A° (b)	D	A°			+	High pH	Quantitative data on K, n, ΔF. Specificity study	(259)
	A° (h, b)	SP	A°			Apparent stoichiom.		Mole ratios for stabilization	(70)
	S (ho, b)	U	S					Tetrachlor-o-biphenol. Antiseptic action also reduced in serum	(6, 7)
	S (h)	E	A, G (?)					Phenols and cresols in uremic serum	(11)
	S (r)	D	S			+		Binds guaiacol, xylol, toluene, which displace other compounds	(14)
V. DYES									
Most dyes	F (various)	Various	A°					Only those of special interest listed below. Dye interaction studies indexed in Table Ia	
Basic dyes, various	S (h)	E	A	G (?)		(+)			(11)
Colloidal dyes, various	S (ho)	P	"G(eu-)"			"—"		Few data and no proof. Cf. (21)	(67)
Evans Blue	F (h)	E	A	G(α)	G(γ)	+			(201)
Phenol Red	S (ho)	U	S			(+)	0.35% phenol (202). Hippuran, diodrast (239). Oleate (214)		See Table Ia
Prontosil	S (h)	E	A		G	+		Complete binding (1.5 mg. %). Some unbound (62.5 mg. %)	(11, 12, 225)

Rose Bengal	F (d)	Sb	A	G	Amino acids, lipids, carbohyd.	Apparent stoichiom.	Salts, taurocholate	G has ½ binding power of A	(211-213)
Sulfonated azo dyes	F (b)	Sy, D	A*		G (γ)	+	High pH. Various other anions	Quantitative data. K, n, ΔF	(136, 138-140)
V. VOLATILE ANESTHETICS									
Chloroform	S (p)	So, VP, FP	S		Ether-extracted lipids	+		Raises conductivity; (by displacing salts?)	(173, 174)
Cyclopropane	Blood (h)	So	Blood					Distribution coefficient greater than in water	(183)
Ether	S (p)	So, VP, FP	S		Ether-extracted lipids	+			(174)
	Blood (h)	So	0					Distribution coefficient less than in water	(114)
Ethylene	Blood (h)	So	Blood					Distribution coefficient greater than in water	(183)
Nitrous oxide	Blood (h)	So	0					Distribution coefficient less than in water	(183)
VI. PHARMACOLOGICALLY ACTIVE ORGANIC ACIDS									
Barbiturates	S (h)	E	A	G(?)		(+)		Some unbound (therapeutic concentrations). Only Evipal to G at high concentration	(11)
	A*(h, b)	U	A*			+		Specificity studies, 17 compounds	(100)
Caronamide	F (d)	D	A			+		β = .68 (3.8 mg.%) and .59 (13.4 mg.%)	(77)
Heparin	F (h, ho)	E, B (cl.), SP	"A" fraction	G, gelatin, protamine	A*	+		Displaces phosphatide of an active lipoprotein in clotting mechanism (?)	(27, 38, 39, 44, 88, 133, 135, 197, 274, 275, 280)

TABLE 1—*Continued*

SUBSTANCE	PROTEINS INVESTIGATED	METHOD	PRIMARY INTERACTION WITH	SECONDARY INTERACTIONS WITH	NO INTERACTION WITH	EASE OF REVERSIBILITY	COMPLEX DISSOCIATED BY	REMARKS	REFERENCES
VI. PHARMACOLOGICALLY ACTIVE ORGANIC ACIDS—*Continued*									
Iodine - containing acids	A (h)	U	A			(+)	Hippuran and diodrast displace phenol red	Skiodan β = .18, hippuran β = .70 (1 mg. %, 4 % A)	(239)
	S (ho)	U	0					No supporting data. (What concentration?)	(84)
	S (h)	P	S			−		Priodax persists in vivo and firmly bound to protein precipitate	(205, 206)
p-amino-hippurate	S (h)	Renal clearance	S			?		β = .17 (25 - 100 mg.%)	(236)
p-amino-salicylate	S (h), A (b)	U	A			+		β = .60 (10 mg. %, 3.5 % A)	(266)
Salicylate	S (h, b)	D	S			+		$\beta \leqq 0.50$ (30 mg. %). Hypersensitive patients bind less.	(250)
	S (h)	P	S						(93)
	S (h)	U	S					β = .75 (10 mg. %) and .50 (20 mg. %)	(237)
	S (h)	E, U	A					Completely bound (45 mg. %), some unbound (180 mg. %)	(11)
Salicylurate	S (h)	D	S					Complete binding (20 mg. %)	(251)
VII. ALKALOIDS AND RELATED COMPOUNDS									
Acetylcholine	F (h) A (d, p)	EA B (f)	G(α) in IV-6 "A"					Plasma cholinesterase Destruction not ruled out	(102) (119)
Acetyl-tryptophane	A* (h, b)	SP	A*			+	High pH		(25)
Adenine Adenosine Adenylic acid	F (b)	D	A*		G (γ)	+		Quantitative data, Δ F	(141)

Aminoacridines (Quinacrine)	F (h) S (h)	? E	A A	G	G			Quinacrine β = .98 (therapeutic concentration) Some unbound (9 mg. %)	(254) (11)
Aminopyrine	S (ho)	U	S			?		Complete binding (0.4 %)	(218)
Atropine	S (b, r)	D, B (c. g.)	S			+	Citrate, peptone	Bound much less than pilocarpine	(14, 246)
Caffeine	S (ho)	D	S			(+)			(186)
Choline	S (various)	D	0					No data on concentration	(14)
Cinchona alkaloids	F (h)	?	A*					β = .50 - .90 (therapeutic concentration)	(125, 254)
Cocaine	S (b, ho, r)	D	S			(+)	High pH with ether	Dialyses from S (b), not from S (r) (0.25%)	(14, 186)
Desoxyribose-nucleate	A (h, egg)	E, SP	A			?			(35, 244)
Epinephrine	S (h) S (various)	Sb D	S 0					Protected against oxidation No data on concentration	(3) (14)
Guanidine	F (h, b)	V	A*	G (γ)			Various interacting anions	Denaturant (2.5 M)	(22, 24)
Histamine	S (various)	D	0					No data on concentration	(14)
Indole - acetate (auxin)	A* (h)	D	A			+	High pH (?), α-naphthalene-acetate, indole - butyrate		(260)
Morphine	S (h, b, r, s)	D	S			+	Citrate, peptone		(14, 248)
Pilocarpine	S (ho, r, b, s)	D, B (c. g.)	S			+	Atropine, ether 0.1%, chloroform 0.05%, aliphatic alcohols	β = .95 - .29 (0.2 - 2.0 mg. %)	(15, 16, 186, 245-248)

TABLE 1—*Continued*

SUBSTANCE	PROTEINS INVESTIGATED	METHOD	PRIMARY INTERACTION WITH	SECONDARY INTERACTIONS WITH	NO INTERACTION WITH	EASE OF REVERSIBILITY	COMPLEX DISSOCIATED BY	REMARKS	REFERENCES
VII. ALKALOIDS AND RELATED COMPOUNDS—*Continued*									
Procaine	S (various)	D	S			+	Various substances		(14)
Prostigmine Physostigmine Carbaminoylcholine	F (h), S (h)	EA, SP	G (α) in IV-6			+	Acetylcholine	Protect plasma cholinesterase against DFP denaturation	(102, 144)
VIII. CARDIAC GLYCOSIDES									
Digitoxin and various other glycosides and aglycones	A (h), F (r)	D, P	A		G (eu-) (pseudo-)	?		Digitoxin β = .50 (1 mg. %; 1% A (h))	(112, 113)
	S (f, r, c)	B (f.h., r.h.)	S				pH > 8.3, saponin, bile salts		(217)
	S (b, r, c)	U, B (f.h.)	S						(150)
	S (h)	B (f.h.)	S		Egg A, lecithin, cholesterol				(182)
	S, F (various)	D, B (f.h.)	A		All G fractions			Saponin, bile salts, high pH do *not* displace	(85, 86)
	F (h)	B (f.h.)	A°		I, II + III, IV-1, IV-4				(215)
Strophanthus glycosides and aglycones	A (h), F (r)	D, P	A		G (eu-) (pseudo-)	+	Ethanol		(112, 113)
	S (b, r, c)	U, B (f.h.)	S						(150)
	S (h)	B (f.h.)	0						(182)
	S, F (various)	D, B (f.h.)	0						(85, 86)
	F (h)	B (f.h.)	0						(215)
IX. MISCELLANEOUS CHEMOTHERAPEUTICS									
Arsphenamine	S (r)	P, SP	G	A		?	Low pH	Also interacts with gum arabic	(179)

Bismuthyl Tartrates	S (b)	U	S					Mono-bismuth tartrate β = .40; di- and tri-bismuth tartrate β = .94 - .96	(228)
	F (ho)	D, P	G (eu-)		G (pseudo-), A	+	High pH		(5)
Diamidines	S (?)	B (bac.)	S			?		Serum protects against antibacterial action	(92)
Naphthoquinones	F (h)	D, So	A*			(+)		Specificity studies	(30, 31)
	F (h), S (various)	D, So	A*	Other fractions except II	G (γ) (II)	±	Low pH	Interactions with A* poorly reversible, other interactions easily reversible	(124)
	F (h)	Color Reaction	A*		G (β, γ)	?	Caprylate		(49)
Neoarsphenamine	S (h)	E	A		G			Complete binding (40 mg. %), some unbound (160 mg. %)	(11)
Suramin (Bayer 205)	S (various)	SP	S					Protects (6-10%) sera of 10 species against coagulation	(143)
	S (ho, r)	P, U	"A" fraction		G fraction	−			(168)
	S (h)	P	S, G (?)	Casein, A(?)	ϕ	−		Smaller hydrolysis products less bound	(20, 66, 241)
X. ANTIBACTERIAL CHEMOTHERAPEUTICS AND RELATED COMPOUNDS									
Chloromycetin	A* (b)	D	A*			(+)		β = .45 - .60 (therapeutic concentrations)	(238)
p-amino-benzoate	S (h)	U	S			+		β = .20 - .30 (1-3 mg. %)	(157)
Penicillins	F (h, b)	D	A*		All other fractions	+		Quantitative data	(261)
	Various	Various	A*		Other fractions	+		β (0.1 γ/cc.) from .56 for benzyl- (G) to .96 for n-heptyl- (K)	(17, 71-73, 75, 142, 203, 258, 261)
Streptomycin	S (h)	?	0						(262)
	S (h)	B (bac.)	S			?		Loss of potency in serum resembling a slow interaction, not destruction	(122)

