PROTEIN BINDING STUDIES BY DIAFILTRATION

by

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A THESIS SUBMITTED IN PARTIAL FULFILMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE IN PHARMACY
in the Division of Pharmaceutical Chemistry
of the Faculty of Pharmaceutical Sciences

We accept this thesis as conforming to the
required standard

THE UNIVERSITY OF BRITISH COLUMBIA
APRIL, 1972
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Date **3-5-72**
A diafiltration technique was used to study drug-protein interactions. Fraction V human serum albumin and plasma and two drugs (phenylbutazone and bishydroxycoumarin) with a high affinity for these substances were used in this investigation. Preliminary experiments were carried out to check for release of foreign substances and for binding of drug to the Amicon diafiltration apparatus. A binding experiment, in the absence of drug, revealed release of a protein-like, ultraviolet absorbing substance from Fraction V human serum albumin. The most suitable method of purification for albumin was by diafiltration with Tris buffer.

Binding curves for bishydroxycoumarin - human serum albumin, phenylbutazone - human serum albumin, and bishydroxycoumarin - plasma interactions were obtained. The $r$ and $r/D_f$ values were calculated and binding parameters estimated by both graphical extrapolation and by a computer non-linear least squares fit analysis. Binding curves were not independent of human serum albumin concentration, but the cause of this effect was not fully resolved.

Results showed the diafiltration technique can yield precise data, can be used over a wide macromolecule concentration range and produces a binding curve, from one experiment, over a wide range of molar binding ratios.

Use of the Amicon diafiltration apparatus in desorption (washout) experiments and equilibrium or direct experiments was also investigated.

Attempts were made to obtain binding data by centrifugation (ultrafiltration) and by a gel filtration technique (Sephadex G-25 batch method).
These methods yielded unsatisfactory results which could not be compared with those obtained by diafiltration.

This abstract represents the true contents of the thesis submitted.


Supervisor
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ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and appreciation to:-

Dr. M. Pernarowski for his encouragement and guidance during my studies.

Dr. A. G. Mitchell for academic advice.

Mr. Rolf Klinger (Electrical Engineering Department) for his valuable assistance in preparing computer programs.

Phyllis Moore for her able typing of this manuscript.
I. INTRODUCTION

Serum albumin is a unique molecule. It binds extensively with most substances. However, lack of specificity distinguishes its binding characteristics from those between an enzyme and its substrate where a high degree of specificity exists. Goldstein, in 1949, indicated the significance of drug interactions with proteins and most of the theory in this area has been developed using serum albumin as the protein model.

Numerous studies, both in vivo and in vitro, have been undertaken to more fully understand the nature of the interactions of ligands with protein molecules. Generally, most of these investigations have been an attempt to quantitatively study how much of a ligand binds, the number of binding sites on the protein, and the free energy changes involved in the process. Qualitatively, studies have emphasized the type of forces involved and the nature of the binding sites.

Methodology, in such studies, has varied widely. In quantitative studies by far the most commonly used method is equilibrium dialysis. The chief disadvantages of this method are that it is time-consuming and limited to low protein concentrations. Furthermore, each experiment yields only one data point for a binding curve.

In this investigation, membrane diafiltration has been studied with respect to its applicability in yielding information on protein binding. Membrane ultrafiltration itself is by no means a new method. However, diafiltration, combining the best features of dialysis and ultrafiltration, is a relatively new technique. A solution of fixed
ligand concentration is passed under pressure through a protein solution and the filtrate is continually sampled for free ligand. Thus, from a single experimental run it is possible to produce an entire binding curve. If the method is to be useful, diafiltration should yield values comparable with those obtained by more conventional methods for the parameters which quantitate the binding of the ligand to the macromolecule. The method was investigated by using human serum albumin (HSA) and two commonly used drugs with a high affinity for albumin, bishydroxycoumarin (BHC) and phenylbutazone (PBZ). The binding characteristics of these two drugs to HSA have been determined by other methods and comparisons can, therefore, be made.

In addition to studying the interaction of BHC and PBZ with HSA by the diafiltration method, two other methods (ultrafiltration using centrifugation and gel filtration) were investigated. These methods yielded erratic results and, therefore, the emphasis in this thesis is on the diafiltration method. Theoretically the diafiltration method could be used to investigate the simultaneous binding of two substances to HSA. It is well established that protein binding is of in vivo significance and that the extent of binding, in some instances, will change when two drugs are administered concurrently. Although a method of analysis was developed for the BHC in the presence of PBZ, no detailed investigations in competitive binding were carried out because of unexpected difficulties with the diafiltration apparatus.
II. LITERATURE

(1) Theory

The importance of the interaction between ligands and plasma protein is now well established. Bennhold (1938) recognized that the plasma proteins are the special transport system for the regulated distribution of both naturally occurring and medicinal substances throughout the body. Goldstein (1949), in his classical review, attributes much of the impetus in this area to the studies by Cohn (1946) who isolated and characterized plasma proteins. Much of the work in the early part of this century was empirical and lacked any clearly formulated theoretical background. It was not until advances were made in drug therapy, and penicillin and sulphonamides were in use, that it became clear that drug-protein interactions were important.

Goldstein reviewed the ways in which data describing protein binding can be quantitatively expressed. The Freundlich isotherm states that at a given temperature the amount of substance absorbed per weight of absorbant is inversely proportional to some power of the concentration of unabsorbed material. However, this is a purely empirical function and has no thermodynamic validity. On the other hand, the Langmuir isotherm can be rationally interpreted. This approach relates the amount of substance absorbed \( x \) per weight of absorbant \( m \) to the concentration \( c \) of unabsorbed material. The constants in the equation are \( a \) and \( b \).

\[
\frac{x}{m} = \frac{bc}{a + c} \quad \text{(Eq. 1)}
\]
As Langmuir noted, simply because an absorption isotherm is produced, it does not necessarily follow that binding forces are physical rather than chemical in nature. In his opinion, it was better to consider all binding forces as being strictly chemical in nature. This concept is similar to that expressed by the Law of Mass Action.

Klotz (1946a) developed his approach to protein binding from the Law of Mass Action. For this approach, several assumptions are necessary:

1. All groups on the protein molecule, capable of interacting, have identical affinities for the ligand molecule.
2. The affinity of any group is unaffected by combination of ligand molecules with other groups.
3. Activities can be replaced by concentration terms.
4. The interaction between groups on the protein with ligand molecules is entirely reversible.

The derivation here is that given by Edsall and Wyman (1958) and the terminology has been adapted to express it in terms of association, rather than dissociation.

A protein molecule (M) combines with a ligand molecule (D), according to the Law of Mass Action,

\[
M + D_f \leftrightarrow M - D_b
\]  

(Eq. 2)

\[
k_{ass} = \frac{[M - D_b]}{[M][D_f]} \quad 1M^{-1}
\]  

(Eq. 3)
where \([M]\) = total protein concentration in moles/1.
\([D_f]\) = concentration of unbound ligand molecule in moles/1.
\([M - D_b]\) = concentration of ligand bound to protein molecules in moles/1.
\(k_{ass}\) = the association constant = 1/
\(k_{diss}\)

If \(\alpha\) is the fraction of unoccupied binding sites on the protein molecule, then

\[
\alpha = \frac{[M]}{[M] + [M-D_b]} \quad \text{(Eq. 4)}
\]

\[
\alpha = \frac{[M]}{[M] + [D_f] k_{ass}[M]} \quad \text{(Eq. 5)}
\]

\[
\alpha = \frac{1}{1 + [D_f] k_{ass}} \quad \text{(Eq. 6)}
\]

Let \(r\) = fraction of occupied sites = \([D_b-M][M]\). Therefore,

\[
r = 1 - \alpha = \frac{[D_b-M]}{[D_b-M] + M} \quad \text{(Eq. 7)}
\]

\[
r = \frac{k_{ass}[D_f]}{1 + k_{ass}[D_f]} \quad \text{(Eq. 8)}
\]

\(r\), in the above equation denotes the average number of ligand molecules bound to a molecule of protein. Eq. 8 describes the situation where the protein molecule has one site capable of binding ligand. This equation can be extended from one to 'n' binding sites.
This approach has been considered in reviews by Klotz (1946 a), Edsall and Wyman (1958), Steinhhardt and Reynolds (1969). The following derivation is general and is that described by Steinhhardt and Reynolds (1969). Consider a number of uniform particles, M, in solution each with n indistinguishable and identical sites. The equilibrium between ligand and sites on the macromolecule, M, can be described by:

\[ [M] + n[D_f] \rightleftharpoons [M - D_{bn}] \]  \hspace{1cm} (Eq. 9)

or else by stepwise addition of ligand:

\[ [M] + [D_f] \rightleftharpoons [M - D_{b1}] \]
\[ [M - D_{b1}] + [D_f] \rightleftharpoons [M - D_{b2}] \]  \hspace{1cm} (Eq. 10)
\[ [M - D_{b2}] + [D_f] \rightleftharpoons [M - D_{b3}] \]
\[ [M - D_{bi-1}] + [D_f] \rightleftharpoons [M - D_{bi}] \]
\[ [M - D_{bn-1}] + [D_f] \rightleftharpoons [M - D_{bn}] \]

where at equilibrium:

\[ k_i = \frac{[M - D_{bi}]}{[M - D_{bi-1}][D_f]} \]  \hspace{1cm} (Eq. 11)

Assume that (1) the association is thermodynamically reversible, and that (2) \([M - D_{bi}], [D_f], [M - D_{bi-1}]\) refers to activities.

The number of possible combinations of n binding sites taken exactly i at a time (or alternatively the number of equally probable
forms of the complex \([M - D_{b_i}]\), i.e.,

\[
P_{M - D_{b_i}} = \frac{n!}{i!(n - i)!} \quad \text{(Eq. 12)}
\]

For example, there are \(n\) probable forms of \(M - D_{b_i}\), but only one form of \(M - D_{b_{bn}}\). By definition, all sites are equal and if present alone must have the same intrinsic association constant, \(k_0\). If the number of equally probable forms of each complex are taken into account, the \(k_i\)'s may be deferred in terms of a single equilibrium constant, \(k_0\). Again, from probability theory, \(M\) contains \(n\) possible sites but \(M - D_{b_1}\) can release only one ligand molecule. Hence, \(k_1 = nk_0\). Similarly, \(M - D_{b_1}\) contains \(n - 1\) possible sites but \(M - D_{b_2}\) can release two ligand molecules. Therefore:

\[
k_2 = \frac{n - 1}{2} \cdot k_0
\]

\[
k_i = \frac{n - 2 + 1}{i} \cdot k_0 \quad \text{(Eq. 13)}
\]

\[
k_n = \frac{1}{n} \cdot k_0
\]

Edsall and Wyman (1958) noted that these statistical factors are always present at equilibrium, whether or not sites are independent and equivalent and therefore binding constants should always be expressed corrected for these statistical factors; i.e., \(k_{i0} = k_i/n\), etc. Hence \(k_{01} = k_{20} = \cdots = k_{n0}\) and any observed differences between these values should indicate intrinsic differences between affinities of the groups, or interactions between groups, or both.
The average number of bound ligand molecules may be expressed as

\[
\text{r} = \frac{\sum_{i=1}^{n} \frac{n!}{(n-i)!i!} x k_i^i x [D_f]^i}{1 + \sum_{i=1}^{n} \frac{n!}{(n-i)!i!} x k_i^i x [D_f]^i}
\]  
(Eq. 15)

As a result of systematically applying Equation 11 to Equation 14 and then substituting the relationships between \(k_i\) and \(k_0\) defined in Equation 13, the following relationship for \(r\) is obtained:

\[
\text{r} = \sqrt[n]{\frac{1}{n!} \sum_{i=1}^{n} \frac{n!}{(n-i)!i!} x k_i^i x [D_f]^i}
\]

The denominator is the general expression of the binomial theorem and the numerator is the first derivative thereof. Hence, after appropriate substitutions and substituting \(K\) for the product of combined activity coefficients and \(k_0\)

\[
\text{r} = \frac{nK D_f}{1 + KD_f}
\]  
(Eq. 16)

This equation, which describes the binding of ligand to \(n\) sites (identical in binding ability) on a protein molecule, may be further extended to a situation where there is more than one set of sites on the molecule. Assume that set one has \(n_1\) sites with intrinsic association constant \(K_1\), and set two has \(n_2\) sites with intrinsic association constant \(K_2\). Then Equation 16 is extended to:

\[
\text{r} = \frac{n_1K_1D_f}{1 + K_1D_f} + \frac{n_2K_2D_f}{1 + K_2D_f} + \cdots + \frac{n_nK_nD_f}{1 + K_nD_f}
\]

(Eq. 17)
Complete independence of sets of sites is assumed. If the values of $K_i$ are separated by a factor of more than $10^4$, then at any particular $D_f$ value only one term of Equation 17 will depend on $D_f$, and in this case $r$ will be determined only by one particular $K_i$.

Equation 8 can be extended to describe a situation where there is only one set of sites but two different ligand molecules, A and B, competing for these sites, each with a different association constant, $K_A$ and $K_B$.

\[
\begin{align*}
    r_A &= \frac{K_A D_f}{1 + K_A D_f} (n - r_B) = \frac{n K_A D_f}{1 + K_A D_f + K_B D_f} \\
    r_B &= \frac{n K_B D_f}{1 + K_B D_f + K_A D_f}
\end{align*}
\]  

Equation 16 is used most often in quantitative binding studies. This assumes a fixed number of binding sites. Frequently, when this equation is used to express binding data and results are plotted in the manner indicated by Scatchard (1950), non-linearity of the plot is observed. Karush (1950 a) found that this non-linearity could not be explained by electrostatic interaction (where binding of ligand molecule may cause an alteration in the net charge on the molecules) or by a Gaussian distribution of free energy of binding. He concluded that $n$ is not necessarily the maximum number of binding sites per molecule.

Non conformity of data to Equation 16 could arise from one or more of several factors:-
(1) Steric interference between bound molecules.

(2) When a charged molecule is bound, electrostatic interaction may arise from the increasing charge on the protein molecule as more ligand binds.

(3) Interaction of the ligand with the molecule may induce conformational charges.

The first of the above factors, steric interference, has not often been discussed in the literature but could be considered on a structure-activity basis.

Electrostatic interaction has been mentioned frequently. Various models have been proposed to describe the above. For example, Tanford (1965) assumed that both ligand and protein molecules were rigid spheres. Similarly, Steinhardt and Reynolds (1969) concluded that there is an additional contribution to the free energy from electrostatic interactions when the ligand added to the protein is charged. This additional contribution is the difference in charging energy between \( \text{MD}_{i-1} \) and \( \text{MD}_i \), i.e., the electrical work done in increasing the charge on the surface when one charged ligand molecule is added.

A function \( f(r) \) is defined such that \( f(r) = 0 \) at \( r = 0 \) and thereby yields an equation which varies with the number of bound ligands

\[
\begin{align*}
\therefore \quad K_0^1 &= K_0 \cdot e^{-f(r)} \quad \text{(Eq. 19)} \\
\Delta F^1 &= -RT \ln K_0 + RT \ln f(r) \quad \text{(Eq. 20)} \\
\therefore \quad r &= \frac{n \cdot K_0 \cdot e^{-f(r)} \cdot x \cdot D_f}{1 + K_0 \cdot e^{-f(r)} \cdot x \cdot D_f} \quad \text{(Eq. 21)}
\end{align*}
\]
From the Debye - Hückel Theory:

\[ e^{-f(r)} = e^{-2wz} \]

where

\[ w = \frac{e^2}{2DRT} \frac{1}{b} - \frac{K}{1 + K_a} \]

A more realistic model for electrostatic interactions was proposed by Tanford (1957) and Tanford and Kirkwood (1957). This model takes into account the location of charge density on a non-conducting surface in terms of distance between the charged sites and the position of these sites relative to the interface between the binding surface and the medium in which it is immersed.

Induced conformational charges in the protein molecule have also been mentioned in the literature in an attempt to explain non-linearity of binding data. Configurational adaptability was first suggested as a possibility by Karush (1950). This suggestion was supported by the observations that on binding of small molecules and ions to macromolecules, conformational changes occurred; i.e., conformational changes as manifested by changes in viscosity, optical properties and anomalies in the shapes of binding curves. Investigations in this field, in more recent years (Steinhardt and Reynolds [1969]), suggest that the forces responsible for bringing about these conformational charges may be one or more of the following:-

(a) electrostatic repulsion between bound ligand molecules, including the net charge on the molecule. Tanford (1965), on investigating
the effect of increased net charge on the macromolecules and applying Equation 21, found that an increase in the distance between binding sites will decrease $w$ and consequently the free energy of the system. Therefore, as the number of sites occupied by a charged ligand increase, the molecule will tend to decrease the free energy of the system.

(b) penetration of a hydrophobic tail into a polar region of the macromolecule with the resultant replacement of the conformation stabilizing segment-segment forces by ligand-segment interactions. Kauzman (1959) suggested that hydrophobic bonds, hydrogen bonds and dipole moments are important for stabilizing protein structure. If such segment-segment interactions are replaced by ligand-segment interactions on binding of ligand, the stability of the protein may be decreased. There are no proven examples of this.

(c) a ratio of the number of binding sites and their association constants in the native form to those in the unfolded form of the macromolecule which is favourable to unfolding. This factor was first suggested by Foster and Aoki (1958) when discussing the $N \rightarrow F$ ($N =$ native form $\rightarrow F =$ unfolded form) transition which occurred at pH 4 with bovine serum albumin. Steinhardt and Reynolds (1969) described a binding isotherm when such a transition occurred. The complete expression is:

$$r = \frac{D_f}{1 + FD_f} \left[ \frac{nK_o}{1 + K_oD_f} + \frac{F(D_f) m J}{1 + J(D_f)} \right]$$

(Eq. 22)
where $n =$ number binding sites on native form
$m =$ number binding sites on unfolded form
$K_Q =$ intrinsic affinity constant for native form
$J =$ intrinsic affinity constant for unfolded form
$F(D_f) =$ ratio of protein present in the two states.

Although the above and other authors have attempted to explain deviations from the expected binding data, it is unlikely that more progress will be made in this field until more is known about HSA at the molecular level.

(2) Physical and Biological Properties of Bishydroxycoumarin

Chemically known as 3,3'-methylene-bis-(4-hydroxycoumarin), BHC is a white crystalline or amorphous powder with a molecular weight of 336.29 and a melting point of 287-293°C (Merck Index). Its structure is:

![Structure of BHC](image)

Cho (1970) suggested that the low water-solubility of the unionized molecule arises from the formation of intramolecular bonds as shown below:

![Intramolecular Bonds](image)
On the basis of his data, $pK_a_1 = 4.44$ and $pK_a_2 = 7.83$. After the first ionization has occurred, solubility would increase only slightly because one intramolecular bond is still present. After the second ionization has occurred a considerable increase in solubility should occur, because intramolecular hydrogen bonds are no longer present to maintain the molecule in a rigid position.

Coumarin drugs gained popularity in anticoagulant therapy following the observation of the anticoagulant effect of spoiled sweet clover hay on cows. The active principle causing the anticoagulant effect was a coumarin type compound (Link [1944]). BHC, one of the coumarin drugs, has been used therapeutically for approximately thirty years.

Weiner and others (1950) were the first to report the binding of BHC to HSA. At levels of 5-100 mg. BHC/l. BHC was 99% bound to albumin, 20% to $\beta$ and $\gamma$ globulins and 50% to $\alpha$ globulins. At therapeutic dosage levels, the free BHC concentration was only 20$\mu$g/ml. indicating its high affinity for plasma and partially explaining the long persistence of the drug in the body. Only very small amounts of BHC were found in the brain, red blood cells and cerebrospinal fluid. O'Reilly and others (1962) also reported that BHC was 99% bound to albumin. The same authors (1964)
found that for all doses of warfarin and high doses of BHC, their long elimination half-lives were due to their small volumes of distribution, which are very similar to that of HSA. However, Solomon and Schrogie (1967) showed that the magnitude and duration of the biological response to BHC was not related to its half-life. They suggested that the variation in response amongst patients was a result of variable absorption and metabolic rates, possibly being explained by a difference in affinity of BHC for receptor sites. O'Reilly and Aggeler (1964b), however, had a different explanation for this variation in response to BHC. They said this discontinuous response to the drug must be genetically determined (i.e., the distribution of the response to anticoagulant therapy instead of being normal or unimodal was multimodal).

The above studies have shown that BHC has a very high affinity for plasma. Hence, protein binding must influence the distribution and metabolism of BHC. Levy (1970) concluded that the increased retention of BHC up to a point where saturation of binding sites occurred could be accounted for by a conformational change in HSA on initial combination with BHC, thus making more sites available.

Interaction of other drugs with anticoagulants has been extensively investigated. Clinical observations have shown that BHC blood levels change when certain other drugs are administered. Most literature references deal with warfarin but these interactions would be expected to apply to BHC. Examples given here will be restricted to interactions of warfarin or BHC with PBZ or to interactions of drugs with BHC.
Weiner and others (1950) showed a decreased disappearance rate for BHC from the plasma, on concurrent administration of oxyphenylbutazone (oxy-PBZ). They suggested that the resultant enhanced hypoprothrombinaemia was due to oxy-PBZ inhibition of the hepatic enzymes. Aggeler and O'Reilly (1967), noting that this result (i.e., of Weiner and others) was contrary to that observed with warfarin, said these results were probably incorrect as the BHC levels calculated were not corrected for oxy-PBZ present.