TABLE 1—*Concluded*

SUBSTANCE	PROTEINS INVESTIGATED	METHOD	PRIMARY INTERACTION WITH	SECONDARY INTERACTIONS WITH	NO INTERACTION WITH	EASE OF REVERSIBILITY	COMPLEX DISSOCIATED BY	REMARKS	REFERENCES
X. ANTIBACTERIAL CHEMOTHERAPEUTICS AND RELATED COMPOUNDS—*Continued*									
Steptomycin	A* (b)	D, B (bac.)	0 or A*					Bacteria protected by A* (0.05%) from streptomycin (0.5 mg. %) but no binding by dialysis	(142)
Sulfonamides	A* (h)	D	A*			+		Specificity study of substituted sulfadiazines; 41 compounds	(264)
	Various	Various	A*		Other fractions	+	PABA and other anions	β (10 mg. %) from .20 for sulfanilamide to .93 for sulfamethylthiadiazole	(1, 54, 55, 64, 65, 76, 98, 120, 157, 185)
Sulfones	A* (h)	So	A*					4 - amino - 4' - β - ethoxyethyl - aminodiphenyl sulfone	(132)

"Proteins Investigated": S, serum (or whole plasma); F, plasma fractions; A, albumin fraction (impure); A*, crystalline albumin.

(b) bovine; (c) cat; (ch) chicken; (d) dog; (f) frog; (g) goose; (ho) horse; (h) human; (p) pig; (r) rabbit; (s) sheep.

"Methods" (see pp. 104-111): B, biological action; C, conductivity; D, dialysis; DA, differential adsorption; Di, diffusion; E, electrophoresis; EA, enzyme activity; EM, electrophoretic mobility; FP, freezing point; OP, osmotic pressure; P, precipitation; Sb, stabilization (of drug); So, solubility; SP, stabilization (of protein); SR, sedimentation rate; ST surface tension; Sy, spectrophotometry; U, ultrafiltration; V, viscosity; VP, vapor pressure.

(bac.) bacteria; (c.g.) cat gut; (cl.) clotting time; (f.h.) frog heart; (hem.) hemolysis; (m.a.) mouse assay; (r.h.) rabbit heart.

"Primary, secondary, no interaction with": Under "primary interaction" is listed that of highest affinity—*i.e.*, the interaction observed at lowest drug concentration. Under "secondary interaction" are listed others simultaneously demonstrable at higher drug concentration. Under "no interaction with" are listed only those instances in which adequate evidence allows certain proteins to be ruled out—*i.e.*, no interaction observed at any reasonable concentration of drug.

0, none; S, interaction, in an experiment with serum, where the specific protein interacting was not further identified; A, A*, interaction, with albumin (impure) or crystalline albumin; G, interaction, with globulin fraction, further specified as to (α, β, γ) and Harvard fraction if data available.

"Ease of Reversibility": +, definitely reversible by simple means, *e.g.*, dilution; (+), probably reversible by simple means, not proved definitely; −, apparently not reversible by simple means.

"Complex dissociated by": Lists special reagents which seem to dissociate the complex without denaturation of the protein.

"Remarks": Pertinent data and conditions of experiments. As in text: β = fraction of total drug bound at the stated concentration; r = moles bound drug per mole total protein under the experimental conditions; n = maximum value of r, at infinite drug concentration.

A general discussion of recent advances in the fractionation and characterization of the plasma proteins is obviously beyond the scope of this paper. A brief resumé, based largely upon recent reviews by Edsall (78), Cohn (49), and Gibson (96), will suffice to present the facts that are pertinent to a discussion of interaction specificity.

Human plasma is a circulating fluid tissue of some 3000 cc. volume, containing about 7 per cent (or 200 grams) of protein. Over twenty-five distinct proteins

TABLE 1a

References to interactions of dyes with plasma proteins

AZO DYES
- Amaranth (140)
- Azorubin (11)
- Azosulfathiazole (138, 139)
- Azo Violet (11)
- Biebrich Scarlet (200)
- Brilliant Congo Red (11, 127)
- Brilliant Vital Red (68)
- Butter Yellow (12)
- Congo Red (11, 81, 107)
- Diamine Blue (67)
- Diamine Red (67)
- Evans Blue (68, 107, 108, 201)
- Helgoland Yellow (11)
- Methyl Orange (11, 138–140)
- Niagara Sky Blue (107, 201)
- Niagara Sky Blue 6 B (107, 201)
- o-amino-azo-toluol (12)
- Orange I and II (139)
- Salmon Red (11)
- Tropaeolin O (11, 200)
- Trypan Blue (62, 227, 231)
- Trypan Red (11, 62)

NITRO DYES
- Naphthol Yellow (11)
- Picric Acid (259)

PHTHALEIN DYES
- Bromphenol Blue (123)
- Bromsulfalein (26, 212)
- Bromthymol Blue (123)
- Eosin (11, 123)
- Erythrosin (123)
- Fluorescein (33, 61, 62, 123)
- Mercurochrome (126)
- Phenolphthalein (123)
- Phenol Red (PSP) (62, 101, 109, 123, 161, 162, 192, 202, 208, 212, 229, 239)
- Phloxin Red (123)
- Rhodamin (11)
- Rose Bengal (11, 123, 211–213)
- Tetrachlorphenolphthalein (123)
- Tetrabromphenolphthalein (123)
- Thymol Blue (123)

TRIPHENYLMETHANE DYES
- Acid Green (St. Denis) (67)
- Acid Violet (St. Denis) (67)
- Basic Fuchsin (126)
- Brilliant Green (126)
- Crystal Violet (126)
- Gentian Violet (67)
- Malachite Green (67, 126)
- Patent Blue (11)
- Victoria Blue (67)

MISCELLANEOUS DYES
- Acridine Orange (67)
- Indigo Carmine (62, 193, 202)
- Lithium Carmine (62)
- Methylene Blue (6)
- Neutral Red (67)

are represented, differing in size, shape, composition, charge, solubility and chemical reactivity. They can be separated into groups ("fractions") by methods that are sensitive to one or another of the differences in physical properties. As the pattern of similarities and differences is rather complex, those proteins falling into a given fraction in one method may be dispersed among several fractions in another.

In the order of increasing solubility (*e.g.*, in ammonium sulfate), the usual categories are *fibrinogen*, *eu-globulin*, *pseudo-globulin*, *albumin*. As the molarity of

salt chosen as boundary between one fraction and the next is rather arbitrary, all fractions obtained in this way are more or less contaminated with proteins of neighboring fractions. For example, the fraction precipitating between half- and fully-saturated ammonium sulfate consists of several albumins, and also contains certain globulins of similar solubility. If a substance interacts specifically with one of the latter it would naturally be reported to combine with "albumin". A number of such instances can be found in Table 1, where subsequent study with purer proteins served to rule out albumin and implicate a particular globulin.

In order of decreasing rate of migration toward the anode in an electric field, the categories are albumin, α_1- and α_2- globulin, β_1- and β_2- globulin, fibrinogen, γ_1- and γ_2- globulin. Crude electrophoretic apparatus might only permit two fractions to be distinguished—rapidly migrating "albumin" and slowly migrating "globulin." Each component distinguished by electrophoresis contains proteins that happen to migrate at the same rate, but these need not share the same solubility properties. A substance migrating strictly with albumin in an electric field might be a pseudo-globulin in salt precipitation. Bilirubin was observed by Bennhold (11) to migrate with albumin in crude electrophoresis. Bendien and Snapper (10) had earlier reported that a portion of the bilirubin interacts with certain globulins that are retained by a partially permeable ultrafilter. The apparent anomalies in this typical case appear to be resolved by the recent discovery of a tightly-bound bilirubin-protein complex that precipitates as a pseudo-globulin and migrates as an α_1-globulin on electrophoresis (49). Evidently similar considerations apply to all other fractionation methods (*e.g.*, ultracentrifugation) short of complete purification of homogeneous proteins.

The low-salt, low-temperature, ethanol method of fractionation represents a refinement of earlier solubility techniques, which has already yielded several products of high purity, including fibrinogen, crystalline albumin, and a metal-combining β_1-globulin. Preparations of γ-globulins have also been produced but as numerous specific antibodies are represented it must be assumed that, at least from the standpoint of surface configurations, a variety of proteins is present.

The remaining fractions are still mixtures but the components of each have been characterized in some detail. The fractionation method and the full description of each fraction are to be found in Edsall's review (78) and the earlier reports of Cohn and his associates (47, 48). To clarify those entries in Table 1 that refer to the Harvard fractions, the nomenclature is set forth in condensed form in *Table 2*.

Fibrinogen. The principal interaction of this protein with thrombin, in the complex clotting mechanism, is evidently in the class of protein-protein interactions, which will not be discussed here. No specific reactivity of fibrinogen with small molecules has been demonstrated, although certain substances are bound at concentrations higher than those required to demonstrate a primary interaction with another protein. Certain substituted naphthoquinones, for example, interact with most fractions including fibrinogen, but the complexes with the latter are appreciably looser than with the others (124). Methylene blue is bound by fibrinogen, to a lesser degree than by most other fractions (103). Fibrin films bind

a number of dyes (233) but it has not been demonstrated whether soluble fibrinogen would also react with them. The insignificance of interactions with fibrinogen is further indicated by the observation that the same interaction data are generally obtained in serum and in plasma.