Aggeler and others (1967) found that in man PBZ competes with anticoagulants for binding sites on plasma albumin, resulting in an increased delivery of coumarins to the liver.

Schrogie and Solomon (1967) showed that for doses of D-thyroxine, clofibrate and norethandrolone which did not affect the metabolism of BHC nor the levels of Vitamin K dependent clotting factors, the anticoagulant response could be increased. This they attributed to an altered affinity of the receptor sites by the drugs.

O'Reilly and Aggeler (1968) gave results for warfarin and PBZ interactions. Results would be expected to be similar for BHC. They showed by fluorometric assay that PBZ increased the prothrombin time and decreased the warfarin half-life and plasma warfarin levels, leading to an increased amount of drug being available for metabolism in the liver.

Welch and others (1969) found that in dogs and in man PBZ had an opposite effect on anticoagulant activity. O'Reilly and Aggeler (1968) explained such an effect in man by PBZ stimulation of drug metabolism via the hepatic microsomal enzymes. This could lessen the hypoprothrombinaemic affect of anticoagulants in the dogs but enhance it in man.
Another interaction investigated was that of heptobarbital by Levy and others (1970). Heptobarbital decreased the response to BHC in man. In oral doses, heptobarbital did decrease the absorption of BHC but irregardless of the route of administration, prothrombin time, plasma levels and half-life were all decreased.

O'Reilly and Levy (1970), who studied warfarin and PBZ, gave a more probable explanation for such interactions. The interaction, they concluded, may be the net result of several effects and its duration and magnitude may be dependent on the dosage of PBZ and the duration of its administration. The increased elimination rate and potentiation of pharmacological action of warfarin would both seem to be due, primarily, to a change in distribution.

More recent studies on BHC have been concerned with the nature of the binding of BHC to serum albumin using various techniques. Chignell (1970) investigated the binding of BHC to HSA by circular dichroism and found that after three moles of BHC had bound there was no further change in molar ellipticity (i.e., no further change in the difference spectra).

Perrin and Idsvoog (1971) studied the binding of BHC to bovine serum albumin (BSA) by circular dichroism and differential absorption spectroscopy and found no major conformational changes occured on binding, suggesting little or no departure from the α-helical structure of BSA at pH 7.4. However, the changes observed in molar ellipticity as BHC binds to BSA do not parallel those observed by Chignell (1970),
suggesting that binding results with BSA cannot always be extended to HSA.

Cho and others (1971) used equilibrium dialysis, spectrophotometry and solubility analysis to study the binding of BHC to HSA in more detail. Their results suggested that the α,β unsaturated lactone structure on the BHC molecule is involved with binding. The main binding energy was shown by thermodynamic studies to be derived from non-ionic forces. The binding sites appeared to be hydrophobic regions on the albumin molecule whose van der Waals contours are selective for the BHC molecule.

O'Reilly (1971) investigated the interaction of a number of coumarin compounds with HSA by equilibrium dialysis and made several general observations about the effect of structure on binding. Precursor type of compounds showed a one to one maximum binding ratio, whereas mono-coumarins and dicoumarins showed a two to one ratio. The strength of binding to albumin was related to the hydrophobic nature of the substituents introduced to the coumarin molecule. Thermodynamic studies suggested that coumarin drug binding to HSA involved both hydrogen bonds (from favourable enthalpy changes) and hydrophobic bonds. A decrease in the number of binding sites as the pH is changed from 7.4 to 10, suggested that binding sites are not rigid but rather are influenced by environmental factors. One other observation made was that there was little difference in binding energies for (-) S-warfarin and (+) R-warfarin, and yet the (-) S form has far greater anticoagulant activity in man. As a result of this observation, O'Reilly suggested plasma albumin might be an inadequate model for studying binding to intracellular receptors for any drug, the inadequacy arising perhaps
from the two dimensional nature of plasma albumin as compared to the three dimensional nature of intracellular receptors, which can distinguish optical isomers.

Chignell and Starkweather (1971) studied the interaction of PBZ, flufenamic acid and BHC with acetylated HSA, HSA which had been previously incubated at 37° for 24 hours, and untreated HSA, by equilibrium dialysis and circular dichroism. They found that with acetylated HSA and incubated HSA, the affinity of BHC was increased several fold. They concluded that, in the former case, the binding site was altered. With incubated HSA, they suggested that the HSA conformation had changed and thus much more BHC could be bound.

(3) Physical and Biological Properties of Phenylbutazone

Phenylbutazone is a white crystalline powder, insoluble in water and has a melting point of 104.5 - 106.5°C. Its molecular weight is 308.37 (Merck Index).

Decomposition in aqueous solutions at room temperature has been observed. The kinetics of this decomposition has been investigated by Pawelcyzk and others (1968), (1969). Their results indicated that the decomposition of PBZ (in ammonium acetate buffer at pH 8.6) after 24 hours at 37°C would be insignificant. Solutions can be kept at room temperature for up to one week. However, if stock PBZ solutions are stored at 4°C they can be kept for several weeks without significant decomposition.
PBZ has a pK<sub>a</sub> value of 4.5 and, hence, at pH 7.4 it would be expected to exist in the enol form (von Rechenburg [1962]).

The above anion will have definite hydrophilic properties.

Burns and others (1953) showed that at plasma levels of 50-150 mg/l, PBZ is 98% bound but at levels greater than 250 mg/l, less than 88% is bound. With increased doses a plateau in plasma levels was reached. Pharmacokinetic studies indicated the excretion of PBZ was not increased nor were the PBZ tissue levels increased when the plateau in plasma levels was reached. Their explanation for the lack of dependence of blood levels on dose (at high dosage levels) was an increased PBZ metabolism rate. Brodie (1956) explained this lack of relationship between blood levels and dose, at high dosage levels, on a basis of saturation of PBZ binding sites. Under these conditions the amount of free drug, which is rapidly metabolized, increases. Von Rechenburg (1953) explained this phenomena in a similar manner. Brodie, and others (1954), showed that PBZ was bound to plasma proteins, particularly the albumin and α-globulin fractions. Pulver, and others (1956) found, by dialysis, that, for dilute solutions, PBZ was 99% bound to serum but for more concentrated solutions was only 94% bound. Wunderly (1956) using equili-
brium dialysis, showed that with dilute protein solutions, an equilibrium between bound and free drug exists and this equilibrium constantly changes with PBZ concentration. He considered PBZ was bound to HSA not by chemical forces but rather by being held within the hydration shell enveloping the protein molecule. Thorp (1964) and Solomon, and others (1968), the former using equilibrium dialysis and the latter an ultrafiltration technique, found similar values for n and k_{ass}. Chignell (1969) investigated the nature of the binding of PBZ by HSA by means of optical studies of the PBZ-HSA complex. On binding of PBZ, a positive elliptical band occurs at 287 μm in the circular dichroism spectrum. This he explained resulted from perturbation of the n-π* transition in the carbonyl chromophore of PBZ by an asymmetric locus at, or near the albumin binding site. As was shown by displacement of a fluorescent probe for hydrophobic regions, hydrophobic interactions are important for maintaining a rigid drug-protein complex. With the introduction of hydrophilic groups into PBZ, the magnitude of the induced optical activity is considerably reduced. Chignell and Starkweather (1970) found, by equilibrium dialysis, that HSA had an additional two binding sites of low affinity for PBZ. They also noted that the affinity of PBZ to acetylated HSA or incubated HSA (incubated at 37° for 24 hours) was altered when compared to that for normal untreated HSA. The number of binding sites was increased and it appeared that these treated HSA samples had altered conformation. Chignell and Starkweather (1971) gave the symmetry rules for the n-π* transition in the carbonyl group. This could obey either the quadrant rule or the octant rule. However, at present these changes in extrinsic optical
activity cannot be interpreted in terms of specific alterations at their protein binding sites, although it could be said that acetylation of HSA or incubation at 37° for 24 hours alters the nature of the binding sites. Rosen (1970) gives quantitative binding data from circular dichroism studies on the binding of HSA and PBZ. From the changes in molar ellipticity as HSA is titrated with PBZ, he could determine the amount of bound drug and free drug present. Thus he calculated n and k values.

Studies on the clinical interaction of PBZ with anticoagulants were outlined in the previous section.

(4) Physical and Biological Properties of Human Serum Albumin

Plasma contains approximately 4% w/v HSA. Values reported in the literature are 46.2 g/l (Geigy Scientific Tables), 4.62% w/v (Riva [1960]), 3.575% w/v (Hitzig [1960]), 3.5 - 4.5% w/v (Schultze and Heide [1960]). Its physical parameters are described by Cohn, and others (1947). It has a molecular weight of 69,000 and its molecular shape is ellipsoidal, being approximately 150Å in length and 38Å in diameter. Schultze, and others (1962) determined its amino acid composition and the terminal groups are characterized but little is known about its primary structure. A knowledge of such structure would answer questions about the heterogeneity of the HSA molecule. It is known, however, that the amino acid residues are linked by a single peptide chain which is crosslinked by disulphide bridges.
Rapid advances in protein binding studies were made after Cohn (1946) produced his scheme for the fractionation of plasma. Fraction V contains 95% or more albumin and also small amounts of transferrin, α₁-acid glycoprotein and α₁-antitrypsin. Commonly, a Cohn-type method is used to produce HSA commercially. Recrystallized HSA is used in some binding studies, but there appears to be little difference in binding characteristics between crystalline HSA and Fraction V (Kostenbauder, and others [1971]).

Foster (1960) summarized the types of molecules bound by HSA. Divalent and polyvalent cations bind more strongly than monovalent, as would be expected because of the net positive charge on HSA. The transition element metals bind more strongly but differ in that they are capable of forming coordinate covalent types of bonds. Anions are bound much more strongly than cations (Klotz and Ayers [1953]). Significant differences have also been observed in studies involving albumin-isomer interactions (Klotz and Ayers [1952]).

Klotz (1947) studied the binding of dyes and found that generally their binding is greater than that of the anions. Surface active agents exhibit even higher affinities for the protein. Binding is cooperative and a large number of ions are bound (Foster [1960]). Karush and Sonenburg (1949) suggested that cooperative binding was due to a structural change in the protein and hence a change in the nature of binding occurred as ligand concentration increases. Yang and Foster (1953) electrophoretically investigated the binding of alkylbenzene sulfonate
to albumin. He observed a single boundary similar to that of the native protein, which corresponded to a distribution of the molecules in a statistical manner over all the available albumin molecules. However, there was also a separate, faster moving boundary. The authors explain this as an all-or-none reaction where the molecule partially unfolds allowing more ligands to bind. There is a third phase to this binding process where the binding ratio apparently increases without limit but presumably affinity to the sites is much lower.

Klotz and Urquart (1949) investigated the significance of thermodynamic changes occurring during binding of organic molecules to albumin. The large positive entropy change could not be due to unfolding and disorientation of the protein molecules, since the change in entropy was small and negative. He explained this in terms of the ice-like structure of water surrounding the macromolecule; i.e., there are several polarized water groups attached to the carboxyl and quaternary ammonium groups. When the ligand molecule binds to one of these groups, water molecules are then displaced. Therefore, an increase in the number of molecular species occurs, causing an increase in entropy. This view is also supported by an increase in volume. Major contributions to free energy of binding must come from the release of solvent molecules from the complex.

Lovrien (1963) studied the binding of detergent molecules to BSA and showed that even at low $D_f$ values, conformational changes occurred, resulting in a decrease in hydrodynamic volume. Klotz and Ayers (1952) noted an increase in the binding capacity of BSA for anions as the pH increases. This is the reverse of what would be expected if the charge
on BSA is taken into account. A change in the protein molecule occurred above pH 7. This they described as a definite change in the conformation of the molecule, i.e., a reversible swelling or unfolding produced by strong internal electrostatic repulsions. Klotz and Ayers (1953) suggested that, for neutral molecules, the very act of complexation makes more sites available. On the basis of the above, it is obvious that the mechanism of binding of ligands to albumin has not yet been fully resolved.

Steinhardt and Reynolds (1969) state that HSA and HSA are microheterogeneous and a solution of albumin, therefore, contains a mixture of components which are not spontaneously interconvertible. These components can be separated because of their varying degree of susceptibility to partial unfolding by acid (Peterson, and others [1965], Sogami and Foster [1968], Peterson and Foster [1965 a,b]). The effect of this heterogeneity on binding properties has not as yet been determined. Its implications in preventing any simple thermodynamic analysis of the \( N \rightleftharpoons F' \) equilibrium are clear. Both HSA and BSA show anomalous hydrogen ion titration behaviour below the isoelectric point and beginning at about pH 4. Yang and Foster (1954) studying this effect and its influence on optical rotation and viscosity, concluded that isotropic expansion of the molecule occurred in acid solution as the positively charged ammonium groups repulsed each other. Tanford and others (1955 b, 1956) confirmed and suggested that this expansion occurs through an intermediary expandible "F" form.
Sophisticated methods of analysis (such as gel electrophoresis, ion exchange chromatography, etc.) have indicated that albumin may not be a single molecular species (e.g., Pederson [1962]). The apparent heterogeneity of albumin may be caused by:—

(i) the binding of endogenous molecules to the macromolecule. Binding of anionic substances increases the electrophoretic mobility of albumin, as is observed when bromophenol blue dye is used as a trace for albumin. Similarly, the mobility of albumin is altered in the nephrosis, perhaps because of binding of fatty acids.

(ii) the association with other globular proteins in the serum.

(iii) the polymerisation of albumin. Hughes (1947) showed that only 70% of the HSA molecules would form mercaptalbumin dimers. Pederson (1962) and others have shown that dimers and higher polymers are present in HSA solutions under various conditions.

(iv) true microheterogeneity in the HSA molecule. This is substantiated by the formation of mercaptalbumin dimers (Hughes [1947]).

(v) bisalbuminaemia. In certain diseases, e.g., diabetes mellitis, tuberculosis, hepatitis, electrophoretic analysis of the serum has occasionally shown two, rather than the one expected albumin peak.

A more detailed account of possible factors giving rise to heterogeneity is given by Schultze and Heremans (1962).
In spite of the above factors, albumin is a suitable model for binding studies in that it is less susceptible to denaturation than other similar proteins. Its denaturation is readily reversible because molecules aggregate whilst still in their folded state. This is in contrast to ovalbumin where disaggregation would result in a highly unfolded and disoriented molecule.

As a result of its high affinity for a wide range of molecules, problems may arise in binding studies. For example, Karush (1951) showed that the phosphate ion bound competitively with BSA. Thus even buffer ions may compete with the ligand being examined and care must be taken in choosing a solvent system.

Another consequence of the high affinity of albumin for ligands is that the macromolecule still has one or two equivalents of endogeneous fatty acids attached to one or more of its higher energy sites. Various techniques have been described in the literature for the removal of these fatty acids. Goodman (1958) passed the HSA solution through a mixed ion exchange bed and then treated the eluate with iso-octane and glacial acetic acid. Chen (1967) removed the fatty acid by using mild acid and charcoal. However, Sogami and Foster (1968) found small but definite differences in stability and UV absorption characteristics between untreated and fatty acid free albumin. These fatty acid residues could significantly alter binding results, particularly for fatty acid-like neutral molecules. The fatty acid molecule might occupy one, or part of one of the binding sites for the molecule in question. Therefore, at low molal binding ratios, discrepancies in data would be likely to occur when different protein preparations are used.
(5) Methodology

Any quantitative investigation of ligand binding to protein must yield numerical values of bound and free drug. Such studies must, therefore, depend on changes in the properties of the interacting molecule or in changed properties or behaviour of the macromolecule. Reviews on methodology have been given by Goldstein (1949), Meyer and Guttman (1968), and Steinhardt and Reynolds (1969). Some methods measure changes in properties of the ligand molecule on binding to the macromolecule. Examples of methods mentioned here will be those which have been used in PBZ or BHC binding studies.

Cho (1971) measured the binding of BHC to HSA by the depression of the absorption maxima of the BHC spectra in the presence of macromolecule. Perrin and Idsvoog (1971) measured changes in extrinsic Cotton effects (expressed as differential ellipticity) as increasing amounts of BHC were added to serum albumin. They also investigated the effects of the binding of BHC to HSA by differential absorption. Chignell and Starkweather (1971) also used this method and studied the binding of BHC and PBZ to HSA. Their investigations and those of Perrin and Idsvoog (1971) resulted in qualitative data only. However, Rosen (1970), assumed that differences in the circular dichroism spectra (as HSA was titrated with PBZ) were proportional to the amount of bound PBZ and thus reported quantitative binding data.

Generally, methods which measure free ligand concentration give more precise results. The most commonly used approach is equilibrium dialysis. A membrane is used which is permeable to all components of
the system, except the macromolecule. Thus, at equilibrium, the chemical potential of all the components except the macromolecule are equal and hence the free ligand concentration can be determined. The free ligand concentration can be measured in a variety of ways. O'Reilly (1971) measured free coumarin drug concentrations spectrophotometrically. Chignell (1969) used a similar approach for the analysis of PBZ. Aggeler, and others (1967), measured free warfarin levels using radioisotopic $^{14}$C-warfarin.

Problems associated with the use of equilibrium dialysis for binding studies are outlined by Steinhardt and Reynolds (1969). These include membrane binding and Donnan effects. Corrections can be made for the former; the Donnan effect is avoided by using higher ionic strengths. Reproducible results can, therefore, be obtained. However, lower protein concentrations are necessary to avoid volume changes; it is a slow method and a considerable time is necessary for the diffusion of ligand molecules across the membrane to achieve equilibrium.

Other methods are analogous in principle to equilibrium dialysis. A useful method and one which is amenable to automation and computer calculation is the dynamic dialysis technique (Meyer and Guttman [1968]). The rate of disappearance of a small molecule from the dialysis cell is proportional to the free drug concentration. This method is more rapid. Cho (1970) used the dynamic dialysis to study BHC - PVP interactions. In ultrafiltration methods, the free ligand is filtered through the membrane by force, either by use of pressure or centrifugation. This is a rapid method but its accuracy is less than that obtained by other methods.
because the macromolecule concentration increases as ultrafiltration proceeds. Solomon, and others (1968) measured the displacement of warfarin $^{14}$C by PBZ $^{14}$C from HSA by ultrafiltration using cellophane bags and centrifugal force. McQueen and Wardell (1971) studied the effect of PBZ on the distribution of sulphadoxine in plasma using Visking dialysis tubing and pressure (5% CO$_2$ in oxygen at 1080 m bar pressure). It was noted that in control experiments with large plasma volumes, even when as much as 60% of the initial plasma volume had been removed, no progressive change in sulphonamide concentration in the ultrafiltrate could be observed. However, no change in the sulphonamide would be detected at high plasma sulphonamide levels, since changes would be small.

All the above methods, except dynamic dialysis, yield only one data point per experiment. Many experiments, therefore, are necessary to graph a binding curve.

Membrane ultrafiltration is by no means a new method. It was first introduced by Martin in 1896 and Grollman (1926) reviewed the use of this method using collodion membranes. He reported agreement for the determination of binding of phenol red to albumin by ultrafiltration with results achieved by dialysis. Thus it can be seen that the macromolecular surroundings of protein solutions can be altered with permselective membranes either by using conventional dialysis, ultrafiltration, or, finally, simultaneous dilution and ultrafiltration. This latter case can also be called diafiltration.

Diafiltration and its use in protein binding studies was first mentioned in the literature by Blatt, Robinson and Bixler (1968). They
described an adaption of an Amicon ultrafiltration cell which permitted the diafiltration of different volumes of macromolecule solution against ligand solutions of varying composition. There are a number of advantages in the use of this method:-

First, a range of biologically inert polymeric membranes with varying molecular weight cut-offs are available. Because of the range of membranes available, it is possible to attain faster filtration rates without loss of macromolecule. These membranes have a much higher solvent permeability relative to regenerated cellophane membranes (the most commonly used membranes in equilibrium dialysis). Secondly, use of pressure with this Amicon cell allows for microsolute exchange in a protein solution to be achieved much more rapidly. Thirdly, the volumes of macromolecule solutions can be maintained at a constant level.

A description of the Amicon ultrafiltration cell is given in the experimental section. Ligand solution of a fixed chosen concentration from a reservoir tank passes at a constant rate (determined by applied pressure) into the Amicon cell containing a volume of macromolecule solution. The filtrate passes through the filter at the same constant rate and thus the cell volume remains constant.

Blatt, Bixler and Robinson (1968) show that there are several different ways in which this method can be used.

(a) Wash-in Experiments: Here the macromolecule solution is placed in the cell and the ligand solution in the reservoir. The instantaneous ligand concentration in the filtrate can be predicted as
a function of cumulative filtration volume. Where the molecule of ligand can pass the membrane unhindered, this experiment can be expressed mathematically:

\[
\ln \frac{C_f}{C_f - C} = \frac{v - v^1}{V_0}\]  \hspace{1cm} \text{(Eq. 23)}

where 
- \( C_f \) = concentration in reservoir
- \( C \) = cell ultrafiltrate ligand concentration
- \( \bar{V}_0 \) = average cell volume during the experiment
- \( v \) = cumulative filtrate volume
- \( v^1 \) = apparent void volume of system.

(b) Wash-out Experiments: These can be similarly performed with solvent (e.g., buffer solutions) in the reservoir and macromolecule and bound ligand in the cell. The wash-out experiment can be expressed mathematically:

\[
\ln \frac{C_0}{c} = \frac{v - v^1}{V_0}\]  \hspace{1cm} \text{(Eq. 24)}

where \( C_0 \) = initial [ligand] in the cell. For binding studies, these authors showed two possible ways in which experiments could be done.

(i) Direct Method: A wash-in experiment is performed and run until diafiltration has been completed, i.e., equilibrium is reached (i.e., \([\text{ligand}]_{\text{reservoir}} = [\text{ligand free}]_{\text{cell}} = [\text{ligand}]_{\text{ultrafiltrate}}\)
Hence, for this experiment, a value for $r$ can be obtained

$$r = \frac{C_+ - C_f}{M} \quad \text{(Eq. 25)}$$

where $C_+$ = total concentration of ligand in cell

$C_f$ = concentration in reservoir

$M$ = free ligand concentration in cell

$M$ = macromolecule concentration.

Such a method has no particular advantage over equilibrium dialysis except that it is run under pressure and hence equilibrium is reached more rapidly. Each experimental run would yield only one point on a binding curve. Difficulties may also arise when it is necessary to measure ligand in the presence of protein.

(ii) Diafiltration Equilibrium: In some instances, plots of $\log (C_f/C_f - C)$ versus $(v - v^1)$ are linear. In this case, a differential material balance within the cell would yield the relationship

$$r = K_B C_f \quad \text{(Eq. 26)}$$

where $K_B$ is determined from the slope, the value of which is $0.4343/(1 + K_B M)^{\bar{v}_0}$. Therefore,

$$D_b = K_B M C \quad \text{(Eq. 27)}$$
This indicates the molar binding ratio, $D_b/M$ (i.e., $r$) is directly proportional to $C$. However, this is only an approximation, being true only at low $D_f$ values. Again the diafiltration technique used in the above manner, has few advantages over equilibrium dialysis and, indeed, is possibly more restricted in the range (of ligand concentration) in which it can be used. Furthermore, it yields only one data point for a binding curve from an experiment.

Blatt, Robinson and Bixler (1968) gave some experimental data for the binding of methyl orange to HSA and the binding of Ca$^{2+}$ to HSA, but most of their data was achieved by the direct method. Their results did not agree well with other literature values.

The authors did derive, for wash-in experiments, an equation based on the Law of Mass Action, but they did

$$\frac{v - v^1}{v_0} = \left[ 1 + \frac{kMn}{(kD_f + 1)^2} \right] \ln \frac{C_{\text{res}}}{C_{\text{res}} - D_f} + \frac{kMn}{kC_{\text{res}} + 1} \left[ \frac{kD_f}{kD_f + 1} + \ln(kD_f + L) \right]$$

(Eq. 28)

not apply this to any ligand-macromolecule interaction. This equation is not amenable to simple linear plotting and computer analysis of data would be necessary.

The experimental section of this thesis will show how the Amicon cell, run under diafiltration conditions, can yield an entire binding
curve from one experiment. At any instant in time, the concentration of ligand in the filtrate will indicate the concentration of free ligand in the cell. A necessary assumption in this case is that the ligand binds in a reversible equilibrium with the macromolecule in the cell instantaneously. The validity of this assumption is not well substantiated in the literature (for the equilibrium occurs too rapidly to study by conventional methods used for binding studies). However, Robbins and others (1965) found, by a method involving the quenching of the fluorescence of albumin by interaction with thyroxine, that association is complete within 150 msec. The dissociation process occurs in two steps with a half life of 0.1 sec and 7 secs, respectively. Froese (1962), by a temperature jump technique, found the rate constants of association of albumin for two azo dyes were $0.36 \times 10^6$ and $2.1 \times 10^6 \text{ moles}^{-1} \text{ sec}^{-1}$.

Ryan and Hanna (1971) studied steroid HSA interactions by diafiltration. Their experimental arrangements were similar to those used in this study. Crawford and others (1972) also used this method to study the binding of bromosulphthalein to serum and albumin. However, these authors, in contrast to Ryan and Hanna (1972), gave no data to substantiate the usefulness or disadvantages of the apparatus.
III. EXPERIMENTAL

(1) Apparatus

(a) Beckman Du Spectrometer
(b) Beckman Du-2 Spectrometer
(c) Bausch and Lomb Spectronic 505 Recording Spectrophotometer
(d) Hitachi 124 Coleman Double Beam Spectrophotometer with Hitachi 165 Recorder
(e) Perkin-Elmer Double Beam Spectrophotometer (Coleman 124) with Perkin-Elmer Recorder (Coleman 165)
(f) Fisher Digital pH meter
(g) Haake R21 Thermoregulator
(h) Isco Fraction Collector - Model 326
(i) Amicon Diafiltration Apparatus
(j) International Centrifuge, Size 1, Model SBV.

(2) Chemicals and Reagents

(a) Bishydroxycoumarin, U.S.P. The melting point of this chemical was 288-289°C. The drug was obtained from Abbott Laboratories Ltd., Montreal, Quebec.
(b) Phenylbutazone, U.S.P. The melting point of this drug was 104.5°C. The drug was obtained from Geigy (Canada) Ltd., Montreal, Quebec.
(c) Human Serum Albumin (Cohn Fraction V). The albumin was obtained from Pentex Inc., Kankanee, Ill. No loss of weight on drying was found. The substance was stored at 4°C.
(d) Tris (hydroxymethyl) aminomethane (Tris). Fisher Reagent Grade.
(e) Sephadex G-25 (fine), Pharmacia, Uppsala.
(f) Blue Dextran '2000', Pharmacia, Uppsala.
(g) Diethylaminoethyl (DEAE) cellulose, Baker Reagent grade. The capacity of the anion exchanger was 0.7 mg./g.
(h) Copper sulphate; 5H2O, Reagent Grade; (h) sodium carbonate, Reagent Grade; potassium dihydrogen tartrate, Reagent Grade.
(i) Folin Phenol Reagent 2N.
(j) Plasma, TA-2 Transfer Pack, Fenwal Labs., Ill. This was kept frozen.
(k) Nitrogen, "G" Grade.

(3) Preparation of Tris Buffer.

Titrate 150.0 ml. of IN HCl solution to approximately pH 7 with 0.5 molar Tris solution. Dilute to almost one litre with distilled water and adjust to pH 7.4 with 0.5 molar Tris solution. Make up to one litre with distilled water. The pH of this buffer at 37°C was 7.1.

This buffer was used in all experiments unless otherwise stated and was prepared daily. The 0.5 molar Tris solution was stored at room temperature, for not more than one week. Its ionic strength is 0.15 and its buffer capacity 0.021, capacity being calculated by a method given by Bates (1961). He claimed that the useful range of this buffer was from pH 7 to pH 9.
Since Tris has intramolecular bonds, it should be relatively inert (Benesch and Benesch [1955]). Chloride ions could bind to HSA but Scatchard and others (1950) showed this ion had a very low affinity for the macromolecule. Hence binding results would not be significantly affected by a buffer containing chloride ions. Few other buffers are available for studies at pH 7.4. Phosphate buffer covers this range but Klotz and Urquart (1949) showed that phosphate ions interfere with the binding of small molecules to protein. Phosphate buffer has also been reported to interfere with the spectrophotometric analysis of BHC.

(4) Determination of Absorptivity Value of Bishydroxycoumarin

Accurately weigh 100 mg. BHC and make up to 100.0 ml. with 0.1N sodium hydroxide solution. Dilute 5.0 ml. of this solution to 100.0 ml. with Tris buffer. Dilute 8.0, 10.0, 12.0, 16.0, and 20.0 ml. aliquots of the Tris solution to 100.0 ml. with Tris buffer. This gives a series of solutions of concentration 4, 5, 6, 8 and 10 mg. BHC/1., respectively. Read the absorbance of these solutions at 310 μm. On the basis of 25 determinations, the absorptivity value for BHC was 59.47 ± 0.54.

Preliminary spectral characteristics of a 5 mg./1. and 10 mg./1. solution of BHC were determined on a Spectronic 505 recording spectrophotometer. See Figure 1. An absorbance maxima occurs at 310 μm and this wavelength was chosen for all subsequent analyses.
Figure 1. Spectral Characteristics of Phenylbutazone (---) and Bishydroxycoumarin (---).

Wavelength $\mu$m
(5) Determination of Absorptivity Value of Phenylbutazone

Accurately weigh 100 mg. PBZ and make up to 100.0 ml. with 0.1 N sodium hydroxide solutions. Make dilutions as in the procedure for BHC in (4) above. This gives a series of solutions of concentration 4, 5, 6, 8 and 10 mg./l. Read the absorbance of these solutions at 264 m\textmu. On the basis of 20 determinations, the absorptivity value for PBZ was 67.58 ± 0.78.

Preliminary spectral characteristics of a 5 mg./l. and 10 mg./l. solution of PBZ were determined on a Spectronic 505 Recording Spectrophotometer. See Figure 1. An absorbance maxima occurred at 264 m\textmu and this was the wavelength chosen for all subsequent analyses.

(6) Analysis of Mixtures of Bishydroxycoumarin and Phenylbutazone by the Absorbance Ratio Method

(a) Determination of Q:310:264 for BHC

Prepare a series of solutions containing 4, 5, 6, 8 and 10 mg. BHC/l. as in (4) above. Read absorbances of these solutions at 310 m\textmu and 264 m\textmu. On the basis of 25 determinations the Q:310:264 value for BHC was determined to be 1.95 ± 0.06.

(b) Analysis of Mixtures of Phenylbutazone and Bishydroxycoumarin in Solution

Weigh 100 mg. BHC and make up to 100.0 ml. with 0.1 N NaOH. Similarly, weigh 100.0 mg. PBZ and make up to 100 ml. with 0.1 N NaOH. Dilute 5.0 ml. aliquots of each of these solutions to 100.0 ml. with Tris buffer. Dilute aliquots of these stock solutions to 100.0 ml. with Tris buffer in the manner indicated below:
<table>
<thead>
<tr>
<th>Solution</th>
<th>ml. PBZ Solution</th>
<th>ml. BHC Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.0</td>
<td>22.0</td>
</tr>
<tr>
<td>2</td>
<td>8.0</td>
<td>22.0</td>
</tr>
<tr>
<td>3</td>
<td>10.0</td>
<td>12.0</td>
</tr>
<tr>
<td>4</td>
<td>10.0</td>
<td>12.0</td>
</tr>
<tr>
<td>5</td>
<td>10.0</td>
<td>8.0</td>
</tr>
<tr>
<td>6</td>
<td>10.0</td>
<td>8.0</td>
</tr>
<tr>
<td>7</td>
<td>16.0</td>
<td>4.0</td>
</tr>
<tr>
<td>8</td>
<td>16.0</td>
<td>4.0</td>
</tr>
<tr>
<td>9</td>
<td>20.0</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>20.0</td>
<td>-</td>
</tr>
</tbody>
</table>

Read the absorbance of each of these solutions at 264 mµ and 310 mµ and calculate the amount of PBZ and BHC present in these mixtures (Pernarowski [1969]). From the spectral characteristics shown in Figure 1, 264 mµ and 310 mµ were found to be convenient wavelengths for the analysis of each drug in the mixture. At 264 mµ, both PBZ and BHC contribute to the absorbance whereas at 310 mµ only BHC contributes. Thus, since $A_{310}$ is measured and $a_{310}$ has already been determined in this study, the concentration of BHC in the mixture is readily determined. $Q_{BHC} = \frac{a_{310}}{a_{264}} = \frac{A_{310}}{A_{264}}$. This is a constant and is equal to 1.95. At 264 mµ,

$$A_T = A_{PBZ} + A_{BHC}$$
where $A_T = \text{total absorbance}$ and $A_{PBZ} = \text{absorbance due to PBZ}$, and $A_{BHC} = \text{absorbance due to BHC}$. Therefore,

$$A_{PBZ} = A_T - A_{BHC}$$

Thus, the absorbance at 264 m\(\mu\) due to PBZ may be calculated by measuring absorbance of the mixture at 264 m\(\mu\) and absorbance at 310 m\(\mu\) (due to BHC only).

Results of the analysis of solutions containing mixtures of PBZ and BHC are given in Table 1. Agreement between calculated and expected concentrations is satisfactory. Preliminary objectives include competitive binding. This method was developed in anticipation of such studies but, because of difficulties in the diafiltration technique, was not used.

(7) The Diafiltration Apparatus

A diagram of the apparatus is shown in Figure 2.

The 50 litre glass waterbath was maintained at 37°C (unless otherwise stated) by a Haake pump and thermostat. The Amicon reservoir tank (12 litre capacity), with a maximum pressure capacity of 100 psi, was made of stainless steel (epoxy coated). It was fitted with a fill port, pressure relief valve, and inlet and output connectors.

The Amicon ultrafiltration cell (Model 52, 50 ml. capacity, 43 mm. diameter) is shown in Figure 3. The cell was seated in a water-jacketed
### Table 1. Analysis of Bishydroxycoumarin and Phenylbutazone in Solution by the Absorbance Ratio Method

<table>
<thead>
<tr>
<th>Solution</th>
<th>Expected PBZ Conc. mg/l.</th>
<th>Expected BHC Conc. mg/l.</th>
<th>$A_{310}$ of mixture</th>
<th>$A_{264}$ of mixture</th>
<th>Calculated PBZ Conc. mg/l.</th>
<th>Calculated BHC Conc. mg/l.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.95</td>
<td>12.0</td>
<td>0.720</td>
<td>0.640</td>
<td>3.99</td>
<td>12.06</td>
</tr>
<tr>
<td>2</td>
<td>3.95</td>
<td>12.0</td>
<td>0.723</td>
<td>0.635</td>
<td>3.91</td>
<td>12.09</td>
</tr>
<tr>
<td>3</td>
<td>5.93</td>
<td>6.0</td>
<td>0.363</td>
<td>0.595</td>
<td>5.99</td>
<td>6.07</td>
</tr>
<tr>
<td>4</td>
<td>5.93</td>
<td>6.0</td>
<td>0.362</td>
<td>0.594</td>
<td>5.99</td>
<td>6.06</td>
</tr>
<tr>
<td>5</td>
<td>5.93</td>
<td>4.0</td>
<td>0.241</td>
<td>0.530</td>
<td>5.97</td>
<td>4.05</td>
</tr>
<tr>
<td>6</td>
<td>5.93</td>
<td>4.0</td>
<td>0.243</td>
<td>0.530</td>
<td>5.96</td>
<td>4.04</td>
</tr>
<tr>
<td>7</td>
<td>7.90</td>
<td>2.0</td>
<td>0.124</td>
<td>0.610</td>
<td>7.98</td>
<td>2.07</td>
</tr>
<tr>
<td>8</td>
<td>7.90</td>
<td>2.0</td>
<td>0.123</td>
<td>0.608</td>
<td>7.58</td>
<td>2.06</td>
</tr>
<tr>
<td>9</td>
<td>9.88</td>
<td>0.0</td>
<td>0.005</td>
<td>0.695</td>
<td>9.97</td>
<td>---</td>
</tr>
<tr>
<td>10</td>
<td>9.88</td>
<td>0.0</td>
<td>0.006</td>
<td>0.699</td>
<td>9.98</td>
<td>---</td>
</tr>
</tbody>
</table>
Figure 2. The Diafiltration Apparatus
beaker maintained at 37°C and the contents stirred by use of a magnetic stirrer. The cell is assembled and clamped together with a snap metal clamp. The cylindrical sleeve is transparent and graduated. A stirring assembly, a pressure relief valve (and fill port), and inlet port and outlet port are built into the cell. O-ring gaskets prevent leakage. Materials used in the cell are Delrin, Teflon or other inert plastics. The sleeve is made of polycarbonate plastic. A polyethylene porous disc acts as a support for the membrane. Polyethylene tubing was provided with this cell but, for reasons given later, was replaced with Teflon tubing in all parts of the system. The membrane used in these studies was Diaflo PM-10 (43 mm diameter) as recommended by the manufacturers for binding studies with HSA. These membranes are made from biologically inert polymeric material and prevent passage of molecules of molecular weight greater than 10,000. This anisotropic diffusive membrane consists of an extremely thin (0.1 - 10μ) layer of dense polymer supported on a much thicker structure of porous open cell structure. This membrane is resistant to most common chemicals and solvents. The ultrafiltrate from the cell outlet port is passed via Teflon tubing to an Isco Fraction Collector (Model 326). An Amicon C/D (Concentration/Dialysis) Selector is connected as shown in the diagram. This allows gas or liquid to flow from the reservoir to the cell as desired and thus the solution in the cell will be concentrated or dialysed respectively. Operation of this apparatus is explained later.
Figure 3. Amicon (Model 52) Diafiltration Cell
(8) General Procedure for the Determination of Drug - Human Serum Albumin Binding using the Diafiltration Technique

(a) Prepare an HSA solution of the desired concentration by dissolving the macromolecule in Tris buffer. Concentrations used are given in the latter part of this section.

(b) Prepare solution of the drug in Tris buffer from a stock solution containing 100 mg. drug / 100.0 ml. of 0.1 N NaOH. Place this solution in the reservoir tank and allow to equilibrate at 37°C.

(c) Prior to apparatus assembly, soak the Diaflo membrane in 100 ml. distilled water for 30 minutes. Discard the water and repeat this process with a further 100 ml. water.

(d) Place a known volume of purified HSA solution (see section on purifications of HSA) in the diafiltration cell and allow to equilibrate at 37°C.

(e) Connect the cell to the reservoir tank as shown in Figure 2. Turn the stirrer on. The stirring rate used was that suggested by the manufacturer, i.e., the vortex should not be more than one third the total depth of liquid in the cell.

(f) i) For operation without the C/D selector: Close pressure relief valves on the reservoir tank and the cell. Open the pressure relief valve on the nitrogen cylinder until the desired pressure (on pressure gauge on the cylinder) is achieved.
ii) For operation with the C/D selector: Put the C/D selector in the 'GAS' position, thus permitting gas to pass from the reservoir tank to the cell. With the pressure relief valves on the tank and the cell both in the open position, open the pressure valve on the nitrogen cylinder slowly. Shut both pressure valves and open the cylinder pressure valve until the desired pressure is achieved on the cylinder gauge. Allow two minutes for pressure to equalize in the tank and the cell. Shift the C/D selector switch to the 'LIQUID' position. Very slightly open the pressure relief on the cell to allow the solution to begin to flow into the tubing connected to the cell. If this valve is opened too much, solution flow will be excessive and a volume increase will occur in the cell.

(g) Sample Collection: During preliminary experiments samples were collected manually. In later experiments samples were collected automatically by an Isco Fraction Collector. Samples can be collected on a time basis (i.e., x minutes per fraction). This approach was used in preliminary studies, but was discarded because flow rates of the filtrate were not constant. In subsequent experiments, samples were collected by volume (i.e., 10 ml. fractions). This procedure involved the use of a volume collecting device but checks on volumes delivered indicated that the device tended to be inaccurate. Hence, all fractions collected were checked independently for volume.
(h) Record the volumes of successive fractions and their absorbance readings until equilibrium is reached or until approximately 500 ml. of filtrate has been collected. (This takes approximately 8 hours, depending on HSA concentration.)

(i) Record the final volume in the cell. Determine the concentration of drug in the reservoir tank.

[Note: The above mentioned stock solutions of BHC and PBZ were stored in the dark at 4°C for not more than one week. There have been reports on the decomposition of PBZ solutions by oxidation and by hydrolysis (see Section II, 3). The extent of this decomposition occurring in solutions was investigated using methods given in a paper by Beckstead and Kaistha (1968). Solutions containing 100 mg. PBZ / 100 ml. of Tris buffer and 0.1 N NaOH, respectively, were prepared. These solutions were placed in a water-bath at 37°C in the presence of light. After 4 days, traces of n-(2-carboxy-2-hydroxy-caproyl-hydrazobenzene) and 4-hydroxyphenylbutazone were found by a thin layer chromatography technique. Qualitatively, the PBZ solution in 0.1 N NaOH contained more decomposition products. By the quantitative simultaneous equation method of Beckstead and Kaistha, after 4 days at 37°C, it was shown that neither solution contained appreciable amounts of decomposition products. After 4 weeks at room temperature, in the presence of light, a solution of PBZ in 0.1 N NaOH contained 9% of decomposition products. Thus, it was concluded that in drug
binding studies where a diafiltration experiment is completed within 8 hours, decomposition of PBZ solutions would be insignificant. In addition, solutions stored at 4°C in the absence of light, for not more than a week, would not decompose significantly.]

(9) Phenylbutazone - Human Serum Albumin Binding Studies by the Desorption (Washout) Diafiltration Technique

Complete a PBZ-HSA binding experiment in the normal manner. Remove ligand solution from the reservoir tank and after rinsing, Tris buffer was placed in the reservoir tank. Rinse tubing to and from the cell and reconnect. Run the experiment in the same manner as for the wash-in diafiltration procedure. Analyse fractions of the filtrate spectrophotometrically at 264 μm.

(10) Bishydroxycoumarin - Human Serum Albumin Binding Studies by the Equilibrium or Direct (Diafiltration) Technique

Place 30 ml. of an HSA solution in the diafiltration cell and solutions (500 ml.) of various BHC concentrations in the reservoir tank. Run the experiment at 25 psi in the same manner as for diafiltration experiments. Analyse fractions of the filtrate until the A₃₁₀ value for the filtrate equals that of the reservoir solution. At this point, final equilibrium is considered to have been reached. Record the final volume in the cell and correct the HSA concentration for any volume increase. Determine the total BHC concentration in the cell by measuring the cell contents spectrophotometrically at 310 μm.
Drug Binding Studies by the Centrifugation (Ultrafiltration) Method

These studies were carried out using Amicon Centriflo membrane cones (CF 50), together with Centriflo supports and tubes (see Figure 4) and an International Centrifuge (Model SBV, Size 1). In principle this is similar to membrane ultrafiltration, with centrifugal force instead of pressure being used to push the drug through the membrane. Since the forces involved are greater, the filtrate is collected rapidly.