γ-globulins. The γ-globulins appear to be involved almost exclusively in those highly specific protein-protein interactions known as immune reactions. Hydrogen ion, certain alkali-earth cations (69, 106), and various denaturants evidently react with all proteins, and the γ-globulins (and fibrinogen) are no exception. As in the case of fibrinogen, various substances that interact with more than one protein may be bound to γ-globulin at high concentration. But these are hardly to be considered specific interactions. The thermal denaturation of γ-globulin is slowed by sucrose and glycine (46) but these are the only reported instances of interaction with small molecules. It must be concluded that these proteins are

TABLE 2

*Nomenclature of the Harvard fractions**

FRACTION	MAJOR COMPONENTS
I	Fibrinogen (175)
II	γ-globulins (180)
III-0	β_1-lipoprotein (180)
III-1, 2, 3	Isoagglutinins, thrombin, plasmin, complement (C'_1) (180)
IV-1, IV-4†	Various α- and β-globulins, including an α_1-lipoprotein, α_2-glycoproteins, and several enzymes (252)
IV-7	Metal-combining β_1-globulin (199a, 251a)
V-1	Bilirubin-containing α_1-globulin
V	Albumin (with about 3% globulin impurity)
Crystalline albumin	

* After Cohn (49).

† IV-2, 3 and IV-5, 6, 7 are subfractions of IV-1 and IV-4, respectively.

endowed with a low order of chemical reactivity, as usually understood, and that the forces responsible for combination with antigens are somehow unique. Configurational factors and surface charge distribution presumably play a role (188) but the intimate mechanisms are still poorly understood.

α- and β-globulins. Purification of most of the α- and β-globulins is far from complete but a characteristic pattern of interaction specificity is beginning to emerge. Most of the members of this group are either enzymes, or occur in the plasma as conjugated proteins carrying smaller molecules in close association.

The enzymes include thrombin, plasmin, several peptidases, amylase, lipase, alkaline phosphatase and choline esterase. The specificity of their interactions finds expression in differing affinities for various substrates and inhibitors. The plasma cholinesterase, which proves to be an α-globulin appearing in fraction IV-6, has a specific affinity for choline esters as substrates and for carbamates of quaternary ammonium compounds related to choline as inhibitors (102). Interaction with the inhibitors has been demonstrated not only by the classical methods

of enzymology but also by their ability to protect the enzyme against a specific denaturant (144). The stabilizing action of plasma albumin against heat denaturation of this enzyme (102) is probably to be attributed to protein-protein interaction. In the same category would be the diastase-albumin interaction, demonstrated by electrophoresis (12). Each of the other enzyme globulins has its own pattern of substrate and inhibitor specificity reflecting, on the whole, rather precise requirements in molecular structure of the interacting compounds. It is not improbable that some of these enzymes combine with metallic or other prosthetic groups and there seems little reason to place such interactions in a special category.

Several pigment proteins have been obtained. A blue-green α-globulin is found in fraction IV-2 (49). Fraction III-0 contains a blue pigment that may be hemocuprein and a pale yellow hematin-protein conjugate, neither of which is associated intimately with the lipoproteins (181). Fraction V-1 contains a stable complex of bilirubin with an α-globulin (49). Bilirubin also combines with albumin but whereas the albumin complex is easily reversible (165), the globulin compound cannot be dissociated by prolonged and repeated dialysis (103). This difference in ease of reversibility was first observed by Hoover and Blankenhorn (130) who found that the serum of patients with bilirubinuria ("obstructive jaundice") yielded bilirubin in a dialysate while that of other jaundiced individuals ("hemolytic jaundice") yielded none. Nevertheless all the pigment in either case can be extracted with acid 50% methanol in the indirect van den Bergh reaction (50, 160). It also appears likely, as pointed out by Bennhold (11), that the non-dialyzable form of bilirubin can be excreted by the liver, implying that the organ can split the complex, but this supposition has not been directly verified.

The metal-combining β_1-globulin in fraction IV-7 forms complexes with iron, copper, and probably zinc, and evidently performs an important physiological function in iron transport (36, 96). The equilibria are shifted toward increased binding by alkaline conditions (49). Macheboeuf (158) has proposed an interesting model in explanation of the observed molar combining ratio in the copper-protein interaction.

Fraction IV-6 contains two or more glycoproteins whose properties had long been under discussion. The carbohydrates, which include hexoseamine and other sugars, are firmly conjugated to the globulin moieties (171, 252).

Finally there are at least two lipoproteins, a large β_1-globulin in fraction III-0 and a smaller α_1-globulin in fraction IV-1 (78, 180). These are found to contain 75% and 35% lipid, respectively, including steroids, phospholipids, and other lipoid-soluble molecules occurring normally in the plasma (49). At least a portion of these are not readily extracted by ordinary lipid solvents, presumably because they are conjugated firmly to the carrier globulins (40). Cholesterol and its esters are found in reversible combination with these lipoproteins (28, 49, 170) and the specific interaction of plasma estrogen (estriol) with fraction III-0 is also easily reversible (19, 207). The carotenoid precursors of vitamin A are associated with the β_1-lipoprotein (11, 181) but the interaction of the vitamin itself has not been studied. It can be assumed that steroid hormones other than estriol will be found to interact with these same globulins.

There remain a group of compounds whose interactions with globulins have not yet been fully analyzed. Shubert (226) studied various vitamins by a crude electrophoretic technique and concluded that riboflavin interacted primarily with globulin (which proved to be a eu-globulin by solubility behavior). Thiamine, on the other hand, displayed no affinity for either albumin or globulin, but its oxidation product, thiochrome, migrated with eu-globulin. Bennhold (12) reported that nicotinamide migrated primarily with globulin, and at higher concentration also with albumin. It is interesting to note that thiamine, which behaved so differently from the other vitamins, was studied at unphysiologically high concentration. It is at least possible that further study might reveal an interaction with globulin at low concentrations. In view of the known role of these substances as enzyme prosthetic groups it would seem reasonable for them all to interact with globulins.

Moore and Roaf (173, 174) showed that the volatile anesthetics ether and chloroform were more soluble in plasma than in saline. Aware of the possibility that plasma lipids might be responsible, they demonstrated the integrity of the interaction in "lipid-extracted" plasma, and consequently attributed the phenomenon to protein interaction. It now seems evident that some lipoprotein remained intact in these experiments, and furthermore, the smallest molar combining ratio that can be calculated from their data on chloroform is still absurdly large. These facts and the high lipoid-solubility of the volatile anesthetics make it appear probable that physical solution in the lipid component of the lipoproteins is responsible, rather than a specific protein interaction. Haggard (114), subsequently, could not confirm the above findings with respect to ether, in whole blood. But the same considerations probably apply in the case of ethylene and cyclopropane whose solubilities are definitely increased in the presence of plasma (183). The question could be settled by studies with purified proteins but this has not yet been attempted.

As reference to Table 1 will show, remarkably few drugs display a primary affinity for globulins. It has been claimed that a number of colloidal dyes are precipitated preferentially with globulins (67). However, some of the same dyes have been shown repeatedly to interact with albumin, and the validity of the experiments can be questioned on other grounds (21). Thorotrast (colloidal thorium dioxide) migrates exclusively with globulin (11) and displays a peculiar affinity for the reticulo-endothelial cells in the body. Colloidal gold and silver salts, which also are taken up by the reticulo-endothelial system, are bound to plasma protein (265) but it has not yet been established whether the globulins are responsible. All three bismuthyl tartrates interact exclusively with eu-globulins (5). Arsphenamine is something of a border-line case which might lead to further understanding of the minimal requirements for interaction with globulins. It is bound primarily to globulin, but also to albumin (179), while the closely related neoarsphenamine interacts only with albumin (11). The very large aromatic polypeptide trypanocide, suramin (Bayer 205), is tightly bound by the plasma proteins (143), and the evidence favors primary interaction with globulins (20, 66, 241).

In summary, the α- and β-globulins of the plasma interact as enzymes with

certain small molecules meeting a rigid specificity requirement, and as carrier proteins in a number of tight complexes with diverse normal plasma constituents. Certain water-insoluble compounds of physiological importance are bound more or less reversibly in fractions containing lipoprotein. The functional value of such arrangements in the transport of insoluble substances has been discussed at length by Bennhold (11, 12), Rothlin (216) and others (57). The very few drugs that have been proved to interact with plasma globulins contain heavy metals or are poorly soluble in water, or both.

Albumin. That the plasma albumin shows the greatest diversity of all the proteins in its capacity for interaction should be apparent from a cursory inspection of Table 1. A phenomenal array of substances foreign to the body, whether endowed with pharmacological activity or not, interacts with plasma proteins and (with few exceptions) all those that have been adequately studied prove to combine with albumin.[3] To what common features, then, do so many different compounds owe their reactivity toward this protein and what molecular properties enhance or diminish affinity for albumin?

Titration data provide an indication of the total acid- and base-combining capacity of plasma albumin and the actual numbers of ionic groups in the charged state at any pH (45, 224). These data are reasonably well confirmed by amino acid analysis, on the assumption that the sum of the diamino acids (lysine and arginine), histidine, and the terminal amino groups is equal to the potential number of cationic residues; and similarly that the sum of the dicarboxylic acids (glutamic and aspartic), tyrosine, the terminal carboxyl groups, and possibly cysteine, is equal to the potential number of anionic residues (after correction for amide-covered carboxyl groups). This leads to a figure of 109 cationic and 120 anionic residues, assuming a molecular weight of 69,000 (78).

The addition or removal of hydrogen ion is an instructive exercise because a titration is the elemental form of interaction experiment, yielding groups of different affinity (pK) as the pH is varied. The unique feature of a titration is the range of concentration (of H^+) explored—a range that cannot be duplicated with any other interacting molecule. Very likely all ionic interactions are to be regarded as competitions with hydrogen ion for the particular protein groups concerned.