![Centriflo Centrifugation (Ultrafiltration) Apparatus](image)

**Figure 4. The Centriflo Centrifugation (Ultrafiltration) Apparatus**

i) Calibration of the Speed of the Centrifuge to Relative Centrifugal Force (r.c.f.)

The relationship between r.c.f. and r.p.m. is as follows:

$$ r.c.f. = 0.00001118 \times r \times S^2 $$

where $r$ = the rotating radius in cm. and $S$ = the rotating speed in
r.p.m. The r.c.f. value for the Centriflo apparatus at 1100 r.p.m. was found to be 108.9 x G (r = 9 cm. and S = 1100 r.p.m.).

ii) Binding Studies

Soak the membrane cones for two hours in distilled water. Spin the empty cones in the centrifuge at 2000 r.p.m. for 10 minutes to remove residual water. Failure to remove residual water would result in an inaccurate determination of drug in the ultrafiltrate.

The maximum amount of solution which could be placed in the cones was 3.5 ml.

Studies indicated that several washings of the Centriflo apparatus were required to remove a foreign substance which absorbed ultraviolet energy at 264 m\text{u}, the analytical wavelength for PBZ.

Experiments with 'purified' HSA solutions (1.2 x 10^{-3} M) at 500, 1100, and 1500 r.p.m. indicated that substantial amounts of a protein-like substance passed through the membrane cone. The ultraviolet spectra of the ultrafiltrate showed that this substance was the same as that found during the diafiltration process.

(12) Phenylbutazone - Human Serum Albumin Binding Studies by a Molecular Sieve Technique using Sephadex G-25

This technique involves the molecular sieving effect which occurs when swollen gel particles of certain polymers come into contact with a mixture of molecular species dissolved in the swelling medium. The solute molecules partition between the solvent external to the gel particles and the solvent imbibed by the gel, as described by the distribution coefficient
(K_D) which is equal to C_i/C_o, where C_i and C_o are the solute concentrations in the internal and external compartments, respectively. The pore size of the gel should be such that for protein molecules, K_D = 0, and for ligand molecules, K_D = 1. Thus protein and protein-ligand complexes can be separated from free ligand. Wood and Cooper (1970) reviewed the ways in which gel filtration can be applied to the protein binding studies.

The simplest approach is to use the Batch method. A known volume of sample solution of known protein and ligand concentrations is added to a known weight of gel. The mixture is allowed to equilibrate and the gel and external phases are separated by centrifugation or filtration. Protein and total ligand in the solution are determined. This method has been frequently used in HSA and plasma binding studies and its applications have been reviewed by Wood and Cooper. Scholtan (1965), however, was the only author to compare his results (on the binding of sulphonamides to serum albumin) with another method.

Chromatographic gel filtration techniques can also be used in protein-drug binding studies. The most commonly used chromatographic technique is zonal separation, but results from this procedure are often difficult to interpret quantitatively when binding is reversible. A technique avoiding this problem of dissociation of the protein-ligand complex was devised by Hummel and Dreyer (1962). They equilibrated the column with the same concentration of ligand solution as was present in the protein-ligand mixture applied to the column. Frontal analysis has been used more recently and appears to give results comparable with those obtained
by other methods (Cooper and Wood [1968], Burke [1969]). The general validity of this method remains to be demonstrated but it is expected to be applicable to the binding of a wide range of ligands to HSA and plasma.

In these studies, PBZ interactions with HSA are studied by the Batch method. Sephadex G-25 (fine grade) has a nominal water regain of 2.5 g. water per g. dry gel.

To prepare the swollen gel, add 4.0 g. of dry gel to 15.0 ml. Tris buffer so that the total volume of liquid in the gel is 25.0 ml. Equilibrate the system by shaking for one hour at 37°C. Remove a sample from the external phase and centrifuge the sample to precipitate any gel particles. Analyse the supernatant fluid.

Determination of the external volume of the gel: Sephadex G-25 has a nominal water uptake of 2.5 g. ± 0.2 g. per g. dry gel but this value does not give the external volume accurately. Using Blue Dextran, the external volume can be determined with greater precision. Blue Dextran 2000 has an average molecular weight of 2,000,000 and remains in the external phase. Prepare a 0.4% w/v solution of Blue Dextran 2000 in Tris buffer. From this solution, make a series of solutions by dilution and record their A₆₂₀ values. The resultant calibration curve is shown in Figure 5. Add 5.0 ml. of an 0.2% Blue Dextran solution to the swollen gel. Add sufficient Tris buffer to make a total volume of 25.0 ml. of solution added to the gel. Equilibrate the gel for one hour. Remove a sample from the external phase and analyse at 620 m$log$ for Blue Dextran. The Blue Dextran concentration could be found from the calibration curve.
Figure 5. Calibration Curve for Dextran Blue 2000
The mean external phase volume (on a basis of six determinations) was found to be 15.32 ml.

Protein Adsorption to the Gel: Prepare a 3.16% w/v HSA solution. Dilute this solution 1 in 2.5, 1 in 5, 1 in 10, 1 in 20 and 1 in 40, respectively. Add 5.0 ml. of each of these HSA solutions to swollen gel samples. Make the total volume of solution added to the gel up to 25.0 ml. with Tris buffer. Equilibrate the gel for one hour at 37°C, with shaking. Remove the entire external phase with gentle suction in a Buchner funnel. Wash the gel well with Tris buffer and analyse the washings for HSA at 280 μm.

The results showed no HSA was adsorbed onto the gel.

Adsorption of Phenylbutazone onto the Gel: Add 5.0 ml. of a range of PBZ solutions (12.81, 20.04, 29.99, 60.70, and 155.30 mg./l.) to the swollen gel. Make the total volume of liquid added to the gel up to 25.0 ml. with Tris buffer. Equilibrate the gel for one hour at 37°C, with shaking. Allow the gel to settle and remove a sample from the external phase. Centrifuge the sample. Analyse the supernatant at 264 μm for PBZ.

The concentration of PBZ in the external phase is known. Thus, since the total amount of drug initially added to the gel is known, the amount of PBZ associated with one g. of gel can be calculated. (The drug associated with the gel is the sum of adsorbed drug and of drug in solution in the internal phase. It is unnecessary to distinguish between these two effects or to know the internal volume.) The results are
plotted in Figure 6. It can be seen, however, that PBZ is not linearly adsorbed onto the gel. Linearity is preferable for easy determination of binding parameters. Thus calculations of binding data, on the basis of data in Figure 6, may be inaccurate.

PBZ-HSA binding using the Batch method: Add 5.0 ml. of an HSA solution and 5.0 ml. of a PBZ solution to the swollen gel. A range of HSA solutions and PBZ solutions were added. Equilibrate the gel for one hour at 37°C, with shaking. Sample and analyse as above for PBZ. Binding results are given in the Results and Discussion Section.
Figure 6. Phenylbutazone Adsorption to Sephadex G-25
IV. RESULTS & DISCUSSION

1. Preliminary Check on Diafiltration Apparatus

A) Release of Foreign Substances from Apparatus

Distilled water was run through the apparatus and samples of the filtrate were collected every hour for three hours. Spectra of these samples were run on the Bausch and Lomb 505 Recording Spectrophotometer. These spectra indicated that the apparatus released a substance with maximum absorbance at 241 µm. To determine the source of this substance, the various parts of the apparatus were soaked overnight in distilled water. Spectra of these solutions were run on the Perkin-Elmer Recording Spectrophotometer. The yellow latex tubing (used for collecting the filtrate) was found to be the source of this substance. The polyethylene tubing used in other parts of the apparatus, on the other hand, yielded no foreign substance. Hence the latex tubing was replaced by polyethylene tubing.

B) Binding of Phenylbutazone and Bishydroxycoumarin to the Apparatus Components

Various concentrations of solutions of PBZ and BHC, as indicated in Tables 2 and 3, respectively, were prepared. Twenty-five ml. of each solution was placed in the Amicon cell and 500 ml. of the same solution was added to the reservoir tank. The experiment was run for each concentration of drug, for 75 minutes. After this period
of time, the concentration of drug in the filtrate was determined spectrophotometrically. In all experiments there was a lower concentration in the filtrate than in the original solution in the cell. Thus, the amount of drug bound can be calculated. Percent drug bound at various concentration levels for PBZ and for BHC are given in Tables 2 and 3, respectively. The results given in these tables indicate that for PBZ only a small percentage of the drug is bound and that this percentage decreases as PBZ concentration increases. For BHC, on the other hand, the percent binding is higher and more variable. Since BHC binding values were high, further investigations on the binding of BHC to the apparatus were carried out.

C) Binding of Bishydroxycoumarin to the Diafiltration Cell

Two diafiltration cells with membrane supports in place, were filled with 50.0 ml. of a $5.60 \times 10^{-5}$ M BHC solution, and the openings were sealed. The cells were rotated on a tumbler at 30 r.p.m. in a water-bath at 37°C. The cell contents were sampled at 2, 4, 8 and 24 hours and the BHC concentration determined spectrophotometrically. A decrease in the BHC concentration was noted with respect to time. The loss of BHC from the solution was expressed as percent BHC bound. The results are given in Table 4. Up to 8 hours, binding, though significant, is still with a reasonable range. At 24 hours, however, the binding of BHC to the cell more than doubled, but since experi-
Table 2. Binding of Phenylbutazone to Apparatus after 75 minutes of Diafiltration

<table>
<thead>
<tr>
<th>Concentration of PBZ in Reservoir Tank moles/l.</th>
<th>% Drug Bound to Apparatus *</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.319 x 10^{-5}</td>
<td>4.30 %</td>
</tr>
<tr>
<td>6.505 x 10^{-5}</td>
<td>3.13 %</td>
</tr>
<tr>
<td>1.191 x 10^{-4}</td>
<td>1.76 %</td>
</tr>
<tr>
<td>1.869 x 10^{-4}</td>
<td>1.63 %</td>
</tr>
<tr>
<td>3.070 x 10^{-4}</td>
<td>1.53 %</td>
</tr>
<tr>
<td>4.310 x 10^{-4}</td>
<td>1.27 %</td>
</tr>
<tr>
<td>6.040 x 10^{-4}</td>
<td>1.34 %</td>
</tr>
</tbody>
</table>

* Each value is the average of two determinations

Table 3. Binding of Bishydroxycoumarin to Apparatus after 75 minutes of Diafiltration

<table>
<thead>
<tr>
<th>Concentration of BHC in Reservoir Tank moles/l.</th>
<th>% BHC Bound at this Concentration of BHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.068 x 10^{-6}</td>
<td>10.79 %</td>
</tr>
<tr>
<td>1.111 x 10^{-5}</td>
<td>6.04 %</td>
</tr>
<tr>
<td>2.781 x 10^{-5}</td>
<td>10.22 %</td>
</tr>
<tr>
<td>2.405 x 10^{-5}</td>
<td>10.49 %</td>
</tr>
<tr>
<td>4.120 x 10^{-5}</td>
<td>8.37 %</td>
</tr>
<tr>
<td>5.070 x 10^{-5}</td>
<td>11.19 %</td>
</tr>
</tbody>
</table>
ments were not expected to run more than 8 hours, this is not a significant factor in studies of this type.

D) Binding of Bishydroxycoumarin to Polyethylene Tubing

A 40 cm. length of polyethylene tubing was placed in a glass flask containing 100.0 ml. of 5.60 x 10^{-5} M BHC solution. The flask was sealed and rotated in the water bath as in C) above. The flask contents were analysed spectrophotometrically at 2, 4, 8 and 24 hours. Percentages of BHC bound to polyethylene tubing with respect to time are shown in Table 5. These results show that binding of BHC to the tubing is very high even after only 8 hours of exposure to the drug. Therefore, other types of tubing had to be investigated.

E) Binding of Phenylbutazone and Bishydroxycoumarin to Teflon and Skimatco Tubing

Teflon and Skimatco tubing were exposed to PBZ and BHC as in D) above. After 12 hours at 37°C, the flask contents were analysed spectrophotometrically. Flasks, containing drug solutions only were used as controls. The results in these experiments showed:-

(a) Skimatco tubing released a substance which absorbed ultraviolet radiation significantly at 264 m\text{\mu}, but not at 310 m\text{\mu}. This, therefore, would not be a satisfactory tubing in that it would interfere with PBZ analysis.
Table 4.  Binding of Bishydroxycoumarin to the Diafiltration Cell

<table>
<thead>
<tr>
<th>Time in hours</th>
<th>% BHC Bound to Cell Components *</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>5.45</td>
</tr>
<tr>
<td>4</td>
<td>5.66</td>
</tr>
<tr>
<td>8</td>
<td>6.42</td>
</tr>
<tr>
<td>24</td>
<td>14.02</td>
</tr>
</tbody>
</table>

* Results given are the average of results from the two diafiltration cells.
Initial BHC concentration in cells was 5.60 x 10^{-5} moles/1.

Table 5. Binding of Bishydroxycoumarin to Polyethylene Tubing

<table>
<thead>
<tr>
<th>Time in hours</th>
<th>% BHC Bound to Polyethylene Tubing</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2.55</td>
</tr>
<tr>
<td>4</td>
<td>5.19</td>
</tr>
<tr>
<td>8</td>
<td>19.95</td>
</tr>
<tr>
<td>24</td>
<td>37.35</td>
</tr>
</tbody>
</table>

* Results given are the average obtained from two 40 cm. sections of tubing (½" diam.)
Initial BHC concentration in the flasks was 5.60 x 10^{-5} moles/1.
(b) Teflon tubing, on the other hand, did not bind either BHC or PBZ and did not release any UV absorbing substances.

As a result of these investigations, Teflon tubing appears to be the tubing of choice for the diafiltration apparatus when PBZ and BHC are being used in studies.

F) Binding of Bishydroxycoumarin and Phenylbutazone to Components of the Apparatus (Connected with Teflon Tubing)

Binding studies were now repeated as in 1 (B) above, except that the experiments were continued for two hours. Approximately 450 ml. filtrate were collected. The filtrate, in this series of experiments, was sampled as indicated in Tables 6 and 7. PBZ and BHC concentrations are also given in these tables. These results show that with both BHC and PBZ, binding to components of the apparatus occurs. The percent drug bound decreases as filtration volume increases, and also as drug reservoir concentration increases. Because of binding variability, corrections are difficult to make during a diafiltration experiment. At best, an average value would have to be used. However, such values may be in error, because results obtained in these experiments may not be due to binding but rather to membrane rejection. (See IV, (1), G). It should be noted that these binding experiments were continued until a volume of filtrate similar to that in drug - HSA binding studies had been collected. Drug - HSA binding experiments, however, took about 8 hours for complete diafiltration.
G) Membrane Binding and Rejection: Diafiltration of Phenylbutazone and Bishydroxycoumarin in the Absence of Human Serum Albumin

An alternative method (to those mentioned in IV (1), F), can be used to study binding and/or rejection. This is based on a comparison of theoretical and experimental dilution curves in the absence of HSA. The following experiments were carried out:

For PBZ, a $8.77 \times 10^{-5}$ M solution of PBZ was placed in the reservoir tank and diafiltered through 30.0 ml. of Tris buffer in the diafiltration cell. Twelve ml. fractions were collected and analysed spectrophotometrically until diafiltration was essentially complete.

For BHC, a $2.046 \times 10^{-4}$ M BHC solution was similarly diafiltered through 25.0 ml. Tris buffer. Ten ml. fractions were collected and analysed spectrophotometrically until diafiltration was essentially complete.

The experimental dilution curves are shown in Figures 7 and 8. Theoretically dilution curves can be plotted and are shown in these figures. A simple exponential expression can relate the filtrate concentration to the reservoir tank concentration (Ryan and Hanna [1971]):

$$[S_n] = [S_o] \left[ 1 - (1 - \alpha)^n \right]$$

where $[S_n]$ and $[S_o]$ are the concentrations in moles/l. of the filtrate and reservoir solutions, respectively, $n$ is the number of fractions collected and $\alpha$ is the ratio of the volume of the fraction to cell volume. As shown in Figures 7 and 8, experimental dilution curves for both BHC and PBZ differ from theoretical
Table 6. Binding of Phenylbutazone to Components of the Apparatus
(Connected with Teflon Tubing)

<table>
<thead>
<tr>
<th>Volume of Filtrate</th>
<th>Reservoir Concentration 3.40 x 10^{-5} moles/l.</th>
<th>Reservoir Concentration 6.91 x 10^{-5} moles/l.</th>
<th>Reservoir Concentration 1.15 x 10^{-4} moles/l.</th>
<th>Reservoir Concentration 4.18 x 10^{-4} moles/l.</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 ml.</td>
<td>7.43 %</td>
<td>7.55 %</td>
<td>5.93 %</td>
<td>--</td>
</tr>
<tr>
<td>120 ml.</td>
<td>6.66 %</td>
<td>6.56 %</td>
<td>4.64 %</td>
<td>--</td>
</tr>
<tr>
<td>180 ml.</td>
<td>6.09 %</td>
<td>6.56 %</td>
<td>3.64 %</td>
<td>3.42 %</td>
</tr>
<tr>
<td>300 ml.</td>
<td>5.44 %</td>
<td>5.96 %</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>450 ml.</td>
<td>5.44 %</td>
<td>4.55 %</td>
<td>3.64 %</td>
<td>3.87 %</td>
</tr>
</tbody>
</table>

Table 7. Binding of Bishydroxycoumarin to Components of the Apparatus
(Connected with Teflon Tubing)

<table>
<thead>
<tr>
<th>Volume of Filtrate</th>
<th>Reservoir Concentration 1.49 x 10^{-5} moles/l.</th>
<th>Reservoir Concentration 3.08 x 10^{-5} moles/l.</th>
<th>Reservoir Concentration 1.78 x 10^{-4} moles/l.</th>
<th>Reservoir Concentration 4.18 x 10^{-4} moles/l.</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 ml.</td>
<td>7.75 %</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>120 ml.</td>
<td>--</td>
<td>8.37 %</td>
<td>--</td>
<td>4.09 %</td>
</tr>
<tr>
<td>180 ml.</td>
<td>--</td>
<td>7.82 %</td>
<td>6.50 %</td>
<td>3.40 %</td>
</tr>
<tr>
<td>300 ml.</td>
<td>6.35 %</td>
<td>5.99 %</td>
<td>6.00 %</td>
<td>1.60 %</td>
</tr>
<tr>
<td>450 ml.</td>
<td>5.37 %</td>
<td>5.30 %</td>
<td>6.00 %</td>
<td>--</td>
</tr>
</tbody>
</table>
Figure 7. Theoretical (—) and Experimental (○—○) Dilution Curves for Phenylbutazone.
(Reservoir Conc - 8.77 x 10^{-5} M PBZ)
Figure 8.
Theoretical (---) and Experimental (- - -) Dilution Curves for Bisacodyl.
dilution curves. This difference is more marked for BHC. This indicates that with both BHC and PBZ, binding and/or rejection of the drug must be occurring in the apparatus.

Blatt, Robinson and Bixler (1968) showed that plots of log $C_f/(C_f - C)$ versus $v$ (see Eq. 23), can give more useful information than the dilution curves shown in Figures 7 and 8. Data from these experimental dilution curves is plotted in the manner indicated by Blatt, Robinson and Bixler in Figures 9 and 10. As predicted, a linear relationship exists (in the absence of HSA). The slope of these plots gives $0.4343 / \bar{V}_q$ and thus the apparent cell volume $\bar{V}_q$ can be calculated. For phenylbutazone, in the absence of HSA, (Figure 9) the apparent cell volume is 34.6 ml., which is larger than the experimentally determined value of 30.0 ml. For bishydroxycoumarin, in the absence of HSA, (Figure 10) the apparent cell volume is 33.1 ml., also larger than the experimentally determined value of 25.0 ml. Blatt, Robinson and Bixler suggest such apparent increases in cell volume arise from solute rejection.

However, neither the experimental dilution curves, nor the plotting method indicated by Blatt, Robinson and Bixler, enable a distinction to be made between membrane (or apparatus) binding or rejection. In both cases, the concentration of ligand in the filtrate at any point during diafiltration is lower than that theoretically calculated. In the case of membrane rejection, however, the concentration of ligand in the cell is higher than that in the filtrate at any instant of time. A distinction could be
Figure 9. Plotting of Phenylbutazone - Human Serum Albumin Binding Results in the Manner of Blatt, Robinson and Bixler (1968).

\((\bullet \bullet) = 9.77 \times 10^{-5} \text{ M PBZ reservoir concentration in the absence of HSA,}\) 
\((\triangle \triangle) = 9.19 \times 10^{-5} \text{ M PBZ reservoir concentration, where HSA concentration = } 1.385 \times 10^{-4} \text{ M,}\) 
\((\square \square) = 9.09 \times 10^{-5} \text{ M PBZ reservoir concentration where HSA concentration = } 4.772 \times 10^{-4} \text{ M.}\)
Figure 10. Plotting of Bishydroxycoumarin - Human Serum Albumin Binding Results in the manner of Blatt, Robinson and Bixler (1968).

( • • ) = 2.046 x 10^{-4} M BHC reservoir concentration in the absence of HSA, ( ▲ ▲ ) = 2.876 x 10^{-5} M BHC reservoir concentration where HSA concentration = 0.420 x 10^{-4} M, ( ■ ■ ) = 2.885 x 10^{-4} M BHC reservoir concentration and HSA concentration where HSA concentration = 6.129 x 10^{-4} M HSA.

\[ \bar{V}_0 = 33.1 \text{ ml.} \]
made, however, if cell and filtrate were sampled simultaneously. Ryan and Hanna (1971), thus reported UM-10 membrane rejection of testosterone solutions. Testosterone concentrations in the filtrate rose steadily towards the reservoir concentration as diafiltration proceeded, but simultaneous sampling of cell contents showed that cell testosterone levels rose above the reservoir concentration. Such experiments were not carried out in this work and therefore it can only be concluded that membrane binding and/or rejection is occurring.

Blatt, Robinson and Bixler (1968) also suggested that changing the membrane area exposed to the ligand solutions, whilst keeping the sample volume constant could be another method of distinguishing between membrane binding or rejection. These authors defined a reflection coefficient ($\sigma$):

$$\sigma = 1 - \frac{C}{C_1}$$

where $C_1$ is solute concentration in the cell. For a small molecule, ideally $\sigma$ should be one. Equations 23 and 24 can be modified, by multiplying the right hand side by $\sigma$. Thus, these experimental dilution curves could be corrected for membrane rejection. However, whether membrane rejection occurs in the same manner in the presence of macromolecule is not known and could not easily be resolved.

Saturation binding to the membrane could also take place (Blatt, Robinson and Bixler [1968]). When the ligand has a very high affinity for the membrane, the membrane will become saturated at low ligand filtrate concentrations. This would give an apparent increase in the void volume of the system, but once the membrane has become
saturated by ligand, Equations 23 and 24 will be obeyed by the diafiltration process.

This discussion, based on current literature, is not likely to significantly affect the binding results in these studies. In a later section [2. F, (ii)], it will be shown that the percent binding found has little effect on the final binding curve.

H) Membrane Retention of Human Serum Albumin: Diafiltration of Human Serum Albumin in the Absence of Drug

This experiment checks membrane retentivity of the macromolecule. Twenty-five ml. of $2.9 \times 10^{-6}$ M HSA were placed in the diafiltration cell and Tris buffer in the reservoir tank. The experiment was run at 25 psi. The filtrate was collected for one hour and its UV spectrum determined on a Perkin-Elmer recording spectrophotometer. This spectrum is shown in Figure 8 and showed that a substance, with a maximum absorbance at 280 nm, was passing through the membrane. As shown also in this figure, this substance did not have the same spectrum as an HSA solution.

i) Effect of Time on the Appearance of Unknown Substance in the Filtrate

Tris buffer was run through the diafiltration cell, containing 25.0 ml. of a $5.8 \times 10^{-4}$ M HSA solution at 25 psi. Spectra of samples of the filtrate collected at 1, 2, 3 and 4 hours were recorded on the Perkin-Elmer recording spectrophotometer. The spectra obtained were identical to that shown
in Figure 11. The $A_{280}$ values of these samples of filtrate collected were plotted against time (Figure 12). Little of the substance was present in the filtrate after three hours.

ii) The Effect of the Unknown Substance in the Filtrate on Phenylbutazone and Bishydroxycoumarin Analysis

Tris buffer was run through 30.0 ml. of a $1.45 \times 10^{-4}$ M HSA solution in the diafiltration cell for 3 hours at 35 psi. The filtrate was collected in 1 hour fractions. Solutions containing 87.35 mg BHC / 100 ml. 0.1 N NaOH and 86.6 mg PBZ / 100 ml. 0.1 N NaOH, respectively, were prepared. These solutions were then diluted 1 in 100 with each hourly fraction of filtrate. Control solutions were prepared by diluting the BHC and PBZ solutions 1 in 100 with Tris buffer. Concentrations of BHC and PBZ were determined spectrophotometrically. Results are shown in Table 8. This series of experiments was carried out to determine how much effect the unknown substance would have on the analysis of BHC and PBZ in the filtrate. Results show that the unknown substance affects the analysis of PBZ but does not affect the BHC analysis. At low BHC concentrations, however, which do occur at the beginning of a binding experiment, the substance could affect BHC analysis in the filtrate. It is necessary, therefore, to remove the unknown "impurity", prior to a binding experiment.
Figure 11. Ultraviolet absorption spectra for a $3.49 \times 10^{-6}$ M HSA solution (---) and for the Unknown Substance in the Filtrate (——).

Figure 12. Binding Experiment in the Absence of Drug: Appearance of Unknown Substance in Filtrate During Experiment.

HSA concentration = $5.8 \times 10^{-4}$ moles/l.
Table 8. The Effect of the Unknown Substance in the Filtrate on the Analysis of Bishydroxycoumarin and Phenylbutazone

<table>
<thead>
<tr>
<th>Solution</th>
<th>Diluent</th>
<th>$A_{264}$</th>
<th>$A_{310}$</th>
<th>Drug Concentration in moles/l.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBZ</td>
<td>Control</td>
<td>0.578</td>
<td>0.002</td>
<td>$2.78 \times 10^{-5}$</td>
</tr>
<tr>
<td>PBZ</td>
<td>1 hr. filtrate</td>
<td>0.625</td>
<td>0.022</td>
<td>$3.00 \times 10^{-5}$</td>
</tr>
<tr>
<td>PBZ</td>
<td>2 hr. filtrate</td>
<td>0.608</td>
<td>0.012</td>
<td>$2.92 \times 10^{-5}$</td>
</tr>
<tr>
<td>PBZ</td>
<td>3 hr. filtrate</td>
<td>0.580</td>
<td>0.003</td>
<td>$2.78 \times 10^{-5}$</td>
</tr>
<tr>
<td>BHC</td>
<td>Control</td>
<td>0.275</td>
<td>0.519</td>
<td>$2.59 \times 10^{-5}$</td>
</tr>
<tr>
<td>BHC</td>
<td>1 hr. filtrate</td>
<td>0.310</td>
<td>0.519</td>
<td>$2.59 \times 10^{-5}$</td>
</tr>
<tr>
<td>BHC</td>
<td>2 hr. filtrate</td>
<td>0.294</td>
<td>0.516</td>
<td>$2.58 \times 10^{-5}$</td>
</tr>
<tr>
<td>BHC</td>
<td>3 hr. filtrate</td>
<td>0.280</td>
<td>0.519</td>
<td>$2.59 \times 10^{-5}$</td>
</tr>
</tbody>
</table>
iii) Purification of the Human Serum Albumin (Fraction V)

(a) Purification by Sephadex G-25:
A Sephadex G-25 column (1 cm. by 30 cm.) was prepared and 5 ml. of a $5.9 \times 10^{-4}$ M HSA solution was applied to the column. The HSA solution was collected from the column and transferred to a diafiltration cell. Tris buffer was then run through the cell and the filtrate collected for one hour. A spectrum of the filtrate obtained on the Perkin-Elmer recording spectrophotometer showed that the same unknown substance was present in the filtrate. Sephadex G-25 does not, therefore, purify HSA solutions.

(b) Purification by Dialysis with a Cellophane Membrane:
Dialysis cells were set up as described by Cho (1970), one set having Visking cellophane as a membrane and another set having a Diaflo PM-10 membrane. In one side of each set of cells, 10.0 ml. of $1.45 \times 10^{-4}$ M HSA solution was placed and, on the other side, 10.0 ml. of Tris buffer. The cells were then rotated at 30 r.p.m. in a 37°C water bath for 24 hours. Spectra of the contents of the compartment originally containing Tris buffer were then obtained on the Perkin-Elmer recording spectrophotometer. These showed that the unknown substance could pass through the Diaflo PM-10 filter but did not pass through the cellophane
membrane. Hence, dialysis with cellophane membranes (either in dialysis cells or in a dialysis sac) could not be used to purify the HSA.

(c) Purification by DEAE Cellulose:

The column was prepared in the following manner. The exchanger (1 g. dry weight / 15 ml. HCl) was poured into 0.5 N HCl with gentle stirring and allowed to stand for 30 minutes. The supernatant fluid was decanted and the exchange gently washed in a Buchner funnel with distilled water until the pH of the effluent was approximately 4. The exchanger was then treated with 0.5 N NaOH as indicated above. It was washed with distilled water until the pH of the effluent was approximately 7. Equilibration was attained by pouring the exchanger into 0.5 M NaCl and titrating slowly to pH 7.4 with 0.5 M HCl. The supernatant fluid was removed and replaced several times with Tris buffer to ensure that the pH remained at 7.4. The exchanger was poured into Tris buffer to make a 5-10% w/v suspension and then poured into the column (1 cm. x 25 cm.). After use of this column, it was washed with Tris buffer and re-equilibrated. Ten ml. of a 5.9 x 10^{-4} M HSA solution were then applied to the column. The fraction containing HSA (as determined by A_{280} values) was then collected and placed in the diafiltration cell. Tris buffer was then
run through the cell and the $A_{280}$ values of the filtrate measured as diafiltration proceeded. Ten ml. of untreated HSA solution ($5.9 \times 10^{-4}$ M) required a diafiltration volume of approximately 250 ml. Tris buffer to produce a zero $A_{280}$ value. The treated HSA solution only required about 75 ml. Therefore, the DEAE column partially purified the HSA solution and purification was completed by diafiltration of the treated HSA with Tris buffer. If this method of purification is used, some loss of the original HSA in the solution would be expected. Therefore a simple method for determining the final HSA concentration was necessary. The absorbance of the purified HSA solution was measured at 280 nm and concentration of the HSA was then read off a calibration curve prepared from standard solutions containing the macromolecule.

Calibration Curve for Measuring Human Serum Albumin Concentrations:

With proteins containing tyrosine and tryptophan, measurement of absorbance at 280 nm, (the UV absorbance maxima) can be used as a quantitative measure of protein concentration (Schultze and Heremans [1966]). Since the spectrum of HSA has an absorbance maxima at 280 nm, this approach can be used to analyse solutions containing HSA (or protein, in general).
Figure 13. Calibration Curve for the Determination of Human Serum Albumin at 280 nm.
the filter was not established. Several general observations could be made about its nature from the above experiments.

(a) The ultraviolet spectrum of the substance in the filtrate was that characteristic of a protein containing tryptophan or tyrosine. The substance, however, was not albumin. (See Figure 11)

(b) Since the albumin solution could not be purified by use of the Sephadex G-25 (which separates molecules of molecular weight 5000 or less), this suggests the molecular weight of the substance must be over 5000. If the PM-10 membrane does indeed cut off at a molecular weight of 10,000, as the manufacturers claim, the substance should have a molecular weight between 5000 and 10,000. However, it is likely that molecular shape determines the molecular weight cut-off level.

(c) Two other tests for protein were carried out on the unknown substance to determine if they were more sensitive than the $A_{280}$ spectrophotometric method:

The Biuret Test: This is based on the formation of a reddish-violet complex, copper ions from a dilute solution of CuSO$_4$ complex with amide linkages. This test was unsatisfactory because Tris buffer interfered to form a dee-blue coloured cupri-ammonium complex with copper ion.

The Folin Ciocalteau Test: This test is not as reproducible or specific as the Biuret Test. Any peptide
The calibration curve was prepared by measuring the $A_{280}$ of a series of standard solutions containing 10, 15, 25, 40 and 100 mg. of Fraction V HSA per 100 ml. of Tris buffer. The calibration curve is shown in Figure 13. Beer's Law is obeyed.

Percent of Recovery of Human Serum Albumin Purified by This Method:

Calculations showed that approximately 33% of the HSA was lost, either on the column or in the diafiltration procedure when 10 ml. of $5.9 \times 10^{-4}$ M HSA solution was purified by this method. Because of these losses the procedure is not satisfactory.

(d) Purification Using the Diafiltration Cell:

The HSA solution was placed in the diafiltration cell and Tris buffer diafiltered through the cell until the $A_{280}$ value for the filtrate was zero. The HSA concentration in the cell was then determined by use of the calibration curve shown in Figure 13. For a $5.9 \times 10^{-4}$ M HSA solution, this diafiltration procedure took approximately 250 ml. Tris buffer and four hours at 25 psi. This method was subsequently used for purifying HSA prior to use in all binding experiments.

iv) Nature of the Unknown Substance

The chemical structure of the substance passing through
bond will yield some colour. The procedure used was that described by Lowry and others (1951).

Reagent A: Sodium Carbonate, 5 gm./250 ml. 0.1 N NaOH
Reagent B: Aqueous Cupric Sulphate, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ Solution, 0.5% w/v
Aqueous Potassium Dihydrogen Tartrate Solution, 1% w/v
Reagent C: Mix 1 ml. of Reagent B with 50 ml. Reagent A. (Discard after 1 day)
Reagent E: Folin Phenol Reagent (2N), diluted 1 in 2.

To 1.0 ml. of standard HSA solution or of filtrate, add 5 ml. Reagent C, mix well and allow to stand 10 minutes or longer, at room temperature. Add rapidly 0.5 ml. Reagent E and mix immediately on a vortex mixer. Allow to stand 30 minutes or longer at room temperature. Read the absorbance of the solutions at 500 $\mu\text{m}$.

A standard curve was prepared using HSA solutions of known concentrations; 0.314, 0.0314, and 0.0031% w/v. A Tris buffer control was also prepared and used as a blank. The standard curve is shown in Figure 14.

This test was not as sensitive or reproducible as that which depended on absorbance measurements at 280 $\mu\text{m}$.

v) Purification of Fraction V Human Serum Albumin and Plasma by the Diafiltration Method

Both a 25.0 ml. plasma sample (undiluted) and 25.0 ml. of $5.9 \times 10^{-4} \text{M}$ HSA solution were diafiltered at 25 psi in the diafiltration cell with Tris buffer until the solutions were
Figure 14. Calibration Curve for Protein Determination by the Folin Ciocalteau Method.
essentially purified. The filtrate was collected in 20 ml. fractions on the Isco Fraction Collector. The spectrum of each fraction was recorded on the Hitachi Recording Spectrophotometer. $A_{280}$ values were measured and plotted against diafiltration volume in Figure 15. This figure shows that considerably more protein was released from the Fraction V HSA than from the plasma. Also spectra of fractions of filtrate from plasma were not identical with those from Fraction V HSA. It would be expected that plasma would release more protein-like material than Fraction V HSA, since there are proteins of lower molecular weight than HSA in plasma. Thus it would seem likely that the protein-like substance released from Fraction V HSA is some impurity present in the HSA powder supplied by the manufacturers. Pentex HSA of a different lot number revealed this same impurity on diafiltration with Tris buffer. From these studies, it seems unlikely that the "impurities" arise as a result of the diafiltration procedure itself. Figure 15 shows that similar diafiltration volumes are required for purifying plasma and the Fraction V HSA ($5.9 \times 10^{-4} \text{ M}$). However, the time for diafiltration of the plasma sample was prolonged (7 hours) compared to that of the HSA (4 hours), because the flow rate through the plasma was slower. The increased protein present in the plasma must cause this decrease in flow rate.
Figure 15. Disappearance of Protein-Like Substance from Plasma (---) and Fraction V HSA (---) on Diafiltration with Tris Buffer.
I) Void Volume in the Diafiltration Apparatus

With the diafiltration apparatus used in these studies, void volume was negligible (i.e., the volume between the membrane and outlet port in the cell). After purification of an HSA sample in the diafiltration cell, the tubing collecting the filtrate was always emptied prior to a binding experiment. Thus, the correction for void volume described by Blatt, Robinson and Bixler (1968) and Ryan and Hanna (1971) was not necessary.

J) Fluctuations in the Cell Volume during Diafiltration

When the diafiltration procedure (see Experimental Section II, 8) without the C/D selector was followed, an initial increase in volume of approximately 5 ml. occurred. This was due to different pressures in the reservoir tank and the cell. Once this initial increase occurred, the cell volume remained constant. As a result, the HSA concentration in the cell would be decreased and HSA concentrations were always corrected for this dilution factor (by multiplying the HSA concentrations by $V_i/V_f$).

When the diafiltration procedure using the C/D selector, was followed, increases in cell volume were rarely observed. However, in the case of an increase in volume, the HSA concentration was always corrected for this dilution factor.

[Note: (1) The volume of the reservoir tank is very large compared to that of the cell and presents difficulties in obtaining equal pressures in both containers. (2) Ryan and
Hanna (1971) eliminated the problems of cell volume fluctuations by filling the cell to capacity with macromolecule solution. In this case, stirring efficiency may be reduced.

2. Drug Binding Studies by the Diafiltration Technique

A) Calculation of Results

Drug binding data was obtained in a manner similar to that of Ryan and Hanna (1971), though in this study it was unnecessary to calculate areas under the curve (when drug filtrate concentration is plotted versus cumulative filtrate volume). Calculations were done by computer. A Fortran IV program "DRUGFIT" was prepared for calculating $r$ and $r/D_f$ values (see Table 9). Plotting routines, and a modified non-linear least squares fit (LQF) analysis (used by Meyer and Guttman [1968 b]) were included as subroutines. Thus plots of the fitted line and the experimental data points could be obtained, and $n_1$, $k_1$, $n_2$ and $k_2$ values computed. For each set of experimental data, a series of data cards were typed.

(a) Preparation of General Data Card:
Table 9. "DRUGFIT" Program

<table>
<thead>
<tr>
<th>Function</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>REAL KK</td>
<td>REAL numbers</td>
</tr>
<tr>
<td>KUKE</td>
<td>Logical flag</td>
</tr>
<tr>
<td>REAL W</td>
<td>REAL numbers</td>
</tr>
<tr>
<td>INTEGER</td>
<td>Integer numbers</td>
</tr>
</tbody>
</table>

Example: `REAL W(100), E(14), RF(100)`
Table 9. (Cont'd.)