The isoelectric point of albumin is at pH 4.9 so that at pH 7.3 the molecule carries a net negative charge. The early supposition that only cations would interact under these circumstances was soon shown to be incorrect. Ionic combination between groups of opposite charge occurs irrespective of the net charge on the protein, and a great many more anions than cations have actually been proved to interact at physiological pH. Some of these ionic combinations will now be reviewed from the standpoint of interaction specificity.

Interaction of the lower fatty acids (C_2–C_{10}) is clearly favored by increasing length of the hydrophobic carbon chain (2, 23, 25). This is not a function of acid

[3] For the purpose of this discussion the evidence for the existence of more than one albumin will be disregarded.

strength, which differs but slightly among the compounds investigated. The primary bond is presumably electrostatic but the resulting complex is probably stabilized by van der Waal forces through close approximation of the nonpolar residue to similar portions of the adjacent protein surface. The exceedingly strong binding of oleic acid (56, 58) is entirely consistent with this picture.

Penicillin interactions allow an extension of the same principle. These compounds may be regarded as modified fatty acids containing a peculiar cyclic dipeptide as part of the long chain (59). Penicillin K with the longest aliphatic chain (n-heptyl) shows the greatest affinity, followed, in turn, by dihydro-F (n-amyl), G (benzyl) and X (p-hydroxybenzyl) (261). The difference between X and G can be attributed to the polar group of the former which is presumably attracted away from the protein toward the water phase.

The barbiturates are also substituted carboxylic acids with an intervening ring between the anionic and substituent groups. Ultrafiltration studies (100) show that binding again increases with chain length, in the order barbital < ipral < neonal < pentobarbital = amytal < ortal. Compounds with cyclic side-chains, like phenobarbital, phanodorn, cyclopal and evipal all have about the same affinity as neonal. Allyl substituents confer somewhat greater affinity than the corresponding saturated groups and thiobarbiturates are bound much more firmly than their oxy-analogues.

Binding of sulfonamides to albumin increases in the order sulfanilamide = sulfacetimide < sulfapyridine < sulfadiazine < sulfathiazole < sulfamerazine = sulfamethazine, when the drugs are compared at the same concentration (10 mg. %) (54, 55, 98). As each of the acetyl conjugates of these drugs is bound to the same degree as the parent compound (64, 65, 98), it may be inferred that the p-amino group is not involved in the interaction. This is in striking contrast to its role in antibacterial action, for acetylation abolishes or markedly diminishes this primary drug effect. The widely divergent affinities of the sulfonamides might be attributed either to differences in acid strength or to specific influences of various rings upon the bond energy of the drug-albumin complex. By analogy to the fatty acids, and to the experiments described below, the latter explanation would appear more probable. The equal binding of sulfanilamide and sulfacetimide appears paradoxical since the former is little ionized and the latter completely ionized at physiological pH, for it is hardly possible that the negatively charged SO_2 group plays no part in the primary combination (98). The facts can be interpreted to show that sulfacetimide actually has considerably greater affinity for albumin than does sulfanilamide, since it is bound to the same degree despite its much lower effective ionic concentration.

A searching comparative study of 41 substituted sulfadiazines has been reported by van Dyke and his associates (264). The substituents were alkyl, methoxy and ethoxy groups, simple alkyl ethers, and hydroxy or phenoxy radicals. Almost any substitution on carbon atom 4, 5 or 6 increased the binding to albumin and substitution in position 4 or 6 was more effective than in position 5. The affinities of the resulting compounds differed widely, from sulfadiazine itself (β = .32, at 10 mg. %) to 4-methyl, 6-phenoxy sulfadiazine (β = .92). The

increased binding of sulfamerazine (4-methyl) over the parent compound was confirmed, as well as the failure of a second methyl group in position 6 (sulfamethazine) to enhance the binding further. There was no obvious relation between affinity for albumin and either solubility or acid strength nor was it obvious what kind of substituent most enhanced the binding. However, the acid strengths of most of the compounds were not determined so that the degrees of ionization remained unknown. Consequently some of the data, based upon total concentrations, may be misleading.

The influence of positional factors is brought out with great clarity by Teresi and Luck (259), who determined K and n for a series of aromatic anions interacting with bovine albumin. Some molecular alterations were reflected in distinct differences in the maximum binding capacity (n) of the albumin, but others produced a change in affinity (K) without affecting the value of n. For example, 25 moles of phenylacetate could be maximally bound per mole of albumin ($K = 23 \times 10^{-5}$) but the maximum binding capacity for phenoxyacetate was only 7 ($K = 1.35 \times 10^{-5}$). The introduction of a single oxygen atom between the ionic group and the aromatic ring makes 18 previously available groups no longer accessible. At the same time the strength of binding to the remaining groups is significantly increased. Similar phenomena were noted with the nitrophenolates. When the nitro group was in the m- or p- position the value of n was 22 to 25, but in the o- position it was reduced to 6. That the ortho substituent actually abolishes the possibility of interaction with 16 to 19 otherwise available groups is made plain by the fact that a para substitution in the same molecule (2, 4-dinitrophenol) no longer has the usual effect, the value of n remaining fixed at 6. Nor does a third nitro-group (picrate) alter matters. On the other hand albumin complexes with di- and tri-nitro compounds have higher bond energies (greater affinities) than with the mono-substituted anions.

In a series of iodinated anions investigated by Smith and Smith (239), affinity for albumin decreased in the order hippuran > iopax > diodrast > neoiopax > skiodan. Unfortunately the sequence entails several simultaneous molecular changes. Between hippuran and neoiopax there is an increase from one to two iodine atoms, shift of a ketone group from a position near the carboxyl to the opposite end of the molecule, and finally (in neoiopax) a reduction in length of substituent carbon chain and appearance of a second carboxyl. Although it is not clear to which of these structural differences the progressive diminution in binding is to be attributed, it is of some interest that the order of affinity might have been predicted from the general priciples elucidated in more complete investigations of other anions. Priodax, containing two aromatic rings, would probably be bound even more firmly than hippuran but its interaction with pure albumin has not yet been studied. A direct comparison between hippuran and hippurate would be particularly revealing with regard to the specific influence of iodination.

The interactions of dyes have been extensively studied, principally because it is so simple to determine their concentrations. Methylene blue was the first substance shown conclusively to interact with a soluble protein (Bechhold, 1907

(6)). As one might expect, both acidic and basic dyes combine with albumin only in their ionized forms (13). Indicators therefore only interact in one colored form, but some (*e.g.*, phenol red) dissociate doubly, carrying a charge in both forms. The great number of permutations commercially available in each dye group provides an unparalleled opportunity for studying the influence upon interaction affinity of molecular size, ring structure, character and position of ionic groups, various polar and non-polar substituents, and steric factors. But most interaction studies with dyes have been incidental to other problems, few systematic investigations having been directed toward the relation of affinity to structure among closely similar compounds. A few of the more interesting investigations are included in Table 1, and numerous other studies are indexed in Table 1a.

It has been suggested as a general rule that affinity is increased by large molecule size, particularly by aromatic rings which can contribute sizable van der Waal forces to the bond energy. The rings should be planar to facilitate close approximation to the protein surface, there should be no steric hindrance to approach, and polar groups should not be placed where their affinity for water can oppose the protein affinity of the whole molecule (138–140, 230).

These ideas emerged from the elegant studies by Klotz on several sulfonated azo dyes. *Orange I and II* contain a single sulfonate group on a phenyl ring in the para position to the azo link, and a hydroxyl group on the naphthyl ring. In orange I this hydroxyl is para to the azo link but in orange II it is in the ortho position, where an intramolecular hydrogen bond can be formed. As the affinity of orange II is appreciably greater than that of orange I, Klotz concludes that a polar substituent diminishes affinity for albumin unless its position permits reduction of its hydrophilic character (139). Azosulfathiazole consists of a sulfathiazole molecule joined through an azo link to a disulfonated naphthyl ring containing an ortho hydroxy residue. The curves obtained in saturation experiments indicate increasing difficulty of approach for successive molecules after albumin has bound the first few. This is attributed to electrostatic repulsion resulting from the presence of more than a single anionic residue. Nevertheless, this dye is bound about as firmly as *methyl orange*, which contains a single sulfonate group. As the latter contains two phenyl rings and no naphthyl, the data are interpreted to show that the larger molecular size and naphthyl ring of azosulfathiazole stabilize the dye-albumin bond despite the opposing effect of the multiple charges (138, 139). Other examples of the enhancing action of large aromatic rings on binding to albumin are found in a penicillin series recently investigated by Klotz (142). A bromophenylazo-hydroxybenzyl penicillin was bound more strongly than penicillin K, and a naphthylazo analogue showed even greater affinity. Likewise, in the experiments of Thimann and Rothschild (260), α-naphthalene acetate was bound more strongly than indole-acetate. On the other hand, *amaranth*, which contains two azo-linked naphthyl rings, has three sulfonate groups, symmetrically placed on the outer edges of the molecule. Despite the favorable ring structure, this dye shows less affinity than azosulfathiazole and methyl orange (140). Klotz concludes that symmetrical charge

arrangements are, in general, detrimental to interaction with albumin, for the reasons stated above.

Another example of the influence of symmetrical ionic groups is found in the studies by Rawson (201) on Evans blue and three related dyes. Evans blue and trypan blue are tolidine dyes containing two toluene rings azo-linked to two naphthyl nuclei, with two sulfonate groups on each of the latter. Niagara sky blue 6B and Niagara sky blue are dianisidine dyes, differing from the tolidine structure only in the replacement of methyl by methoxy in each benzenoid ring. The difference between the first and second member of each pair lies wholly in the position of the sulfonate groups on the naphthyl rings. In Evans blue and Niagara sky blue 6B these are in positions 2 and 4, along one edge of the molecule, while in the other two they are in positions 3 and 6. There is no striking difference between the binding of tolidines and dianisidines but both 2,4 sulfonates are bound very much more firmly than the more symmetrical 3,6 isomers. Gregersen and Gibson (107) had made the earlier observation that the absorption spectra of these dyes were also influenced by change in position of the sulfonate groups but not by the shift from methyl to methoxy in the phenyl rings.

That symmetrical *non-ionic* substitutions have the opposite effect of increasing affinity is shown by the behavior of a series of triphenylmethane dyes. Binding increased in the order basic fuchsin < malachite green < brilliant green < crystal violet, these dyes differing by the progressive substitution of dialkyl-amino groups in the para positions of all three rings (126).

Further pertinent information bearing on positional factors emerges from the recent work of Bueding (30, 31) showing that 1,2 naphthoquinones are bound more completely than 1,4 naphthoquinones. The introduction of an aliphatic side-chain in position 2 of a 1,4 naphthoquinone diminished the extent of interaction and further substitution of aliphatic groups in position 3 led to a greater reduction of affinity. That the 1-4 edge of the molecule is primarily involved in the interaction was clearly evidenced by the indifference of the binding to aliphatic substituents in the 5-8 positions. It might be predicted, however, that polar groups in the latter positions would diminish the extent of interaction.

The interactions of alkaloids and other organic cations have not been investigated with sufficient attention to the factors determining specificity. The semiquantitative work of Beutner (14, 15) showed that pilocarpine was more firmly bound than other alkaloids studied. It has also been shown that a number of alkaloids combine (with gelatin) only on the alkaline side of the isoelectric point (190), suggesting combination with anionic protein groups. Interactions of alkaloids with pure albumin have not yet been systematically examined.

The cardiac glycosides are outstandingly important drugs whose binding to albumin is not obviously of an ionic character. Cholesterol and other steroids combine primarily with globulins. The bile salts, which contain additional hydroxyl groups and an aliphatic carboxyl residue in position 17, combine primarily with albumin (11, 49). The active principles of the cardiac glycosides closely resemble both cholesterol and the bile acids, sharing with the former its lack of a distinct ionizing group, and with the latter their content of more than a single

hydroxyl. Like the bile salts they are bound by albumin and unlike other steroids they do not interact with globulins (85, 112, 215). The unsaturated lactone in position 17 is essential to the pharmacological activity but its specific influence on binding affinity is unknown.

Most investigators agree that a particular glycoside and its aglycone are bound to the same degree (85, 112). If this is correct, it follows that the sugar residue is of no importance in interaction, and the same must be true of the hydroxyl in position 3, which is involved in the glycoside linkage (83, 89). However, few investigators have directed their experiments toward decisively settling this question. Pairs of glycosides containing the same aglycone and different sugars should be studied (*e.g.*, digitoxin *and* digilanid A, k-strophanthin-β *and* cymarin) as well as the corresponding aglycones themselves.

The most complete comparative investigation of binding showed that affinity decreases in the order oleandrin > digilanid B > digitoxin > k- or g-strophanthin > "digitalin" > digilanid C > k-strophanthosid (112). As the various aglycones differ chiefly in the number and position of hydroxyl groups it would be interesting to correlate affinity for albumin with this single variable. No quantitative data on the binding of pure aglycones are available, and the frequent use of impure mixtures has led to considerable confusion.

If the difference in sugar residue can be ignored, the greater binding of oleandrin than of digilanid B must be caused by the difference in a single group. Oleandrigenin differs from gitoxigenin only in acetylation of its hydroxyl in position 16, adjacent to the lactone ring (89). The marked reduction of affinity from digilanid B to digilanid C (containing the same sugars) may be caused by the shift of a hydroxyl from position 16 to the exposed position 12, on the "upper" edge of the molecule. It may also, however, be connected with the unique steric arrangement (α) of the 3-hydroxyl of digoxigenin which forces the sugars of digilanid C into a different plane from those of digilanid B (89). Complete removal of the 16-hydroxyl of digilanid B, yielding digitoxin, does not lead to enhanced binding but to a slight diminution. However, digilanid B contains a sugar not found in digitoxin.

Several investigators have observed that k-strophanthin is bound poorly or not at all (85, 127, 150, 182, 215), but as the methods employed were biological (frog heart) a rapidly reversible interaction might not have been detected. Employing *in vitro* technique, Haarman (112) found that k- and g-strophanthin were significantly bound but (as confirmed by Rothlin (215)) pure k-strophanthosid was not. If, as seems probable, the aglycone strophanthidin has a very low order of affinity for albumin, it must be attributed to the aldehyde group in position 10. The poor binding of both strophanthidin and digilanid C would seem to show a deterrent effect of polar substituents along the "upper" edge of the phenanthrene nucleus, reminiscent of an analogous effect in the dye orange I (p 141).

Further quantitative investigations of the cardiac glycosides and aglycones should not only clarify the influence of the sugars but should also reveal whether the lactone represents a point of primary attachment to albumin, and whether

the albumin-aglycone bond is to be regarded as entirely non-ionic. If the latter is the case, variation of pH and consequent alteration of charges on the protein should not affect the extent of interaction. In this connection Hoekstra (127) claimed that the digitoxin-albumin combination dissociated above pH 8.3, a finding not confirmed by subsequent investigators (85, 86, 113).

Substances that apparently do not interact with plasma proteins. For the reasons outlined in the discussion of methods, failure to obtain evidence of interaction may often be the result of inadequate technique, particularly when the possibility of binding is not explored at low enough drug concentration. An instructive example of this type of error is to be found in the work of Paget and Vittu (185), who sought to demonstrate the sulfanilamide-serum interaction by change in refractive index of a protein solution. Apparently because the method was not sensitive enough to small changes, concentrations of 250 to 500 mg. % were employed. Of course, no interaction was detected because at these concentrations the *fraction bound* is entirely negligible so that the change in refractive index was essentially that to be expected from the total sulfanilamide concentration.

There is general agreement (11, 224) that sodium and potassium ions are not appreciably bound. From solubility data (183) it is to be concluded that nitrous oxide is not bound in plasma, and the same is true of ether if Haggard's (114) data are accepted over those of earlier workers (174).

Urea is generally regarded as unbound in the plasma. The electrophoresis studies of Bennhold (11) are offered in support of this view but they were carried out at concentrations over 100 mg. %. On the other hand evidence in favor of interaction under physiological conditions has been obtained by three other investigators (153, 159, 214) while the denaturant action of urea on albumin at high concentration is well known (22, 24).

The question of a reversible interaction between glucose and plasma proteins must be left open. Bennhold (11) was unable to observe any migration of glucose with either albumin or globulin in an electric field, but de Haan (60) claimed that in bovine serum about 25 % of the glucose is not ultrafiltrable. Moreover, glucose compounds are found in the glycoprotein conjugates of fraction IV-6 (171, 252).

Streptomycin, like glucose, is highly polar, and interacts weakly if at all. Several investigators (142, 262) have been unable to observe binding by physical methods but antibacterial potency is unquestionably reduced in the presence of albumin (122, 142). This suggests that binding may be found only at concentrations too low to determine by non-biological methods. In explanation of these findings it has been proposed that the hydroxyls arrayed about the streptidine ring outweigh the expected electrostatic attraction of the charged guanidine groups for anionic centers on the albumin (59).

All but one of the porphyrins interact with albumin (3, 11, 50, 97, 164, 165, 210), while bilirubin (49, 165) and hematin (181) also form specific complexes with globulins. The coproporphyrins contain a single carboxyl group at the outer edge of each of the four nitrogenous rings, and these compounds migrate

with albumin. Addition of four more carboxyls leads to the uroporphyrin structure in which every reactive position on the periphery of the molecule is occupied by an ionic group. The uroporphyrins do not interact with either albumin or globulins (11, 97).

The probability that strophanthidin and its glycosides do not interact with albumin has been considered above. The failure to demonstrate any binding of thiamine has also been discussed. It cannot be accepted as proved that this vitamin is entirely unbound at normal plasma concentrations. It has been claimed that histamine and choline do not interact with plasma proteins (14), but while they obviously are not bound as extensively as some other alkaloids, the possibility of some interaction has not been adequately ruled out.

The location of binding sites on the protein surface. Attempts to localize interactions to specific reactive groups of plasma proteins were obviously out of the question until one of them had been completely purified and characterized with respect to content of amino-acids and their acidic and basic dissociation constants. A few bold thrusts at the problem have now been reported in connection with albumin interactions.

Klotz (140) has found that the combination of methyl orange and azosulfathiazole with albumin, as measured by spectral alteration, is constant as the pH is varied between 5 and 9 but complete dissociation occurs between pH 9 and 11.5. This corresponds very closely with the dissociation range of the ϵ-amino groups of lysine, histidine giving up its protons at about pH 7, and arginine guanidinium groups remaining charged up to pH 12.6 It was therefore concluded that these dyes probably combine with the ϵ-amino groups of lysine. It was not obvious why the arginine groups should have been unavailable since protamines, consisting largely of arginine, interact strongly with various sulfonates. On the other hand, these dye interactions yield a value of 22 for n, on the basis of which Klotz (138) had earlier concluded that the combination was with arginine, since the figure corresponded so closely to the total number of residues of this amino-acid. There are 59 lysine residues and if, as the pH variations now suggest, the dyes interact with ϵ-amino groups, only half of them must be involved. This would raise the perplexing question why half the lysine amino groups should be inaccessible.

The experiments of Teresi and Luck (259) with aromatic carboxylic acids and nitrophenols led to values of n in two categories, some falling between 22 and 25, others between 6 and 9. The former corresponds, as stated above, with the number of arginine residues, but it is improbable that these anions would continue to interact up to pH 12.6 since the small change from pH 7.6 to 8.2 led to a diminished affinity in all those cases where the interacting compound itself was fully ionized. Values of n between 6 and 9 correspond roughly to the number of terminal α-amino groups but it seems doubtful that these are specifically involved in the interactions.