48 \[ H = \text{FLOAT}(L)/100. \]
49 \[ CO = \text{CO}^1 \times 10^5 \]
50 \[ M = M^1 - n \times 10^{-1} \times 10^5 \]
51 \[ NVB = 1 \]
52 \[ 13 \]
53 \[ \text{READ}(1,12) \{L(1),1\} \times NVB,NVE \]
54 \[ NU = 21 \times NVB,NVE \]
55 \[ \text{IF}(V(1) = 0.0) \text{GO TO 31} \]
56 \[ \text{CONTINUE} \]
57 \[ NVB = NVE + 1 \]
58 \[ \text{GO TO 13} \]
59 \[ \]
60 \[ \text{WHITE}(6,41) \text{ ICOUNT} \]
61 \[ \text{IF}(K = E+1) \text{GO TO 19} \]
62 \[ \text{WHITE}(6,45) \{A, B, C, \text{CN}, M, L, VX\} \]
63 \[ \text{GO TO 16} \]
64 \[ \text{WHITE}(6,46) \{A, B, C, \text{CN}, M, L, VX\} \]
65 \[ \text{WHITE}(6,42) \]
66 \[ F1 = 0. \]
67 \[ V1 = 0. \]
68 \[ NU = 17 \times 1.4 \]
69 \[ V(1) = V(1) \times 10^5 \]
70 \[ \text{IP}(A(1),E+1) \{A(1) = 1, E = 1\} \]
71 \[ DJ = \text{A(1)} / \text{A(1)} \]
72 \[ DF = DJ / M \]
73 \[ F1 = F1 + UX / V(1) \]
74 \[ VT = VT + V(1) \]
75 \[ S = C \times VT \]
76 \[ DU = 2 \times F1 - VX \times UX \]
77 \[ NV = \text{CIVM} \]
78 \[ R = DBH / M \]
79 \[ \text{KF} = \text{KDF} \]
80 \[ \text{WHITE}(6,43) \{V(1), A(1), UX, DF, F1, S, DBH, R, KDF\} \]
81 \[ \text{KK} = \text{KDF} \]
82 \[ \text{KDF} = \text{DF} \]
83 \[ \text{WHITE}(6,43) \{V(1), A(1), UX, DF, F1, S, DBH, R, KDF\} \]
84 \[ \text{WHITE}(6,43) \{V(1), A(1), UX, DF, F1, S, DBH, R, KDF\} \]
85 \[ \text{KDF} = \text{DF} \]
86 \[ NU = 17 \times KDF, N \]
87 \[ J = 1 - KU \]
88 \[ \text{KK} = \text{KDF} \]
89 \[ \text{KDF} = \text{DF} \]
90 \[ \text{WHITE}(6,43) \{V(1), A(1), UX, DF, F1, S, DBH, R, KDF\} \]
91 \[ \text{WHITE}(6,43) \{V(1), A(1), UX, DF, F1, S, DBH, R, KDF\} \]
92 \[ \text{WHITE}(6,43) \{V(1), A(1), UX, DF, F1, S, DBH, R, KDF\} \]
93 \[ \text{WHITE}(6,43) \{V(1), A(1), UX, DF, F1, S, DBH, R, KDF\} \]
94 \[ \text{WHITE}(6,43) \{V(1), A(1), UX, DF, F1, S, DBH, R, KDF\} \]
95 \[ \text{WHITE}(6,43) \{V(1), A(1), UX, DF, F1, S, DBH, R, KDF\} \]
96 \[ \text{WHITE}(6,43) \{V(1), A(1), UX, DF, F1, S, DBH, R, KDF\} \]
97 \[ \text{WHITE}(6,43) \{V(1), A(1), UX, DF, F1, S, DBH, R, KDF\} \]
98 \[ \text{WHITE}(6,43) \{V(1), A(1), UX, DF, F1, S, DBH, R, KDF\} \]
99 \[ \text{WHITE}(6,43) \{V(1), A(1), UX, DF, F1, S, DBH, R, KDF\} \]
100 \[ \text{WHITE}(6,43) \{V(1), A(1), UX, DF, F1, S, DBH, R, KDF\} \]
101 \[ \text{WHITE}(6,43) \{V(1), A(1), UX, DF, F1, S, DBH, R, KDF\} \]
102 \[ \text{WHITE}(6,43) \{V(1), A(1), UX, DF, F1, S, DBH, R, KDF\} \]
103 \[ \text{WHITE}(6,43) \{V(1), A(1), UX, DF, F1, S, DBH, R, KDF\} \]
104 \[ \text{WHITE}(6,43) \{V(1), A(1), UX, DF, F1, S, DBH, R, KDF\} \]
105 \[ \text{WHITE}(6,43) \{V(1), A(1), UX, DF, F1, S, DBH, R, KDF\} \]
106 \[ \text{WHITE}(6,43) \{V(1), A(1), UX, DF, F1, S, DBH, R, KDF\} \]
107 \[ \text{WHITE}(6,43) \{V(1), A(1), UX, DF, F1, S, DBH, R, KDF\} \]
i
Table 9. (Cont'd.)

```
SUBROUTINE SOMS(N,SXX,SXY,A,ULG,M)

DO 10 I=1,N
SXY(I)=0.

DO 10 J=1,N
SXX(I,J)=0.

DO 30 K = 1,N
CALL UVFY(IK,A,FY,M)
DO 20 1 =1,N
SXY(I) = SXY(I) + FY*DVI(I)
DO 20 J = 1,N
SXX(I,J) = SXX(I,J) + DVI(I)*DVI(J)
CONTINUE
30 CONTINUE

DO W = 1,N
OIAG(I) = 1./SORT ISXX(I,I)
SXY(I) = SXY(I)*DIAG(I)
SXX(I,J) = SXX(I,J)*DIAG(I)*DIAG(J)
CONTINUE

RETURN
END

SUBROUTINE SLVM(SXX,SXY,C,DIAG,OA,S,M)

DO 20 I=1,M
DO 10 J = 1,M
S(I,J) = SXX(I,J)
CONTINUE
20 CONTINUE
M I , I ) + r

CALL CU(S,M,SXY,0,DET)
DO 40 1=1,M
DA(I) = 0.
DO 30 J = 1,M
DA(I) = DA(I) + SXY(I,J)*DIAG(I)
CONTINUE
30 CONTINUE

RETURN
END

FUNCTION SS0E1A...DA...M

DIMENSION A(10),UA(10),TA(10)
DIMENSION XI(150),X2(150),Y(150)
COMMON OA,X1,X2,Y,ZERO,YNIT

CALC(Y(A(1),A(2),A(3),A(4),X1(1)))

RETURN
END
```
<table>
<thead>
<tr>
<th>Line</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>224</td>
<td>RETURN</td>
</tr>
<tr>
<td>225</td>
<td>END</td>
</tr>
<tr>
<td>226</td>
<td>SUBROUTINE DVFY(K,X,Y,M)</td>
</tr>
<tr>
<td>227</td>
<td>DIMENSION U(1),V(1),X(I(150),Y(1)</td>
</tr>
<tr>
<td>228</td>
<td>CUMULATE X,Y,Z, M,N</td>
</tr>
<tr>
<td>229</td>
<td>CALL C1C1A.N.IL.M.DET</td>
</tr>
<tr>
<td>230</td>
<td>DO 5 I=1,M,2</td>
</tr>
<tr>
<td>231</td>
<td>T(K)=(A(I+1))X(K)</td>
</tr>
<tr>
<td>232</td>
<td>DO 4 I=1,M</td>
</tr>
<tr>
<td>233</td>
<td>U(I)=T(I)+A(I+1)*X(I)</td>
</tr>
<tr>
<td>234</td>
<td>5 DO 1=1,M</td>
</tr>
<tr>
<td>235</td>
<td>X(I)=X(I)*T(I)</td>
</tr>
<tr>
<td>236</td>
<td>END</td>
</tr>
</tbody>
</table>

**Example Code: SUBROUTINE C1C1A.N.IL.M.DET**

- RETURN
- ENDD
- SUBROUTINE C1C1A.N.IL.M.DET
- DIMENSION U(1),V(1),X(I),Y(I)
- CUMULATE X,Y, Z, M,N
- CALL C1C1A.N.IL.M.DET
- DO 5 I=1,M,2
- T(K)=(A(I+1))X(K)
- DO 4 I=1,M
- U(I)=T(I)+A(I+1)*X(I)
- 5 DO 1=1,M
- X(I)=X(I)*T(I)
- END
Table 9.  (Cont'd.)

```
296  135  CONTINUE
297  222  DO 3 I=1,N
298       L=L+1
299  19    IF( INDEX(L,1)-INDEX(L,2) ) > 19, 3, 19
300  19    JRM=INDEX(L,1)
301  19    JCOL=INDEX(L,2)
302  549    MU 549 X=1,N
303  549    T=K(JKMC)
304  549    A(K,JMC)=A(K,JC0L)
305  549    A(K,JCOL)=T
306  549    CONTINUE
307       3  CONTINUE
308       31  RETURN
309       301  END
```

END OF FILE

SIG S
The parameters CA, B, C, CO, M, and VX are printed onto the card.

CA = absorptivity of the drug (e.g., 59.47 for BHC)
B = molecular weight of the drug (e.g., 336.29 for BHC)
C = concentration of drug in reservoir solution (e.g., $9.32 \times 10^{-3}$ g./l.)
CO = the reciprocal of the product of VX (the final volume in the cell) and the molecular weight of the drug
M = the concentration of HSA solution in moles/l. (e.g., $5.15 \times 10^{-5}$ moles/l.)
L = a constant normally left blank in binding studies. The use of this constant for varying various parameters of the binding system will be explained in a later section.

VX = the volume in the cell in ml. (e.g., 27.0 ml.)
K = a constant. When $K = 1$, no printer plot will be plotted out. If $K \neq 1$, a printer plot will be given.

KF = a constant. If $KF = 1$, the debug will be on. If $KF \neq 1$, the debug will be off.
KD = in the case of inaccurate initial data, the number of initial r and D_f values to be deleted for the non-linear least square fit. All r and r/D_f values are given on the read-out. Calcomp plots may be obtained instead of printer plots.

(b) Preparation of Experimental Data Card:

\[ V_1, A_1, V_2, A_2 \text{ etc.} \]

Where \( V \) = volume of fraction of filtrate collected (e.g., 9.6 ml.) and \( A \) = absorbance reading of this fraction (e.g., 0.263).

Experimental data is typed onto data cards filling each card up to column 77 and then beginning on subsequent cards until all the data is entered. The card deck is read into the computer and the results are printed out in 10 columns with one row for each given data point. The ten columns are: \( V, A, DX, DF, FI, S, DB, DBB, R, RDF. \)
Where:

\[ V = \text{volume of fraction collected in ml.} \]
\[ A = \text{corresponding absorbance reading for this fraction} \]
\[ DX = \text{concentration of drug in filtrate fraction in g./l. (}= \text{mg./ml.}) \text{ i.e., concentration of free drug} \]
\[ DF = \text{concentration of drug in filtrate fraction in moles/l.} \]
\[ FI = \text{cumulative sum of the amount of drug in the filtrate in mg.} \]
\[ = FI_0 + [(V) \times (DX)] = \Sigma [(V) \times (DX)] \]
\[ S = \text{the total amount of drug in the cell and filtrate in mg.} \]
\[ = C \times VT \text{ (i.e., total filtrate volume is VT at this point)} \]
\[ DB = S - FI - [(DX) \times (VX)] = \text{total bound drug in mg. in the cell where (DX) \times (VX) is the amount of free drug in the cell in mg.} \]
\[ DBB = \text{the amount of bound drug in moles/l.} \]
\[ = DB \times CO \]
\[ R = \frac{DBB}{M} \]
\[ RDF = \frac{R}{DF} \]

\( n_1, k_1, n_2, k_2 \) values are printed out. The percent errors of the experimental data points as compared to the fitted line are given.

B) Plotting of Results

There are several ways of presenting binding data. The method of choice is the one which characterizes binding over as much of the
binding isotherm as possible. Plots vary in their ability to show difference in binding behaviour and to give accurate values of $n$ and $k$. Plotting methods have been described by Goldstein (1949) and Meyer and Guttman (1968). These can be derived from various rearrangements of Equation 8.

An adsorption type of plot, where $r$ (or some parameter proportional to $D_b$) is plotted against $D_f$, gives values of $k$ which are subject to considerable error. It is difficult to ascertain if data obeys the Law of Mass Action.

A double reciprocal plot is frequently used [e.g., Ryan and Hanna (1971), Crawford and others (1972), Solomon and others (1968)]. Here $1/r$ is plotted against $1/D_f$, where the intercept on the $1/r$ axis gives $1/n$ and the slope of the line gives $1/nk$. In this plot there is disproportionate weighing of points at high and low $D_f$ values. Hence, a few low $D_f$ values will outweigh many high $D_f$ values.

Nichol and others (1967), showed that double reciprocal plots could, theoretically, distinguish between the various combinations of polymerization of the macromolecule and ligand binding. The double reciprocal plot could assume six different possible forms:–

i) A straight line with slope and intercept independent of protein concentration, i.e., a single protein or series of polymers with polymerization and binding occurring independently, all sites being equivalent, or non-competitive isomerization with one form being inactive.
ii) A family of straight lines with different intercepts on the 1/r axis but a common intercept on the 1/D_f axis; i.e., non-competitive polymerization and ligand binding, with one form of polymer binding no ligand.

iii) A curve convex to the 1/D_f axis, independent of protein concentration; i.e., non-competitive isomerization and binding, with one form having less affinity for ligand than the other, or a single protein with two sets of sites or two non-interacting proteins with different binding affinities.

iv) A curve convex to the 1/D_f axis with a common 1/r intercept; i.e., non-competitive polymerization and binding with polymeric species having less affinity for ligand than the monomer.

v) A curve initially concave to the 1/D_f axis, independent of protein concentration; i.e., competitive isomerization and ligand binding.

vi) A series of curves initially concave to the 1/D_f axis with a common 1/r intercept; i.e., competitive polymerization and ligand binding.

(In (v) and (vi), the curves may subsequently become convex to the 1/D_f axis).

Although these results are theoretical, the above effects may be causing curvature of the plots, rather than deviations from the Law of Mass Action. (See Literature Section on Theory II (1)).
With albumin, isomerization occurs at low pH (Sogami and Foster [1968]). Various techniques have been used to show that polymerization occurs in HSA solutions. (Schultze and Heremans [1966]). Pederson (1962) showed that a 25% w/v aqueous solution of HSA stored for 24 hours at 4°C was mostly in the monomeric form. Thus polymerization and isomerization could be factors influencing HSA-ligand binding and the shape of binding curves.

Crawford and others (1972) used the classification of Nichol and others (1967). Double reciprocal plots of bromosulphthalein-HSA binding data were indicative of a competition between polymerization of the HSA and binding of the ligand.

In a Scatchard plot, (Scatchard [1949]), r/Df is plotted against r. The intercept on the r axis (obtained by extrapolating the initial linear portion of the curve) gives n1 and extrapolation to the r/Df axis gives n1k1. The slope of the initial straight line portion gives -k1. In this plot, points are more evenly weighted. Frequently when this plot is used to express protein binding data, a curve is obtained. It is possible to resolve this curve graphically to give n and k values for two sets of sites. Accuracy in data decreases when more than three sets of binding sites are considered (Rosenthal [1967]). Relevant papers using Scatchard plots to obtain binding parameters are by O'Reilly (1971), Rosen (1970), Cho (1970) and Chignell (1969).

From the above discussion, the Scatchard plot appears to be preferable for expressing binding data. Binding data from these
studies was, therefore, expressed in Scatchard plots. $n_1$ and $k_1$ were obtained by graphical extrapolation of the initial straight line portion of the curve. No attempt was made to evaluate $n_2$ and $k_2$.

Recently papers have been published on the use of computer techniques to fit macromolecule-ligand binding data to a mathematical model, avoiding the subjective bias of plotting techniques. (Fletcher and Spector [1968], Meyer and Guttman [1968 b]).

In these studies, $n_1$, $k_1$, $n_2$, and $k_2$ values were also obtained from the computer program. A modified version of a non-linear least square fit analysis (Meyer and Guttman [1968 b]) was included in subroutines in the computer program. Binding data was assumed to fit to a relationship which assumes two classes of binding sites exist on the HSA molecule, i.e.,

$$r = \frac{n_1 k_1 D_f}{1 + k_1 D_f} + \frac{n_2 k_2 D_f}{1 + k_2 D_f}$$

$r$ is the dependent variable and $D_f$ the independent variable. Approximate estimates for $n_1$, $k_1$, $n_2$, $k_2$ were put into the program. (See Table 9). The more accurate these estimates are, the fewer the iterations required for the computer calculations. In contrast to graphical extrapolation methods, this LQF method has no subjective bias.
C) Bishydroxycoumarin - Human Serum Albumin Binding Results

These results are shown in Table 10.

i) Precision of Results:

Figure 16 shows a typical calcomp plot for these binding results. The experimental values deviate little from the fitted line, except at low r values. This plot shows that the diafiltration technique yields precise results (i.e., a smooth binding curve with little scatter of data points) except at low molar binding ratios. At low r values, the limits of accuracy of spectrophotometric analysis are exceeded. A more sensitive analytical technique should eliminate this problem.

ii) Reproducibility of Results:

Experiments 4 and 5 (Table 10) indicate that the diafiltration technique can produce reproducible results.

iii) Effect of Reservoir Bishydroxycoumarin Concentration:

Experiment 3 indicates that the ligand reservoir concentration of BHC can influence binding results. Here the LQF $k_1$ value is low compared to results from other experiments in Table 10. When reservoir ligand concentration is high compared to the HSA concentration, fewer data points are obtained at low r values and thus the LQF $n_1k_1$ value is lower. To obtain a wide range of data points, the ratio of HSA concentration reservoir ligand concentration should be unity or less.
Figure 16. Calcomp Scatchard Plot for Bishydroxycoumarin - Human Serum Albumin Binding.

\[ r = \frac{Q}{h} \cdot \frac{1}{A} \]

\( \Delta \) = experimental data points and solid line is non-linear least square fitted line. HSA concentration = \( 3.685 \times 10^{-4} \) M and BHC reservoir concentration = \( 2.821 \times 10^{-4} \) M. (Experiment 6 in Table 10). Calcomp plot reduced in size from original plot.
iv) Effect of Pressure:

The results in Table 10 suggest that pressure does not have a significant effect at 15 psi or above. Operation at the very low pressure in experiments 6 and 7 indicates that high \( n_1 k_1 \) values are obtained.

v) Comparison of \( n \) and \( k \) values obtained by Graphical Extrapolation and by LQF Method:

Table 10 reveals that, in most cases, \( k_1 \) values are higher and \( n_1 \) values are lower, by the LQF method than by the graphical extrapolation method. This difference could be expected. In the graphical extrapolation method, the initial first few points were ignored because of considerable scatter. The percentage error of these first few experimental data points compared to the fitted line was high. However, when the LQF method was used, all experimental data points were included.

vi) Comparison with Literature Values:

Results given in Table 10 do not agree well with the literature values given in Table 11, and are consistently higher. However, results by the diafiltration technique cover a wider range of \( r \) values than do methods commonly used for studying binding. By diafiltration, binding data can be obtained at much lower \( r \) values than can be measured by equilibrium dialysis or by ultrafiltration. Thus \( n_1 k_1 \) values would be expected to be higher. Comparison with literature values is difficult and of little value unless experimental conditions and the range of molar binding ratios investigated are the same.
vii) Effect of Human Serum Albumin Dilution:

Figure 17 shows that HSA dilution has a very noticeable effect on BHC-HSA binding curves. As HSA concentration decreases the binding curves move toward higher \( r \) and \( r/D_f \) values (Figure 17), and \( n_1 \) and \( k_1 \) values increase (Table 10). However, there is no simple relationship between \( n_1 \) or \( k_1 \) and the HSA concentration (Figures 18 and 19). The simple Law of Mass Action approach to protein binding studies suggests that BHC-HSA binding should be independent of HSA concentration. This deviation from the expected behaviour will be further discussed in Section IV, (2), F.

viii) Double Reciprocal Plots:

Binding data from Experiments 2, 8, 9 are plotted as double reciprocal plots in Figure 20. The series of curves are initially convex to the \( 1/D_f \) axis, with a common intercept on the \( 1/r \) axis. Using the classification of Nichol and others (1967) discussed in IV, (2), B, this suggests that noncompetitive polymerization of HSA and binding of BHC is occurring, with the polymer having less affinity for BHC than the monomer.

D) Phenylbutazone - Human Serum Albumin Binding Results

These results are shown in Table 13.

i) Precision of Results:

Figure 21 shows a typical calcomp plot for PBZ-HSA binding. Again, as in IV, (2), C, (i), the diafiltration technique yields
The Effect of Human Serum Albumin Concentration on Bishydroxycoumarin-Human Serum Albumin Binding.

\[ A = 0.420 \times 10^{-4} \text{ M HSA and } 2.875 \times 10^{-5} \text{ M BHC in reservoir}, \]
\[ \triangle = 0.724 \times 10^{-4} \text{ M HSA and } 5.750 \times 10^{-5} \text{ M BHC in reservoir}, \]
\[ \odot = 6.123 \times 10^{-4} \text{ M HSA and } 2.882 \times 10^{-4} \text{ M BHC in reservoir}. \]

(Experiments 9, 8 and 1 in Table 10)
Figure 18. Relationship Between $k_1$ and Human Serum Albumin Concentration for Bishydroxycoumarin - Human Serum Albumin Binding and Bishydroxycoumarin - Plasma Binding. (---) = Fraction V HSA and (-----) = Plasma.
(Data from Figure 17 and Figure 26.)
Figure 19. Relationship between $n_1$ and Human Serum Albumin Concentration for Bishydroxycoumarin - Human Serum Albumin Binding. $x = \text{data from HSA (Fraction V) (from Figure 17).}$

Figure 20. Double Reciprocal Plots for Bishydroxycoumarin-Human Serum Albumin Binding. Symbols and concentrations are as for Figure 17. (Experiments 1, 8 and 9 in Table 10)
Figure 21. Calcomp Scatchard Plot for Phenylbutazone - Human Serum Albumin Binding.

△ = experimental data points and the solid line is the non-linear least square fitted line. HSA concentration = 3.585 x 10^{-4} M and PBZ reservoir concentration = 9.59 x 10^{-5} M.
### Table 10. Bishydroxycoumarin - Human Serum Albumin Binding Results.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>HSA Conc. in moles/1</th>
<th>Reservoir BHC Conc. in moles/1</th>
<th>Pressure psi</th>
<th>Extrapolated n&lt;sub&gt;1&lt;/sub&gt;</th>
<th>Extrapolated k&lt;sub&gt;1&lt;/sub&gt; in l./M</th>
<th>LQF n&lt;sub&gt;1&lt;/sub&gt;</th>
<th>LQF k&lt;sub&gt;1&lt;/sub&gt; in l./M</th>
<th>LQF n&lt;sub&gt;2&lt;/sub&gt;</th>
<th>LQF k&lt;sub&gt;2&lt;/sub&gt; in l./M</th>
<th>Extrapolated n&lt;sub&gt;1&lt;/sub&gt;k&lt;sub&gt;1&lt;/sub&gt; in l./M</th>
<th>LQF n&lt;sub&gt;2&lt;/sub&gt;k&lt;sub&gt;2&lt;/sub&gt; in l./M</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.123 x 10^-4</td>
<td>2.882 x 10^-4</td>
<td>15</td>
<td>3.0</td>
<td>5.00 x 10^5</td>
<td>2.4</td>
<td>4.61 x 10^5</td>
<td>13.6</td>
<td>5.95 x 10^3</td>
<td>1.50 x 10^6</td>
<td>1.13 x 10^6</td>
</tr>
<tr>
<td>2</td>
<td>4.257 x 10^-4</td>
<td>2.901 x 10^-4</td>
<td>15</td>
<td>2.9</td>
<td>5.24 x 10^5</td>
<td>2.4</td>
<td>5.38 x 10^5</td>
<td>12.2</td>
<td>6.50 x 10^3</td>
<td>1.52 x 10^6</td>
<td>1.29 x 10^6</td>
</tr>
<tr>
<td>3</td>
<td>1.602 x 10^-4</td>
<td>2.805 x 10^-4</td>
<td>25</td>
<td>3.0</td>
<td>5.39 x 10^5</td>
<td>2.7</td>
<td>3.24 x 10^5</td>
<td>17.9</td>
<td>5.92 x 10^3</td>
<td>1.62 x 10^6</td>
<td>8.90 x 10^5</td>
</tr>
<tr>
<td>4</td>
<td>2.884 x 10^-4</td>
<td>1.007 x 10^-4</td>
<td>25</td>
<td>2.8</td>
<td>5.92 x 10^5</td>
<td>1.7</td>
<td>1.71 x 10^6</td>
<td>11.4</td>
<td>9.73 x 10^3</td>
<td>1.66 x 10^6</td>
<td>2.86 x 10^6</td>
</tr>
<tr>
<td>5</td>
<td>2.815 x 10^-4</td>
<td>1.186 x 10^-4</td>
<td>25</td>
<td>3.0</td>
<td>5.84 x 10^5</td>
<td>1.9</td>
<td>1.26 x 10^6</td>
<td>268.0</td>
<td>3.92 x 10^2</td>
<td>1.75 x 10^6</td>
<td>2.41 x 10^6</td>
</tr>
<tr>
<td>6</td>
<td>3.685 x 10^-4</td>
<td>2.821 x 10^-4</td>
<td>7</td>
<td>3.5</td>
<td>5.78 x 10^5</td>
<td>2.3</td>
<td>1.46 x 10^6</td>
<td>28.4</td>
<td>3.66 x 10^3</td>
<td>2.02 x 10^6</td>
<td>3.44 x 10^6</td>
</tr>
<tr>
<td>7</td>
<td>0.810 x 10^-4</td>
<td>2.438 x 10^-5</td>
<td>4</td>
<td>2.8</td>
<td>8.32 x 10^5</td>
<td>2.0</td>
<td>2.83 x 10^6</td>
<td>39.7</td>
<td>1.59 x 10^3</td>
<td>2.33 x 10^6</td>
<td>5.55 x 10^5</td>
</tr>
<tr>
<td>8</td>
<td>0.724 x 10^-5</td>
<td>5.749 x 10^-5</td>
<td>15</td>
<td>4.5</td>
<td>8.22 x 10^5</td>
<td>3.1</td>
<td>1.37 x 10^6</td>
<td>8.4</td>
<td>2.30 x 10^4</td>
<td>3.70 x 10^6</td>
<td>4.24 x 10^6</td>
</tr>
<tr>
<td>9</td>
<td>0.420 x 10^-4</td>
<td>2.830 x 10^-5</td>
<td>15</td>
<td>5.2</td>
<td>1.70 x 10^6</td>
<td>3.9</td>
<td>1.53 x 10^6</td>
<td>13.0</td>
<td>1.10 x 10^4</td>
<td>8173 x 10^6</td>
<td>6.00 x 10^6</td>
</tr>
</tbody>
</table>

Note: LQF = values from non-linear least square fit method.
precise results, except at low molar binding ratios (where
wider scattering of data points occurs).

ii) Effect of Reservoir Phenylbutazone Concentration:

Experiments 11 and 15 (Table 13) show that reservoir PBZ
concentration influences binding results. This effect is more
marked than that with BHC, because \( n_1 \) is smaller. Again when
the ratio of PBZ reservoir concentration: HSA concentration
is greater than unity, no data points are obtained at low
molar binding ratios. In experiments 11 and 15 it was impossible
to determine \( n \) and \( k \) values by graphical extrapolation or by
the LQF method.

iii) Effect of Pressure:

Experiments 10, 12, 13 and 14 (Table 13) indicate that
pressure has little effect on the PBZ-HSA binding results.

iv) Comparison of \( n \) and \( k \) Values Obtained by Graphical
Extrapolation and by the LQF Method:

Again, as in IV, (2), C, (v), Table 12 shows \( n_1 \) values
are lower and \( k_1 \) values higher by the LQF method than by
graphical extrapolation. However, as indicated in Table 13,
poor non-linear least square fits were obtained for several
experiments. In these experiments fewer data points are
obtained for the initial straight line portion of the Scatchard
plot. If these few points are widely scattered, then the LQF
will be poor. But elimination of these data points from the
LQF analyses, made it impossible to analyse data by LQF method.
v) Comparison with Literature Values:

Results in Table 13 are higher than the literature values given in Table 12. In the literature, a variety of methods and experimental conditions were used. As explained in IV, (2), C, (vi), comparison of literature values is only valid if the same range of \( r \) values are investigated under the same experimental conditions.

vi) Effect of Human Serum Albumin Dilution on PBZ-HSA Binding Results:

Figure 22 shows that as HSA concentration decreases, there is a shift of PBZ-HSA binding curves to higher \( r \) and \( r/D_f \) values. Table 12 shows \( n_1 \) increases as HSA concentration decreases (both by graphical extrapolation or by LQF method). \( k_1 \), however, by graphical extrapolation appears to remain constant, whereas by the LQF method, it increases except for the lowest HSA concentration used in Experiment 17. Figure 23 shows there is no simple relationship between \( n \) and HSA concentration. The effect of HSA dilution on binding results will be discussed in IV, (2), F.

vii) Double Reciprocal Plots:

Binding data from experiments 12 and 17 is plotted as double reciprocal plots in Figure 24. The curves are initially convex to the \( 1/D_f \) axis, with a common intercept on the \( 1/r \) axis. Using the classification of Nichol and others (1967), (see IV,
Figure 22. The Effect of Human Serum Albumin Concentration on Phenylbutazone - Human Serum Albumin Binding.

- $\Delta = 0.420 \times 10^{-4}$ M HSA and $3.359 \times 10^{-5}$ M PBZ in reservoir
- $\Box = 0.778 \times 10^{-4}$ M HSA and $6.415 \times 10^{-5}$ M PBZ in reservoir
- $\ast = 3.892 \times 10^{-4}$ M HSA and $1.284 \times 10^{-4}$ M PBZ in reservoir

(Experiments 12, 16 and 17 in Table 13)
Figure 23. Relationship Between $n_1$ and Human-Serum Albumin Concentration for Phenylbutazone - Human Serum Albumin Binding.
(Data from Figure 22.)

Figure 24. Double Reciprocal Plot for Phenylbutazone Human Serum Albumin Binding.
(Data from Experiments 12 and 17 in Table 13.)
Table 11. Literature Values for n and k for Bishydroxycoumarin - Human Serum Albumin Binding Studies

<table>
<thead>
<tr>
<th>n</th>
<th>k(1./M)</th>
<th>Temp</th>
<th>pH</th>
<th>[HSA]</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>3.5 x 10^5</td>
<td>20°C</td>
<td>7.4</td>
<td>0.1</td>
<td>Equilibrium</td>
<td>Cho (1970)</td>
</tr>
<tr>
<td>2.8</td>
<td>1.7 x 10^5</td>
<td>40°C</td>
<td>7.4</td>
<td>0.1</td>
<td>Dialysis</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>1.15 x 10^5</td>
<td>27°C</td>
<td>10</td>
<td>0.4%</td>
<td>Equilibrium</td>
<td>O'Reilly (1971)</td>
</tr>
<tr>
<td>3.2</td>
<td>7.5 x 10^5</td>
<td>25°C</td>
<td>7.4</td>
<td>1 x 10^-5M</td>
<td>Equilibrium</td>
<td>Chignell &amp; Starkweather (1971)</td>
</tr>
</tbody>
</table>

* O'Reilly reports $k_1$ value as $2.31 \times 10^5$, but this is actually an $n_1k_1$ value.
** Calculated from data in paper.

Table 12. Literature Values for n and k for Phenylbutazone - Human Serum Albumin Binding Studies

<table>
<thead>
<tr>
<th>n</th>
<th>k(1./M)</th>
<th>Temp</th>
<th>pH</th>
<th>[HSA]</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.17 x 10^5</td>
<td>25°C</td>
<td>7.4</td>
<td>1 x 10^-4M</td>
<td>Ultrafiltration (by centrifugation)</td>
<td>Solomon and others (1968)</td>
</tr>
<tr>
<td>1</td>
<td>1.25 x 10^5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Thorp (1964)</td>
</tr>
<tr>
<td>1.14</td>
<td>2.37 x 10^5</td>
<td>4.56 x 10^4</td>
<td>7.4 (Phosphate Buffer)</td>
<td>0.1% w/v</td>
<td>Circular Dichroism</td>
<td>Rosen (1970)</td>
</tr>
<tr>
<td>1.86</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1 x 10^5</td>
<td>7.4 (Phosphate Buffer)</td>
<td>1 x 10^-5M</td>
<td>Equilibrium</td>
<td>Chignell (1969)</td>
<td></td>
</tr>
</tbody>
</table>
Table 13. Phenylbutazone - Human Serum Albumin Binding Results.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>HSA Conc. in moles/l.</th>
<th>Reservoir BHC Conc. in moles/l.</th>
<th>Pressure psi</th>
<th>Extrapolated $n_1$</th>
<th>Extrapolated $k_1$ in 1./M</th>
<th>LQF $n_1$</th>
<th>LQF $k_1$ in 1./M</th>
<th>LQF $n_2$</th>
<th>LQF $k_2$ in 1./M</th>
<th>Extrap. $n_1k_1$ in 1./M</th>
<th>LQF $n_1k_1$ in 1./M</th>
<th>LQF $n_2k_2$ in 1./M</th>
</tr>
</thead>
<tbody>
<tr>
<td>* 10</td>
<td>$4.775 \times 10^{-4}$</td>
<td>$9.09 \times 10^{-5}$</td>
<td>8</td>
<td>1.1</td>
<td>$4.66 \times 10^5$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>$4.89 \times 10^5$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>** 11</td>
<td>$4.398 \times 10^{-4}$</td>
<td>$6.260 \times 10^{-4}$</td>
<td>15</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>* 12</td>
<td>$3.892 \times 10^{-4}$</td>
<td>$1.284 \times 10^{-4}$</td>
<td>15</td>
<td>1.2</td>
<td>$4.59 \times 10^5$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>$5.51 \times 10^5$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>$3.585 \times 10^{-4}$</td>
<td>$9.59 \times 10^{-5}$</td>
<td>10</td>
<td>1.3</td>
<td>$4.78 \times 10^5$</td>
<td>1.1</td>
<td>8.33 x $10^5$</td>
<td>29.5</td>
<td>5.97 x $10^2$</td>
<td>$6.46 \times 10^5$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>* 14</td>
<td>$1.809 \times 10^{-4}$</td>
<td>$7.240 \times 10^{-5}$</td>
<td>25</td>
<td>1.1</td>
<td>$4.77 \times 10^5$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>$5.25 \times 10^5$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>** 15</td>
<td>$1.495 \times 10^{-4}$</td>
<td>$4.530 \times 10^{-4}$</td>
<td>25</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>$0.778 \times 10^{-4}$</td>
<td>$6.415 \times 10^{-5}$</td>
<td>15</td>
<td>1.5</td>
<td>$4.51 \times 10^5$</td>
<td>1.1</td>
<td>2.86 x $10^6$</td>
<td>3.59</td>
<td>9.12 x $10^3$</td>
<td>$6.81 \times 10^5$</td>
<td>3.16 x $10^6$</td>
<td>3.25 x $10^4$</td>
</tr>
<tr>
<td>17</td>
<td>$0.420 \times 10^{-4}$</td>
<td>$3.359 \times 10^{-5}$</td>
<td>15</td>
<td>1.9</td>
<td>$4.54 \times 10^5$</td>
<td>1.3</td>
<td>8.41 x $10^5$</td>
<td>430 ?</td>
<td>6.44 ?</td>
<td>$8.60 \times 10^5$</td>
<td>1.09 x $10^6$</td>
<td>2.77 x $10^4$</td>
</tr>
</tbody>
</table>

* Poor non-linear least square fit.

** No non-linear least square fit possible.
(2), B), this suggests that noncompetitive polymerization of HSA and binding of PBZ could be occurring, with the polymeric species having less affinity for PBZ than the monomer.

E) Bishydroxycoumarin - Plasma Albumin Binding Results

These results are shown in Table 14. The pressures used in Experiments 18, 19 and 20 were chosen to give similar flow rates. With plasma, diafiltration experiments proceeded more slowly than with Fraction V HSA.

i) Precision of Results:

Figure 25 shows a calcomp plot for plasma albumin - BHC binding. Again, the diafiltration technique yields precise results, except at low r values.

ii) Effect of Pressure:

Experiments 20 and 21 (Table 14) show that the pressure applied does not appreciably affect BHC - plasma binding.

iii) Comparison of n and k Values Obtained by Graphical Extrapolation and by LQF Method:

As in the case of BHC - HSA and PBZ - HSA, the LQF method gave higher k₁ values and lower n₁ values than the graphical extrapolation method.

iv) Comparison of n and k Values with Literature Values:

With BHC - plasma albumin binding, the diafiltration technique again yields higher values for k₁ than given in the
literature (Table 11). Explanations for the higher values have already been given in IV, 2, C, (vi).

v) Comparison of BHC - Plasma Albumin Binding and BHC - HSA Binding:

Comparison of the results given in Tables 10 and 14, indicate that binding is similar, although plasma has a slightly higher affinity for BHC. Plasma proteins other than albumin, may also bind BHC. It has been shown that BHC does bind to other proteins such as α-,β- and γ-globulins; but with less affinity (Weiner and others [1950]). Another explanation could be that differences exist in the conformation of Fraction V HSA and plasma albumin. Endogenous substances may have been removed from Fraction V HSA during the fractionation procedure. It was noted also, in IV, (1), H, (v) that much more protein-like substance was lost into the filtrate on purification of 4% w/v HSA, than from plasma.

vi) Effect of Plasma Dilution:

Figure 26 indicates that shifts of BHC - plasma binding curves occurs to higher r and r/D values as plasma dilution increases. By the graphical extrapolation method n remains quite constant, although it increases slightly by the LQF method, as plasma dilution increases k increases as plasma dilution increases (either by graphical extrapolation or by LQF method). There is no simple relationship between k and the extent of plasma dilution. (See Figure 18). This plasma dilution effect on BHC - plasma binding will be discussed in IV, (2), F.
Figure 25. Calcomp Scatchard Plot for Bishydroxycoumarin - Plasma Binding.

\( r = \frac{D_b}{M} \)

\( \Delta \) = experimental data points and the solid line is the non linear least square fitted line. HSA concentration = \( 4.632 \times 10^{-4} \) M and BHC reservoir concentration = \( 2.185 \times 10^{-4} \) M. (Reduced in size from original.)
Figure 26. The Effect of Plasma Dilution on Bishydroxycoumarin - Plasma Binding.

- $\Delta = 0.516 \times 10^{-4}$ M HSA and $2.768 \times 10^{-5}$ M BHC in reservoir.
- $\blacksquare = 1.208 \times 10^{-4}$ M HSA and $5.100 \times 10^{-5}$ M BHC in reservoir.
- $\bigcirc = 4.632 \times 10^{-4}$ M HSA and $2.185 \times 10^{-4}$ M BHC in reservoir.
<table>
<thead>
<tr>
<th>Experiment</th>
<th>HSA Conc. in Plasma moles/1.</th>
<th>Reservoir BHC Conc. in moles/1.</th>
<th>Pressure psi</th>
<th>Extrapolated ( n_1 )</th>
<th>Extrapolated ( k_1 ) in l./M</th>
<th>LQF ( n_1 )</th>
<th>LQF ( k_1 ) in l./M</th>
<th>LQF ( n_2 )</th>
<th>LQF ( k_2 ) in l./M</th>
<th>Extrapolated ( n_1 k_1 ) in l./M</th>
<th>Extrapolated ( n_2 k_2 ) in l./M</th>
<th>LQF ( n_1 k_1 ) in l./M</th>
<th>LQF ( n_2 k_2 ) in l./M</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>( 4.632 \times 10^{-4} )</td>
<td>( 2.185 \times 10^{-4} )</td>
<td>45</td>
<td>( 2.9 )</td>
<td>( 5.52 \times 10^5 )</td>
<td>( 1.9 )</td>
<td>( 9.863 \times 10^5 )</td>
<td>( 10.6 )</td>
<td>( 6.22 \times 10^3 )</td>
<td>( 1.49 \times 10^6 )</td>
<td>( 1.87 \times 10^6 )</td>
<td>( 6.64 \times 10^4 )</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>( 1.208 \times 10^{-4} )</td>
<td>( 5.10 \times 10^{-5} )</td>
<td>15</td>
<td>( 3.1 )</td>
<td>( 1.22 \times 10^6 )</td>
<td>( 2.7 )</td>
<td>( 1.45 \times 10^6 )</td>
<td>( 381 )</td>
<td>( 1.75 \times 10^2 )</td>
<td>( 3.78 \times 10^6 )</td>
<td>( 3.68 \times 10^6 )</td>
<td>( 6.67 \times 10^4 )</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>( 0.516 \times 10^{-4} )</td>
<td>( 2.768 \times 10^{-5} )</td>
<td>10</td>
<td>( 3.0 )</td>
<td>( 1.92 \times 10^6 )</td>
<td>( 2.5 )</td>
<td>( 5.58 \times 10^6 )</td>
<td>( 289 )</td>
<td>( 2.89 \times 10^2 )</td>
<td>( 5.46 \times 10^3 )</td>
<td>( 1.37 \times 10^7 )</td>
<td>( 8.35 \times 10^4 )</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>( 0.516 \times 10^{-4} )</td>
<td>( 2.888 \times 10^{-5} )</td>
<td>22</td>
<td>-</td>
<td>-</td>
<td>( 2.6 )</td>
<td>( 5.94 \times 10^6 )</td>
<td>( 7.1 )</td>
<td>( 1.34 \times 10^4 )</td>
<td>( - )</td>
<td>( 1.57 \times 10^7 )</td>
<td>( 9.50 \times 10^4 )</td>
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</tr>
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</table>
F) The Effect of Human Serum Albumin Concentration on Binding Results Obtained by the Diafiltration Procedure

On the basis of the binding results shown in the preceding sections, it can not be concluded at this stage that the diafiltration procedure is a satisfactory technique for drug - HSA binding studies. The problem of dependence of binding curves on HSA concentration has to be resolved. This problem is possibly inherent in the diafiltration procedure. However, it is also possible that the simple Law of Mass Action does not adequately describe drug - HSA binding and that binding is not independent of macromolecule concentration. This emphasizes the necessity of determining the effect of HSA concentration on binding curves in all binding studies.

i) Literature Reports on the Effect of Human Serum Albumin Concentration on Binding Results

It is surprising to find that only in rare instances has the effect of protein concentration been studied. Meyer and Guttman (1968 b) when studying the binding of several drugs to BSA by dynamic dialysis, used two concentrations of BSA. They showed only a slight shift in binding curves at different BSA concentrations. The shifts, however, were in the opposite direction to those of BHC binding curves in these studies. A small shift like this could be explained by experimental errors in $D_f$ and/or $M$ (see Theoretical Discussion of this in IV, (2), F, ii).

Cho (1971) using equilibrium dialysis and 0.1, 0.2, 0.4% w/v HSA solutions showed no significant effect of HSA concentration on BHC-HSA binding curves. However, Cho only investigated the effect
of HSA concentration over a very narrow range of HSA concentration.

Crawford and others (1971) studied bromosulphthalein (BSP) - HSA binding by gel filtration. In the 2.7% w/v - 5.5% w/v HSA range, the BSP binding capacity in moles/moles HSA decreased as albumin concentration was increased [according to the equation $y = k + a_1 + b_2x + c_2x^2$, where $y = \log$ (number of BSP molecules/molecule HSA), $k$, $b_1$, and $c$ are constants common to all groups of serum tested, $a_1$ is characteristic of the serum used, and $x$ is log of albumin concentration]. However, since they only used one equilibrium concentration of BSP, they could be effectively obtaining molar binding ratios at a section through a family of binding isotherms.

To avoid this latter problem, Crawford and others (1972) investigated diafiltration as a method whereby an entire binding isotherm could be obtained. Thus, the effect of HSA concentration on BSP binding, both in serum and in isolated HSA could be examined. These authors reported that, in serum, there appeared to be a transition between molar BSP binding capacity increasing with increasing HSA concentration, and a decreasing capacity with increasing HSA concentrations, especially at equilibrium BSP concentrations of about $5 \times 10^{-5}$ M. The molar binding capacities for albumin and serum are very similar in the 40 µM free BSP concentration range but, below this, serum has a lower capacity. At high $D_f$ values, serum has a higher capacity (this must be due to presence of endogenous compounds bound to HSA in serum with a relatively low affinity). However, these
researchers worked only at 1.5%, 2.3 and 3.39% albumin concentrations. At the two lower HSA levels, BSP binding results (on double reciprocal plot) were very similar, whereas at 3.39% the results indicated an increased n value and a resultant decreased $k_{ass}$ value (they published no results for these binding parameters, however). It would have been of interest to see results at lower HSA concentration. It should be noted that these authors give no indication of any preliminary studies on the diafiltration apparatus, e.g., binding studies to apparatus membrane binding or retention, etc., for these factors could indeed be present and affect results. They do not compare their results quantitatively with other literature values.

The effect of BSA concentration on testosterone - BSA binding over a wider BSA concentration range was studied by Ryan and Hanna (1971). Their results were similar to those shown in these studies. Their nk values were always consistently higher than the literature values although the n values were the same. They also encountered the problem of dependence of testosterone binding values on BSA concentration. Their explanations for the anomalous results are given below:-

(a) They suggested that the properties of the membrane may be influenced by the presence of protein.

(b) The binding properties of the protein changes under the conditions of diafiltration.

(c) Values of n and k obtained for steroid-binding by the classical 48 hours equilibrium dialysis are not true values.
There was, however, no evidence given to support these views. Thus, there is little in the literature on this HSA concentration effect. When this subject has been investigated with HSA, studies have not been over a wide range of HSA concentration. To determine whether binding is independent of HSA concentration, a wide range of HSA concentrations (i.e., from 0.1% - 4% w/v) including physiological concentrations, should be used.

ii) Theoretical Investigation of Altered Parameters Involved in Drug-Macromolecule Binding and Their Effect on the Scatchard Plot

The effects of changes in the parameters $M$ and $D_f$ were examined theoretically. Experimental data previously obtained was used and general data cards were appropriately altered (Table 15). The computer program allowed for such changes.