From the standpoint of an approaching small molecule the individuality of the separate amino-acids is probably submerged in the totality of the surface pattern of the protein. Much as the doors and windows of a house cannot be

defined in terms of the characteristics of the component bricks, so may a specific binding site represent a new quality that is more than the sum of amino-acid residues. The characteristics of each such site may depend not only upon the properties of its ionic groups but also upon those of neighboring non-ionic residues, and upon steric hindrances to approaching molecules, and surface configurations affecting the strength of van der Waal forces. If there is any truth in such a concept the occasional correspondence between a value of n and the number of residues of a particular amino-acid must be regarded as fortuitous. The nature of the binding sites would then have to be elucidated by methods considerably more refined than have yet been employed.

PHARMACOLOGICAL SIGNIFICANCE OF DRUG-PROTEIN INTERACTIONS. Most drugs, at therapeutic concentrations, interact with one or more of the plasma proteins. Before any pharmacological significance can be attributed to these interactions, three fundamentally different possibilities must be considered:

(1) Drug bound to plasma protein is pharmacologically active.
(2) Only unbound drug is active but the drug-protein complex can penetrate to the sites of drug action, where special mechanisms transfer the drug molecule to receptor proteins.
(3) Only unbound drug is active or freely diffusible and the drug-protein complex is generally confined to the circulating plasma.

The first possibility can be dismissed at once. Biological methods for the study of interactions owe their usefulness to the diminution of drug activity caused by interaction. Microbiological techniques permit direct proof that the potency of an antibacterial drug in the presence of protein is exactly that of its *unbound* fraction, which can be independently estimated by a non-biological method. The same proof is available for any drug whose potency is assayed against an isolated organ in the presence and absence of plasma protein.

The second possibility—that plasma proteins act as carriers for drugs—requires more serious consideration. Bennhold (11, 12) observed that numerous substances appeared to be completely and irreversibly bound at usual concentrations. At a loss to explain how these compounds could diffuse out of the vascular channels, he proposed a theory of "directed transport" according to which the carrier proteins themselves escape from the blood stream and enter tissue cells. There still remained the problem of intracellular liberation of bound drug, which could be resolved only by postulating special dissociation mechanisms in the various organs.

In support of this theory Bennhold cited experiments of von Jancsó (265), Schulten (227), and Plattner (193).[4] These showed that under very limited conditions certain dyes and colloidal gold suspensions perfused through liver or kidney do not enter the cells of these organs *until* albumin is added to the perfusion fluid. One then observes prompt uptake by reticulo-endothelial cells in the liver, or excretion by the tubules of the kidney. So many anomalous results complicated these experiments that the investigators themselves were unwilling to draw any general conclusions. It is noteworthy that the results were obtained

[4] Incorrectly attributed to Höber and Titagew.

with colloidal substances which are known to be disaggregated by albumin. Certainly the conclusion that albumin combines with the colloidal molecules and carries them over into the cells is not warranted.

Further support for the "directed transport" theory was drawn from the studies of de Haan (62, 63) on the phenomenon of phagocytosis, where there is good evidence that ingestion of particulate and colloidal matter is facilitated by protein. But there seems little justification for extending these observations to other cells and physiological membranes.

Hoekstra (127, 128) found that protection against the toxicity of digitoxin for the frog heart was afforded by rabbit serum but not by frog serum, while in the rabbit heart the efficacy of the two sera was reversed. It was concluded that the complex of digitoxin with homologous protein must enter the heart, a feat of which heterologous protein is incapable. These provoking findings have not been confirmed by other investigators.

While the relative impermeability of the glomerular membrane to protein seems well established (82, 202), certain capillaries must evidently allow passage to albumin molecules (96). The protein content of cervical lymph in dogs may be as high as 2.6% (217), most of which must have escaped from the vascular channels. Gregersen and Rawson (108) attributed the disappearance of Evans blue from the blood stream to escape of albumin since the dye is almost completely albumin-bound, and this disappearance is exceedingly slow. The well-devised experiment of Pfaff and Herold (191) also indicates a negligible escape of albumin. When a mixture of green and blue dyes was injected intravenously in a rabbit and the mesentery observed microscopically, the green dye, which was completely bound to plasma protein, remained well confined within the vascular channels, but the less completely bound blue dye promptly diffused into the tissue spaces. Only when severe inflammation was artificially induced did the green dye appear beyond the capillary walls, presumably still attached to escaping albumin molecules. However, the ultimate mode of disappearance of the green dye from the blood stream was not investigated.

As the chain of logic that forced Bennhold to the theory under consideration originated in the apparent irreversibility of a number of interactions, the nature of the evidence for such irreversibility will be reviewed briefly.

If it is sometimes difficult to demonstrate reversibility, it is almost impossible to prove irreversibility. Most drug-protein complexes, as Table 1 shows, dissociate readily upon simple dilution or dialysis, because an appreciable concentration of unbound drug is in equilibrium with them. When dissociation constants are small and drug concentrations low, apparently stoichiometric complexes are formed. These, too, should dissociate readily whenever the equilibrium is disturbed by rapid removal of unbound drug, no matter how low its concentration. However, such methods as ultrafiltration and electrophoresis that do not markedly alter an equilibrium would not permit the detection of unbound drug in such cases, and irreversibility could neither be proved nor disproved. Therefore Bennhold's frequent observation that *all* of a substance migrated electrophoretically with a protein fraction is not a valid basis for his conclusions about irreversibility.

Even prolonged dialysis does not suffice to rule out simple reversibility because

the rate of diffusion out of the bag is proportional to the concentration gradient across the membrane. If extremely little unbound drug were present none might be detected in the dialysate in any reasonable period of time. Heymann and Fieser (124), finding that the hydroxy-naphthoquinone M-1971 (at 116 mg.%) did not dialyze out of albumin, placed the bag in another albumin solution. This should have trapped any drug diffusing out, preventing back-diffusion. As none appeared in the dialysate under these conditions it is fair to conclude that very little unbound drug was present. However, even this novel technique does not overcome the objections stated above, and irreversibility cannot be regarded as proved. In the capillaries, or in direct contact with microorganisms, circulating plasma is exposed to so phenomenal a surface area for diffusion that conclusions about irreversibility based on dialysis might be entirely invalid.

There would appear to be at least two fairly rigid tests of reversibility. The drug-protein complex might be greatly diluted, followed by separation of the protein (e.g., by ultrafiltration) and determination of drug concentration in the water phase after evaporation to the original volume, or by adsorption techniques. Alternatively an adsorbent of high affinity for the drug might be introduced into the original solution containing the complex. Kimmig and Weselmann (134) were thus able to show that a highly bound sulfonamide that could not be detected in an ultrafiltrate of plasma was easily removed by charcoal. In either technique there must be assurance that the integrity of the protein is not destroyed.

The foregoing remarks are not intended to detract from the fundamental importance of Bennhold's contributions to this field but only to indicate, with Rothlin (216), that a "vehicle function" of plasma protein cannot be accepted in the teleological sense of a specially-designed mechanism for directed transport of various substances, including drugs. The means whereby the several globulin conjugates are split to make the molecules they carry available for metabolic processes are not understood. But the behavior of drug-protein complexes is quite compatible with mass law principles and the main effects of interaction upon the fate of drugs in the body can be analyzed without recourse to special theories of transport or unique mechanisms of dissociation.

The primary effect of interaction, then, as suggested in the third possibility proposed at the beginning of this section, is to confine drug molecules within the blood stream in an inactive form, and thereby hinder their access to the sites of drug action, excretion and metabolism. This concept is illustrated schematically in *Figure 9*. It was first formulated (in extension of Ehrlich's ideas) by Storm van Leeuwen (248, 249) who proposed that drugs combine not only with so-called *dominant receptors* responsible for primary pharmacological action, but also with numerous *secondary receptors* in the body tissues. The effect and fate of drugs *in vivo* was considered to depend upon the *distribution* of drug molecules between the dominant and secondary receptors. In the plasma, the secondary receptors were identified as the plasma proteins.

Drug excretion and diffusion. Protein binding played a pivotal role in the development of current concepts of renal physiology, and the first proof of tu-

bular secretion (Marshall and Vickers (161)) rested upon the large discrepancy between the rapid rate of excretion of phenol red and its poor ultrafiltrability from plasma. Subsequent studies led to the consistent result that the fraction filtered at the glomeruli corresponds to that ultrafiltered *in vitro* (162, 192, 229). That essentially *all* the dye is removed in a single passage of blood through the kidney (162, 235) despite the fact that 80% is in protein combination implies that protein-bound molecules are fully available *for tubular secretion*, and also that such protein complexes can dissociate with extraordinary rapidity.[5]

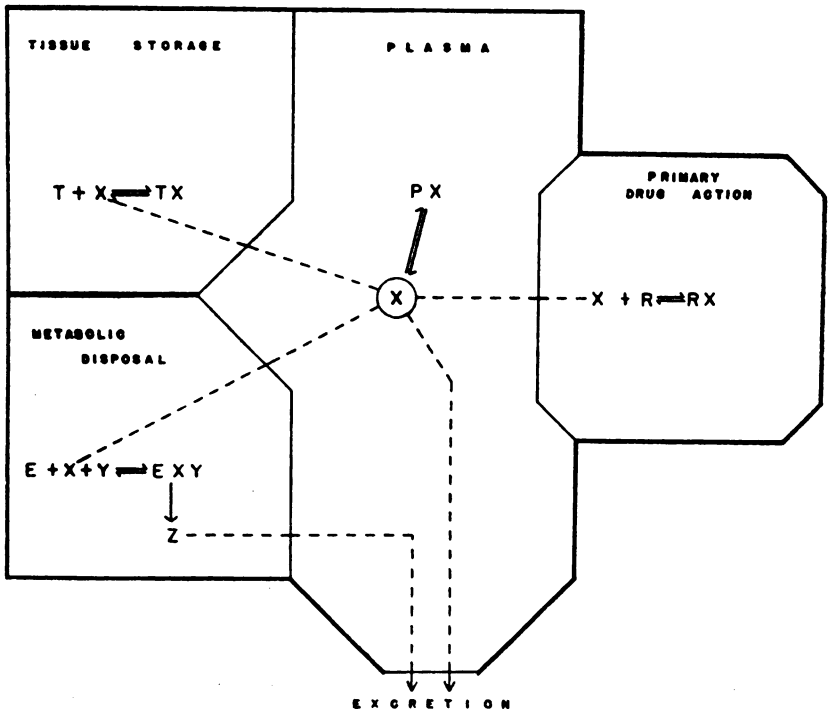

FIG. 9. POSTULATED INFLUENCES OF VARIOUS REVERSIBLE INTERACTIONS, INCLUDING THOSE WITH PLASMA PROTEINS, UPON PRIMARY DRUG ACTION

The divergent effects of interaction upon glomerular and tubular excretion amply confirmed with numerous substances, are entirely reasonable. As no dilution is entailed, the filtration of about one-fifth of the plasma water at the glomeruli does not disturb reversible equilibria. Only when the plasma volume is reconstituted in the lower nephron would bound drug be expected to dissociate. But the rapid removal and excretion of unbound drug by the tubule cells would of necessity lead to dissociation of a drug-protein complex.