Situation 1: Assume that 10% of the original macromolecule $M$, in the cell escaped through the membrane. This macromolecule interfered with measurement of absorbance of the drug in the filtrate by absorbing at the analytical wavelength. This increased the apparent concentration of drug in the filtrate, $D_f$, by 10%. From Figure 27, it can be seen that, in this situation, the curve is moved slightly upwards and across to the right, i.e., for any point on the original curve, the $r$ and $r/D_f$ values are increased. This effect does simulate the shift of binding curves observed in experimental data. However, the experimental shift
Table 15. Changes in General Data Cards

<table>
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<tr>
<th>Situation</th>
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<th>Column 34, 35 on new General Data Card</th>
<th>[HSA] on Original General Data Card</th>
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<tr>
<td>1</td>
<td>[HSA] ↓ by 10%</td>
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<td></td>
<td></td>
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<tr>
<td>2</td>
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<td>↑ by 10%</td>
</tr>
<tr>
<td></td>
<td>$D_f$ ↑ by 10%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>[HSA] ↓ by 10%</td>
<td>--</td>
<td>↓ by 10%</td>
</tr>
<tr>
<td></td>
<td>$D_f$ unaltered</td>
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<td></td>
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<tr>
<td>4</td>
<td>[HSA] unaltered</td>
<td>- 9</td>
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</tr>
<tr>
<td></td>
<td>$D_f$ ↓ by 9%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* When a number, e.g., -9 is put in columns 34 and 35 this means that whilst $D_f$ is decreased by 9%, [HSA] will be increased by 9%. Hence, for the [HSA] to be maintained constant, it is necessary to decrease [HSA] on original data card by 9% on new data card.
Figure 27. Scatchard Plots Indicating the Effect of Changing Parameters on Bishydroxycoumarin - Plasma Albumin Binding

(●●●) = experimental binding curve where reservoir BHC concentration = 2.860 x 10^-5 M and HSA concentration = 0.515 x 10^-4 M. (△△△) = theoretical binding curve where filtrate BHC concentration is increased by 10% and HSA concentration is decreased by 10%.
was much greater than that calculated. To achieve such results theoretically would require a much greater percent change in M and $D_f$. Experimentally, this would be unlikely and this does not seem to be an explanation for the results obtained.

Situation 2: Assume a 10% increase in $D_f$ but an unaltered HSA concentration. This plot (Figure 28) shows this does not alter the Scatchard plot significantly. For a given value of $r$, the $r/D_f$ value is slightly decreased and the curve moved downwards. Thus a 10% error in $D_f$ is unlikely to alter results significantly.

Situation 3: Assume a 10% decrease in HSA concentration but unaltered $D_f$ values. This could occur if the protein became concentrated at the membrane. This might reduce the concentration of HSA available for drug binding. This plot (Figure 29) moves significantly up and to the right. The effect is similar but of lesser magnitude than that observed experimentally. This implies that concentration of protein at the membrane could influence results.

Situation 4: Assume a 9% decrease in $D_f$ but an unaltered HSA concentration. This could occur if, as Blatt, Robinson and Bixler (1968) suggested, the drug became polarized at the membrane and hence was retained. The $D_f$ values in the filtrate would be low. This effect is the reverse of Situation 2 and, the plot
Figure 28. Scatchard Plots Indicating the Effects of Changed Parameters on Bishydroxycoumarin - Plasma Binding.

(●●) = experimental binding curve (data as in Figure 27), and
(■■) - theoretical binding curve where filtrate BHC concentration is increased by 10% and HSA concentration is unaltered.
Figure 29. Scatchard Plots Indicating the Effects of Changed Parameters on Bishydroxycoumarin - Plasma Binding Curves.

(• •) = experimental binding curve (data as in Figure 27) and
(○ ○) = theoretical binding curve where BHC filtrate concentration is unaltered and HSA concentration is decreased by 10%.
(Figure 30) shows that there is a slight movement upwards of the curve.

The net result of this theoretical study was that:

1. It is unlikely that loss of protein molecule through the membrane could account for the observed HSA concentration effects.

2. High $D_f$ values would not affect results significantly.

3. Experimental error in the measurement of M (or an inaccurate HSA concentration due to volume changes) in the direction of a decreased macromolecule concentration is probably the most significant of these four effects. A decreased protein concentration could also be obtained by concentration of the protein at the membrane.

None of these effects, however, completely explain the experimental data obtained at a range of macromolecule concentrations.

iii) Discussion of the Human Serum Albumin Concentration Effect in Terms of Problems Inherent in the Diafiltration Technique

Blatt, Robinson and Bixler (1968), expressed their methyl orange - HSA binding data (using PM-10 filter) in a plot of $\log C_f/C_p - C$ against filtrate volume. Theoretically, this should be a straight line relationship [see Literature Section II, (5)]. Their results showed that as the HSA concentration
Figure 30. Scatchard Plots Indicating the Effects of Changed Parameters on Bishydroxycoumarin - Plasma Binding Curves.

(●●) = experimental binding curve (data as in Figure 27) and
(△△) = theoretical binding curve where filtrate BHC concentration is decreased by 9% and HSA concentration is unaltered.
increased, the curvature of these lines increased and their slope decreased. When binding data from PBZ - HSA and BHC - HSA interactions was plotted in the above manner of Blatt, Robinson and Bixler, similar results were observed. (See Figures 9 and 10.) Thus, binding results of Blatt, Robinson and Bixler also appeared to be dependent on albumin concentration, as were the binding results in this study and those of Ryan and Hanna (1971). All these studies used the diafiltration technique. Therefore, this apparent HSA concentration effect could be an inherent problem with the diafiltration technique. Possible inherent problems will now be discussed:

(a) Ligand polarisation at the membrane:

Blatt, Robinson and Bixler (1968) explained their results in terms of polarisation of the methyl orange dye at the membrane. To overcome this problem they suggested using the lowest possible pressures and HSA concentrations or use of a larger parosity membrane. Work with PBZ and BHC in these studies, suggests that operation at very low pressures gives high $k_1$ values. Also it is preferable to use near physiological HSA concentrations, rather than low HSA concentrations. A larger porosity membrane than the PM-10 Diaflo would only increase the problem of interfering impurities in the Fraction V HSA.
(b) Ligand Binding and/or Rejection by the Diafiltration Apparatus:

The difficulty of distinguishing between ligand binding and rejection was discussed in IV, (1), G. The effect of binding and rejection on free drug concentrations in the cell and filtrate was also discussed here. In IV, (1), F, the binding of PBZ and BHC to the apparatus was shown to average approximately 5-6% (though a relationship existed between the percent drug bound and filtrate volume, and ligand reservoir concentration). The theoretical section investigation in IV, (2), F, (ii), however, indicates that even if 10% of the drug were bound, it could not cause shifts of the curves observed in these studies when HSA concentration decreased. It could be possible also that, in the presence of HSA, ligand binding and/or rejection is altered.

(c) Altered Membrane Properties:

Ryan and Hanna (1971) suggested that membrane properties may change during diafiltration. However, it would seem more likely that apparent altered membrane properties would be caused by increasing HSA concentrations. As the HSA concentration increases, clogging of the membrane may occur and flow rates would, therefore, decrease. Decreased flow rates with increased HSA concentration were observed in these studies and also those of Ryan and Hanna (1971). Decrease in HSA concentration caused by clogging at the
at the membrane is considered in the theoretical investigations in IV, (2), F, (ii). It was shown that this effect was unlikely to cause binding curve shifts of the magnitude observed experimentally on HSA dilution (although it could have some influence on shifts).

(d) Conformational Changes in the Human Serum Albumin Molecule During Diafiltration:

Diafiltration itself could possibly alter the HSA molecule and change its binding parameters, either through conformational changes or by polymerization and/or denaturation. This could be caused by effects of pressure or stirring stress or stirring rate on the HSA molecule. Such changes would also occur during the diafiltration purification procedure. Ryan and Hanna (1971) considered this possibility. They washed BSA in the diafiltration cell for 12 hours with buffer. A subsequent equilibrium dialysis experiment indicated that the washed BSA had a decreased affinity for testosterone.

Chignell and Starkweather (1971) incubated HSA solutions for 24 hours at 37°. Data for binding curves for the interactions of both BHC, and PBZ, with this incubated HSA, were obtained by equilibrium dialysis. Similar shifts of Scatchard binding curves occurred with incubated HSA as did with binding curves at low HSA concentrations in the present studies. Chignell and Starkweather (1971) suggested this might be due
to conformational changes, since the shifts of binding curves with acetylated HSA were similar to those with incubated HSA. Thus, it could be possible that shifts of binding curves at low HSA concentrations in the present studies are due to conformational changes. It would be difficult to show whether such changes were indeed occurring.

(e) Accuracy of the Literature Values:
As has already been discussed in Section IV, (2), C, and D, it is difficult to compare literature values with values obtained by the diafiltration technique because of the wider range of molar binding ratio covered by the diafiltration technique.

3. Phenylbutazone - Human Serum Albumin Binding Studies by Desorption (Washout) Diafiltration Technique

These desorption studies were carried out by using $3.585 \times 10^{-4}$ M HSA and $0.959 \times 10^{-5}$ M PBZ. PBZ was desorbed from HSA molecules as diafiltration proceeded. Hence a decrease of PBZ concentration could be followed in the filtrate.

Results are shown in Figure 31. Binding and desorption patterns were different. Even after 500 ml. of filtrate had been collected in the washout experiment, the absorbance of the filtrate was still 0.258. The reason for the lack of agreement between these two curves is not clear. One possible explanation might be that the length of time required for the
Figure 31. Binding of Phenylbutazone by Human Serum Albumin by the Desorption Method (○○○) and the Diafiltration Method (●●●)

\([\text{HSA}] = 3.583 \times 10^{-4} \text{ M}, \quad [\text{PBZ}] = 0.959 \times 10^{-4} \text{ M}.\)
purification, wash-in and washout experiments may permit denaturation and/or aggregation of the HSA molecule. This may alter binding properties of HSA.

4. Bishydroxycoumarin - Human Serum Albumin Binding Studies by the Equilibrium or Direct Method

In these experiments, the HSA concentration was approximately $0.72 \times 10^{-4}$ M and BHC concentration in the reservoir varied from $1.78 \times 10^{-5}$ M to $1.34 \times 10^{-4}$ M.

Since $D_f$, $D_t$ and M are known, $D_b$ and r can be found by means of Equation 25. The results are shown on a Scatchard plot in Figure 32. It was assumed in these studies, that the unknown substance passing through the membrane (from Fraction V HSA) would not affect BHC analysis in the filtrate at equilibrium.

Results obtained with this method differ from those obtained by the diafiltration technique and are closer to literature values (Table 11). The $n_1$ value was found to be 3.50 and the $k_1$ value $2.69 \times 10^5$ 1/M.

The results have not been corrected for:

i) Depression of BHC absorbance at 310 m\(\mu\) by HSA. Hence, there is a slight inaccuracy in the $D_t$ reading. The inaccuracy is not significant (Cho [1970]).

ii) BHC binding and/or rejection.

This method is equivalent to equilibrium dialysis but the time involved is shorter. Only one point on a binding curve is obtained from each experi-
Figure 32. Human Serum Albumin - Bishydroxycoumarin Binding by the Equilibrium (or Direct) Method
ment. There is, therefore, no real advantage in this procedure, although the results indicate that the binding parameters are similar to those obtained by other methods. (See Table II). It is not possible to cover such a wide range of molar binding ratios as is possible with the diafiltration procedure. Data points are more scattered than those from diafiltration experiments. Thus the equilibrium or direct method does not appear to offer advantages over conventional methods for protein binding studies.

5. Drug Binding Studies by the Centrifugation (Ultrafiltration) Method

These experiments showed that Amicon Centriflo (CF 50) membrane cones cannot be used for binding studies involving Fraction V HSA. Further studies were not carried out, since membrane cones with a lower molecular weight cut-off were not available. It is interesting to note that this method is widely used in clinical studies of drug binding to plasma.

6. Phenylbutazone - Human Serum Albumin Binding Studies by a Molecular Sieve Technique (Batch Method) Using Sephadex G-25

In PBZ - HSA binding studies, the total amount of PBZ ($D_b$ and $D_f$) in the external phase is analysed spectrophotometrically. Since the total amount of PBZ initially added to the gel is known, the amount (mg.) of PBZ associated with one g. of the gel can be calculated. Thus, from Figure 6, the concentration of drug in the external phase can be determined. Therefore, $D_f$ is known and $D_b$ and $r$ can be calculated.
For the PBZ - HSA interactions studied, erratic results were obtained. The free drug concentration was, in some experiments, higher than the initial PBZ concentration in the external phase (in the absence of HSA). It was found that in control binding experiments where Tris buffer only was added to the swollen gel, that a contaminant was present in the external phase. The external phase had a low absorbance reading at 264 mμ, the analytical wavelength for PBZ. This contaminant probably explains the erratic binding results obtained. Results for the determination of PBZ in the external phase were not corrected for depression of absorbance by HSA. However, this would probably have only a small effect on binding results. (Cho [1970] showed the depression of absorbance readings of BHC by HSA was most significant.)
V. SUMMARY AND CONCLUSIONS

1. The Amicon diafiltration apparatus was used to study BHC - Fraction V HSA, PBZ - Fraction V HSA, BHC - plasma interactions.

(a) The polyethylene tubing provided with the apparatus was found to bind BHC with a high affinity. However, teflon tubing was found to release no UV absorbing substances, nor to bind either PBZ or BHC. Teflon tubing was, therefore, used in the diafiltration apparatus.

(b) Binding studies, with both PBZ and BHC, were carried out in the absence of HSA. Both PBZ and BHC bound to the diafiltration apparatus. The percent drug bound decreased with increase in filtrate volume, and with increase in the concentration of drug in the reservoir.

(c) Experimental dilution curves (i.e., in the absence of HSA) deviated from theoretical dilution curves, for both PBZ and BHC. This also suggested binding was occurring in the apparatus.

(d) In these studies it was not possible to distinguish between binding and/or rejection.

(e) Binding studies, in the absence of drug, revealed that a protein-like substance, with maximum UV absorbance at 280 nm, was passing through the Diaflo membrane. Its UV absorption characteristics and
a positive Folin test suggested the substance was protein-like. Its molecular weight appeared to be greater than 5000.

(f) It was not possible to purify the HSA by dialysis with cellophane membranes or by Sephadex G-25. The most satisfactory purification procedure was by diafiltration for several hours, with Tris buffer, until no protein-like substance appeared in the filtrate.

(g) Plasma also yielded protein-like material in the filtrate on diafiltration with Tris buffer. UV spectra revealed this substance differed from that released from HSA.

(h) HSA released more protein-like material into the filtrate than did plasma.

The above indicates that the diafiltration apparatus should always be checked for release of substances which may interfere with analysis. For each ligand used, binding and/or rejection in the apparatus should be determined. In protein binding studies, a membrane should be chosen with maximal retention for the protein and minimal binding or rejection of the ligand.

2. Study of BHC - HSA, PBZ - HSA, BHC - plasma binding by the diafiltration technique had advantages over conventional methods used for protein binding studies:

(a) From one diafiltration experiment an entire binding curve can be obtained.
(b) Except at low molar binding ratios, the binding data was precise (i.e., there was little scatter in data points).

(c) An experiment can be completed in approximately 8 hours (the time is dependent on HSA concentration and ligand reservoir concentration). Less time, therefore, is required for this method than for the equilibrium dialysis technique.

(d) Data for a binding curve was obtained over a wide range of molar binding ratios. Binding data could be obtained at lower molar binding ratios than can be obtained by conventional methods.

(e) A wide range of protein concentrations could be used. On the other hand, equilibrium dialysis, circular dichroism, etc., studies are often restricted to low protein concentrations.

(f) The diafiltration technique is readily amenable to automation (i.e., in-line analysis).

(g) The Fraction V HSA can be purified by the diafiltration technique.

3. Binding parameters obtained by graphical extrapolation differed from those obtained by the non-linear least square fit computer method. The $n_1$ values were higher and $k_1$ values lower by the graphical extrapolation method.

4. Binding parameters for BHC - HSA, BHC - plasma and PBZ - HSA interactions obtained by either graphical extrapolation or by the LQF
computer analysis, differed from the literature values, i.e., $k_1$ values were higher and $n_1$ values lower.

5. Binding curves for BHC - Fraction V HSA and BHC - plasma interactions were similar, but $k_1$ values for BHC - plasma interactions were slightly higher.

6. Binding curves for BHC - HSA, BHC - plasma and PBZ - HSA interactions did not appear to be independent of macromolecule concentration. It is not clear whether the HSA concentration effect is a problem inherent in the diafiltration apparatus or whether the simple Law of Mass Action approach does not adequately describe such interactions.

7. Although the diafiltration technique offers advantages over conventional methods, the problem of HSA concentration effect on binding curves must first be resolved before it can be concluded that the diafiltration technique is suitable for protein binding studies.

8. The desorption (washout) diafiltration technique gave unsatisfactory results for PBZ - HSA interactions.

9. The equilibrium or direct diafiltration method gave values for binding parameters for BHC - HSA interactions which were close to literature values. This method, however, has no real advantage over the conventional methods used in protein binding studies.
10. Two other techniques, centrifugation (ultrafiltration) and a Sephadex G-25 gel filtration method (batch method) were also studied. These yielded unsatisfactory results and were not further investigated.
REFERENCES


(i) Theory of Protein Titration Curves. II Calculations for Simple Models at Low Ionic Strength
(ii) The Location of Electrostatic Charges in Kirkwood's Model for Organic Ions Binding.


APPENDIX 1

Sample Calculation of Results by "DRUGFIT" Computer Program

CA = 59.47, B = 336.29, C = 9.32 x 10^{-3} g./l., CO = 0.11014 x 10^{-3},
M = 0.515 x 10^{-4} moles/l., L = 0, VX = 27.00 ml., (K and KF as desired),
KD = 0. (Results from plasma-BHC experiment). p^{121}

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<td>.367</td>
<td>3.98</td>
<td>2.17 x 10^5</td>
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</table>
APPENDIX 1 (Cont'd.)

<table>
<thead>
<tr>
<th>V</th>
<th>A</th>
<th>R</th>
<th>RDF</th>
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</thead>
<tbody>
<tr>
<td>9.6</td>
<td>.379</td>
<td>4.03</td>
<td>2.12 x 10^5</td>
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<tr>
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<td>.389</td>
<td>4.07</td>
<td>2.09 x 10^5</td>
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<tr>
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<td>4.12</td>
<td>2.06 x 10^5</td>
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<tr>
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<td>.411</td>
<td>4.15</td>
<td>2.02 x 10^5</td>
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<td>.421</td>
<td>4.19</td>
<td>1.99 x 10^5</td>
</tr>
<tr>
<td>9.6</td>
<td>.429</td>
<td>4.23</td>
<td>1.97 x 10^5</td>
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</tbody>
</table>

No data points eliminated from LQF analysis.

\[ n_1 = 2.5 \quad n_2 = 85.7 \]
\[ k_1 = 5.57 \times 10^6 \quad k_2 = 971.2 \]
APPENDIX 2

Results and Calculations for Phenylbutazone - Human Serum Albumin Binding by Equilibrium or Direct Method

<table>
<thead>
<tr>
<th>Wt. of Sephadex (g.)</th>
<th>Ext. Volume (ml.)</th>
<th>HSA added to gel (mg.)</th>
<th>mg. PBZ initially added to gel</th>
<th>Total PBZ ((D_t + D_f)) measured in external phase in mg.</th>
<th>mg. PBZ associated with Ig. gel</th>
<th>Free PBZ in external phase in mg. ((D_f) from Figure 6)</th>
<th>Bound PBZ in m.g.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.00</td>
<td>15.32</td>
<td>161</td>
<td>1.553</td>
<td>0.894</td>
<td>0.165</td>
<td>0.515</td>
<td>0.279</td>
</tr>
<tr>
<td>4.00</td>
<td>15.32</td>
<td>161</td>
<td>0.776</td>
<td>0.282</td>
<td>0.124</td>
<td>0.453</td>
<td>---</td>
</tr>
<tr>
<td>4.00</td>
<td>15.32</td>
<td>161</td>
<td>0.304</td>
<td>0.132</td>
<td>0.043</td>
<td>0.256</td>
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</tr>
<tr>
<td>4.00</td>
<td>15.32</td>
<td>80.5</td>
<td>0.899</td>
<td>0.355</td>
<td>0.133</td>
<td>0.469</td>
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</tr>
<tr>
<td>4.00</td>
<td>15.32</td>
<td>33.4</td>
<td>0.100</td>
<td>0.086</td>
<td>0.003</td>
<td>0.046</td>
<td>0.040</td>
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<tr>
<td>4.00</td>
<td>15.32</td>
<td>33.4</td>
<td>0.064</td>
<td>0.045</td>
<td>0.005</td>
<td>0.064</td>
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</tr>
</tbody>
</table>

Notes:
1  Total Volume = 25 ml.
2  PBZ (mg.) associated with 1 g. gel = [PBZ (mg.) added initially - Total PBZ measured in external phase] × wt. of gel.
3  Bound PBZ (mg.) = Total PBZ in external phase - Free PBZ in external phase.