The behavior of phenol red at the tubules is duplicated by penicillin G which

[5] Whether all drug-protein complexes are capable of dissociating as rapidly is still unknown but would seem unlikely. Study of the kinetic aspects of both combination and dissociation should yield information of prime theoretical and practical importance.

is more than half bound to plasma protein (74), and by diodrast and hippuran (235) which are less completely bound (239). That there is but a single mechanism of tubular secretion is evidenced by the competition of all these substances for excretion (157, 235) and it is of some interest that they also compete for attachment to plasma albumin (239).

Obviously no valid statement can be made about the mechanism of renal excretion of any substance without full knowledge of its fractional binding at the concentration employed. It is incorrect to assume that a drug is filtered and resorbed, simply filtered, or filtered and secreted, on the basis of clearance with respect to that of inulin. Clearance must first be calculated on the basis of *ultrafiltrable* plasma concentration. Fortunately, neither inulin nor creatinine appear to be bound appreciably at concentrations usual to clearance studies.

The sulfonamides, because of their low clearances, had been generally assumed to leave the plasma by glomerular filtration alone, but Lundquist (157) presents sound evidence that the filtrable fractions of sulfathiazole and sulfamethylthiadiazole are not large enough to account for the clearances observed. The conclusion that these drugs are partly excreted by the tubules is confirmed by the effects of diodrast and phlorhizin, both of which reduce the sulfonamide clearance by about two-thirds, to the filtration level. Caronamide, which inhibits the tubular excretion of other substances, was also initially thought to be cleared solely by glomerular filtration but allowance for protein binding again permits the conclusion that some, at least, is excreted by the tubules (77).

The mechanism of hepatic excretion is poorly understood but evidently, as at the renal tubules, both unbound and bound drug can be removed from the plasma. Thus bromsulfalein, despite its high fractional binding, is quantitatively excreted in the usual liver function test (211). Brauer (26) observed, however, that in perfused or sliced rat livers, the *rate* of uptake of the dye was much reduced by albumin.

Diffusion of drugs and other substances into the cerebrospinal fluid has been the subject of numerous investigations that are outside the scope of this review. With respect to most diffusible molecules cerebrospinal fluid is generally regarded as an ultrafiltrate of plasma (60, 61, 64). Davis' (53–55, 57) proof that this is true of the sulfonamides and the practical therapeutic implications of his findings are too well known to require restatement here. The diffusion of a number of dyes from the blood into various body fluids is unquestionably hindered by protein binding (61, 62, 99, 108, 124, 191, 231).

Plasma urate presents a curiously anomalous behavior, for despite an ultrafiltrable fraction of 80%, it appears in cerebrospinal fluid to only 6% of its total plasma concentration, and its renal clearance is correspondingly low (276). It is postulated by Wolfson and his associates (277, 278) that about 70% of the total exists in some unique non-diffusible form, probably polymeric in nature, and some experiments on normal and azotemic chickens (151) lend support to this hypothesis. If such a polymer exists the cerebrospinal fluid urate would presumably be in equilibrium with the monodisperse plasma urate. It is not clear why the supposed polymer should pass so freely through an ultrafiltration membrane.

Drug persistence. Some drugs are selectively concentrated and stored in certain organs, yet the process is obviously reversible since they all slowly disappear from the body. One wonders whether this binding to fixed tissues is not wholly compatible with the principles governing reversible binding in the plasma. The effective intracellular protein concentration in various organs (for example, the liver) is obviously considerably higher than would appear from a calculation of gross protein content. If the affinity of these proteins for a drug is very great, "complete" binding would be expected to result, and the total amount that could be thus bound and stored would be roughly determined by the mass of the organ. An extremely minute circulating plasma level might then be in equilibrium with large amounts of drug so stored. After a large single dose of such a drug one would expect a rapid disappearance from the plasma and excretion into the urine, fractional binding being low while drug concentration is still high. But the excretion rate should soon become negligible while large amounts of drug remain in the body to be released very slowly from tight combination with fixed tissue and plasma proteins. This description will be recognized as typical of the behavior of such substances as emetine, the organic arsenicals and antimonials, quinacrine, and suramin.

The case of suramin is a particularly remarkable one. Although there is no selective storage in any fixed tissue (20), the drug confers protection against trypanosomiasis for weeks to months after a single injection (167), and its actual presence in the plasma for this period has been demonstrated (241). That it is firmly bound to plasma protein is well established but it is not entirely clear whether albumin or a globulin is primarily responsible (66, 143, 168). Spinks (241) has shown that interaction depends upon the enormous molecular size, aromatic polypeptide structure, and terminal sulfonate groups, and that persistence, in a series of related compounds, can be roughly correlated with affinity for plasma proteins. It has been suggested that the colloidal nature of the molecule makes it ineligible for renal excretion (59) but such excretion does occur during the first few days after injection (20, 241).

The iodinated aromatic anion, priodax, also persists in the plasma for months, apparently tightly combined with protein (205, 206). To what extent differences in the persistence of other drugs are also to be explained on the basis of interaction with plasma or tissue proteins cannot be decided from the meager data at hand.

Chemotherapy. The fact that suramin is therapeutically active despite its nearly complete binding might seem paradoxical. In Davis' (59) opinion the contrasting effectiveness of suramin and ineffectiveness of penicillin K in plasma is to be explained on the basis of relative potency. Quinacrine, for example, and related amino-acridines, are bound as much as 98% at therapeutic levels, the unbound 2% evidently sufficing to inhibit plasmodial growth (254). The diamidines are so highly bound in plasma that their antibacterial action is abolished (92), but they remain effective against trypanosomes. The therapeutic action of naphthoquinones against plasmodia (124, 238) and schistosomae (30, 31) is markedly reduced by plasma protein. The impressive inhibition of growth of the tubercle bacillus by oleic acid is nullified by traces of albumin (56, 58).

But penicillin G and sulfadiazine are effective antibacterials despite fractional binding of more than 50%.

The variant and unpredictable influence of plasma protein binding upon the behavior of different chemotherapeutics toward a variety of organisms emphasizes the wisdom of avoiding generalizations in this field. For example, the simple statement that protein binding antagonizes the antibacterial action of penicillin K obscures a possibly important therapeutic advantage. This drug is over 90% bound at concentrations considered adequate for other penicillins (261). If the fractional bindings were not much smaller at very much higher concentrations (cf. Figure 7) a significant therapeutic advantage could be gained. The unbound 10% would exert the desired antibacterial effect and the bound 90% would dissociate as excretion proceeded, to "buffer" the unbound penicillin level. The plasma proteins would provide a flexible reservoir of penicillin serving to damp otherwise rapid fluctuations of level. It may be that if an effective *unbound* concentration can be maintained, the capacity for protein interaction is to be desired in a chemotherapeutic agent.

Displacement of bound drug. A surprisingly large number of casual observations have been reported showing that substances interacting with plasma proteins can be partially or completely liberated by introduction of other substances into the system. The reports are summarized in the eighth column of Table 1.

In some cases there is obvious competition for binding sites, as in the reduction of anion binding by alkali and of cation binding by acid (178, 242); or, conversely, the displacement of titration curves when the character of the anion is varied (243). The depression of the binding of one anion by another has also frequently been observed and may be of some practical importance in the use of buffers for interaction studies. Phosphate, for example, interacts with albumin and may displace other anions (2). Barbital (Veronal) significantly depresses the binding of phenol red (208). A number of carboxylic acids promote dissociation of complexes between anionic dyes and albumin (140a). Hippuran and diodrast displace phenol red from its albumin combination (239).

There are other instances of displacement which can reasonably be attributed to competition between compounds that are known to compete for physiological receptors or that are structurally similar. Atropine antagonizes the binding of pilocarpine (14). Bile salts and saponin were reported to split the digitoxin-albumin complex (127) but neither finding has been confirmed. Carbamic ester inhibitors are displaced by acetylcholine from their combination with the plasma cholinesterase (102) and flavoenzyme inhibitors are displaced by the enzyme prosthetic groups (121).

There remain a number of curious cases where the relation between the antagonistic substances is by no means evident. The displacement of pilocarpine was effected not only by atropine, as stated above, but also by benzene, xylol, several phenols and guaiacol (which itself interacted with serum) (14, 249). A series of aliphatic alcohols and their esters had the same action, hexyl alcohol proving most efficient (16). (Cf. the optimal stabilizing effect of C_7 carboxylic acid (23).)

Bile salts were reported to liberate rose bengal, bromsulfalein and bilirubin from plasma combination (212, 213) but this could not be confirmed, and glycocholate has since proven ineffective as a displacing agent in several cases (85, 124). Heparin replaces cephalin in the combination with thromboplastic lipoprotein (38, 39). The tightly bound naphthoquinone M-1971, that could not be liberated by dialysis, readily dissociated from albumin upon addition of isoamyl alcohol (124).

Finally, ether, at anesthetic concentrations, was reported by Storm van Leeuwen (247) to dissociate the serum protein complexes of a number of alkaloids. Dale (52) showed that the sensitivity of cats to histamine is increased ten-fold by etherization, an observation that might be explained by displacement of histamine from combination in the plasma. However, there is still no good evidence that such a combination exists. It has been reported recently that etherization and dial-urethane both increase the passage of sulfathiazole into the cerebrospinal fluid. The investigators attributed the finding to vascular dilatation but noted that despite equivalent vascular changes chloroform does not duplicate the effect (257). The possibility of dissociation of the highly bound sulfonamide from albumin under the influence of ether was not considered.

These numerous observations, not yet falling into any intelligible pattern, pose stimulating questions for pharmacological study. There are obviously occasions, in the normal or pathological state, when the concentration of a drug may be sufficient to displace other drugs or normal plasma constituents from their protein combinations. If the fractional binding of such a displaced substance were initially great, the effect of the displacement could be a sudden and substantial increase in the unbound plasma concentration. It is not difficult to imagine the possible dangers such episodes might present, but whether they really occur, under what conditions, and with which competing drugs, is entirely unknown.

Species and other differences in binding capacity. That the serum of different species varies considerably in the capacity to protect against drug effects has been appreciated for many years. These differences have frequently been observed with drugs known to be bound exclusively by albumin, so it may be inferred that chemical reactivity of the respective albumins is the principal variant. The superior binding properties of rabbit serum toward various drugs has been almost universally noted. Beutner (14) found the relative effectiveness of several sera in binding pilocarpine to be rabbit > goose > sheep > human > horse > bovine, while pig serum was as ineffective as egg albumin. Storm van Leeuwen (245) reported the sequence rabbit > cat = bovine > dog for the same compound. In both studies the order of affinity was the same for other alkaloids as for pilocarpine. In the binding of cardiac glycosides Farah (85) found the order rabbit > dog > human > frog. Kocian (143) studied the stabilizing effect of suramin against protein coagulation and reported the order carp > duck > human > horse > pig = bovine = rabbit > goose > chicken. Recently Wendel (268) and also Heymann and Fieser (124) showed that naphthoquinone antimalarials are bound to a very different extent by duck and human plasma. Moreover,

the relative binding power of the two sera was different for each compound tested. Most drugs are bound to about the same extent by human, bovine and horse plasma, a fortunate coincidence since these are the common sources of albumin for experimental work. In general, however, the complete unpredictability of species differences and the variation of relative binding power with different drugs dictate caution in transferring interaction data from one species to another.

Differences in the binding capacity of human plasma have been meagerly examined. Farah (85) reported that in the binding of digitoxin by the serum of normal subjects there were distinct differences that proved, upon repeated test, to be characteristic of each subject.

Storm van Leeuwen (250, 251) claimed to have shown that the sera of asthmatics hypersensitive to aspirin had a dimished binding power toward the drug as compared with normals and other asthmatics. The data, however, do not show impressive differences.

The plasma proteins undergo profound alterations that disrupt the normal electrophoretic pattern, in a number of conditions, including even so acute an episode as lobar pneumonia (111a, 279). It is well known that the plasma proteins are grossly disordered in such states as nephrosis, cirrhosis, myeloma, the collagen diseases, and various parasitic infestations. In few of these has the binding capacity of the plasma proteins been studied.

The abnormal loss of congo red from plasma in patients with amyloid disease was regularly observed by Bennhold (11). In nephrotics without amyloid the binding capacity of serum toward the dye was also substantially below normal. It was subsequently shown that nephrotic serum has a depressed binding power toward many other substances and that this depression is disproportionate to the decrease in plasma albumin.

The possibility that impairment of the binding capacity of plasma proteins may be a significant factor in many diseases or may play a part in unusual sensitivity or resistance to drugs has not yet been systematically investigated.

Drug allergy. The vexing question of drug allergy appears to be closely connected with protein interactions in general. Following Landsteiner's method of producing antigens by coupling foreign substances to proteins through diazo linkages, a number of investigators have succeeded in producing artificial drug antigens and obtaining specific antibody responses from experimental animals. Over twenty years ago Mayer and Alexander (170a) did this successfully with atoxyl and since then the experiment has been repeated in standard form with aminopyrine and related compounds (176, 177, 218), strychnine (129), epinephrine (269, 270), aspirin (34), histamine (87, 209), organic arsenicals (232), tyrosine (42), thyroxine (43), sulfonamides (95), and probably others. The drug, coupled firmly to protein, is injected over a period of several weeks into a test animal. The resulting antiserum can then be titrated by a precipitin reaction against the original antigen; or the anaphylactic response of the whole immunized animal or of its isolated organs can be elicited. The protein employed as antigen need not be heterologous. In some cases only albumins were suitable as antigens, purified globulins giving wholly negative results.

The antibody globulins obtained by these techniques can be characterized as follows: They react with the original antigen but the reaction can be blocked by the specific haptene employed, or by closely related compounds—*i.e.*, the drug used for sensitization interacts specifically with the antibody. In the intact animal, or in isolated organs, the anaphylactic response can be elicited only once; if first blocked by the haptene drug it cannot be elicited at all. The extent of interaction between antibody and compounds closely related to the haptene drug may vary considerably. Gerber and Gross (95) showed that while cross-reactions occurred between sulfanilamide, sulfacetimide, and sulfanilic acid and their respective antisera, the specificity did not extend to sulfathiazole, para-aminobenzoic acid, or 2,4- nitroaminophenol.

Although the obvious practical aim in studies of this type would be to obtain antibodies capable of neutralizing certain drug effects *in vivo*, few very successful outcomes in this direction have been reported. Clutton and co-workers (43) presented evidence that antisera against thyroxyl-thyroglobulin protected rabbits against exogenous thyroglobulin or thyroxin, but no alteration of the normal basal metabolic rate was observed. Butler and his associates (34) found that antisera against aspirin-protein complexes protected rats dramatically against the antipyretic action of aspirin when artificial fever was induced. Fell and his collaborators (87) observed slight protection against the anaphylactic action of ovalbumin in guinea-pigs and rabbits by an anti-histamine immune serum. Singer (232) presented data purporting to show that the LD_{50} for mice injected with certain arsenicals was appreciably altered by an anti-arsenical immune serum. Hooker and Boyd (129) calculated the probable amount of antibody globulin produced in typical experiments of this kind and showed the improbability of a titer high enough to neutralize any significant proportion of injected drug, unless the drug were so highly potent that the therapeutic dose contained relatively few molecules.

It is difficult to understand how, if so drastic a coupling procedure as diazotization were required for the production of an antigen, such substances could ever arise *in vivo*, as they must, if drug allergy is to be explained on this basis. It is therefore highly significant that in a few instances antigenic substances have been produced without diazotization. Clutton, Harington and Yuill (42) starting from the premise that both tyrosine and carbohydrate play significant roles in normal antigenicity, linked glucose to tyrosine and the latter through a peptide bond to protein. Potent antisera to this conjugate were produced by rabbits when either horse albumin or globulin was used as carrier. A similar peptide-linked antigen was successfully produced from the isocyanate of histamine, which was presumed to react with the lysine ϵ-amino groups of horse globulin (209).

While the linkages between haptene and protein seem more natural in these compounds than in the azoproteins, they are still far from the reversible bonds typical of drug-albumin complexes. A closer approach to the latter is found in the observation that the Wassermann or Forssman haptenes become antigenic on simple contact with *heterologous* serum, no drastic treatment being required

(267). The only recorded observations of similar spontaneous development of antigenicity with *homologous* serum, are those of Samson and Götz (218). Finding that aminopyrine at 0.4% in horse serum yielded no unbound drug in an ultrafiltrate, these investigators proceeded to sensitize guinea-pigs in the following simple way. Fresh blood was drawn from the animals and allowed to stand with aminopyrine for thirty minutes. The mixture was then injected intraperitoneally, the process being repeated at intervals of 5 to 10 days. At the end of this time an allergic response was obtained upon injecting a similar mixture (containing less drug) intravenously. Adequate controls ruled out non-specific factors but the phenomenon was peculiar in that the pure drug would elicit an allergic response if injected after sensitization. Moreover, pure aminopyrine in aqueous solution (without blood) could be used for sensitizing, but then a response was no longer elicited by the drug itself but only by the drug-blood mixture. No attempt was made to demonstrate antibody in the circulating plasma, and the experiments are open to criticism in other respects, but the observations conform so closely to the spontaneous development of clinical drug allergy that they might be profitably re-examined.

The fundamental question, of course, is whether the common interactions of drugs with proteins in the plasma are capable of giving rise to antigenicity, and if so, under what conditions. There is no particular evidence to suggest a parallelism between affinity for plasma proteins and allergic potentiality but this does not bear directly upon the question. Obviously interaction of a drug with plasma protein is not a *sufficient* condition for allergy, or *all* penicillin-treated patients would become sensitive to the drug. Whether interaction of the kind discussed in this review is a *necessary* condition remains to be proved.

* * *

I would emphasize again, in conclusion, what is probably the most fundamental aspect of the interactions of drugs and plasma proteins. These diverse combinations must be regarded as model systems for elucidating the nature of the primary interaction of each drug with its protein receptor in the body. In isolated instances the affinity for plasma protein appears to parallel the primary potency of a drug. Davis and Wood (53) have shown that this is true in the sulfonamide series. Another parallelism has been indicated in the competition of certain substances for tubular excretion and for binding to albumin. But it is not generally the case. Even among the sulfonamides acetylation abolishes antibacterial action without affecting protein binding. Penicillin K and X, in the absence of albumin, are both more potent antibacterials than G, although the order of affinity for albumin is K > G > X. In the barbiturate series binding cannot be correlated with either therapeutic potency or susceptibility to metabolic destruction in the body.

Simple parallelisms are not to be expected. Only as we elucidate the nature of the forces that are responsible for interaction specificity, and begin to gain insight into the precise nature of the protein surface, shall we also be furthering our

understanding of the remarkable capacity for specialized protein interactions that distinguishes the potent drug molecule from its biologically indifferent kin.

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