Dynamic Dialysis as a Method for Studying Protein Binding II: Evaluation of the Method with a Number of Binding Systems

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Abstract □ A method for determining the concentrations of unbound and bound small molecule in a protein solution is described, based on the fact that the rate of disappearance of small molecule from a dialysis cell is proportional to the concentration of the unbound species. Curves describing the kinetics of dialysis for 8-nitrotheophylline, 8-chlorotheophylline, caffeine, warfarin, methyl orange, and phenol red in the presence and absence of albumin were obtained and analyzed to yield estimates of unbound and bound forms of the compounds. The data were treated by means of Scatchard plots to provide values for the binding parameters, n and K. The values were found to be in agreement with those reported in the literature and/or determined by independent methods. The ease of application and merits of the method were demonstrated by a comprehensive study of the influence of pH on the binding of 8-nitrotheophylline by bovine serum albumin. Utilization of the method for the study of competitive inhibition of protein binding was also illustrated.

Keyphrases □ Dialysis, dynamic—protein binding determination □ Small molecule binding—protein □ Protein binding—competitive inhibition, drugs □ Kinetic equations—dialysis, small molecules □ pH binding profile, small molecule—kinetics □ UV spectrophotometry—analysis

Previous reports in this series (1, 2) have described in detail a new method for the determination of the degree of interaction between proteins and small molecules. The method is based on the fact that the rate of disappearance of small molecule from a dialysis cell is proportional to the concentration of the unbound species. The influence of experimental variables such as pH, temperature, stirring rate, viscosity, size of the membrane, volumes of liquid within the system, and membrane binding of the small molecule have been previously considered (2).

The present communication describes a series of studies designed to further evaluate the dynamic dialysis method as a technique to quantitate protein binding systems, and to obtain values for the fundamental binding parameters of n (the number of binding sites involved in the interaction) and K (the intrinsic association constant characterizing the interaction). The method was also applied to the comprehensive determination of the effect of pH on the interaction between 8-nitrotheophylline and bovine serum albumin over a wide range of pH. Studies were also conducted to demonstrate the potential of the dynamic dialysis method for the study of competitive inhibition of protein binding.

EXPERIMENTAL

Materials—Human serum albumin, (HSA) Fraction V, (Calbiochem), was used in this study. Warfarin sodium (Endo Laboratories) was used, and the remainder of the materials utilized in the study have been previously described (2).

Dynamic Dialysis Studies of Protein Binding—The protocol for the dynamic dialysis system and the treatment and interpretation of data have been discussed previously (1, 2). The method was employed to assess the binding of phenol red, warfarin, caffeine, 8-chlorotheophylline, 8-nitrotheophylline, salicylate, and methyl orange by bovine serum albumin (BSA). Initially, 7 ml. of a control solution, prepared to contain the given small molecule in 0.04 M phosphate buffer at an appropriate pH, was placed into a 7-cm. long dialysis sac. The sac was then immersed in the buffer bathing solution of 0.04 M, phosphate buffer. The system was maintained at 25 ± 0.2°. Each control dialysis was followed for about 2 hr., and the results were used to calculate the apparent permeability rate constant characterizing the escape of small molecule from each sac. The sac was then rinsed thoroughly with distilled water and blotted with tissue to remove excess moisture. Seven milliliters of a small molecule-protein solution, prepared in 0.04 M buffer at the same pH as the control, was then introduced into the sac. The sac was placed into fresh buffer bathing solution and the dialysis of the small molecule from the protein compartment was followed for 5 to 9 hr. The frequency at which 100-ml. samples were removed from the external solution and replaced with fresh buffer solution was dictated by the need to maintain sink conditions, and by the sensitivity of the assay method for the particular small molecule. It was observed from preliminary experiments that sink conditions were approximated if the concentration of small molecule in the external solution was not allowed to exceed one-tenth of that unbound in the internal solution. At the termination of a run, the internal solution was sampled and analyzed for small molecule content. The binding parameters obtained from the dynamic dialysis studies were then compared with values obtained by alternate approaches such as equilibrium dialysis or ultrafiltration, and/or with literature values.

For the study of the influence of pH on the interaction between BSA and 8-nitrotheophylline, three separate experimental systems could be conveniently run at one time, each maintained at a different pH. For these determinations, a series of solutions were prepared containing 8-nitrotheophylline in the presence and absence of BSA. The solutions covered a pH range of 3.0–10.9, in 0.04 M phosphate buffer. The protein systems contained approximately 0.75% BSA. The external buffer bathing solution was also prepared at the same pH and buffer concentration as the internal solution. The dialysis was followed for a sufficient period of time to fully characterize the kinetics of each system. The pH's of the internal and external solutions were redetermined at the termination of each experimental run and were found not to have changed.

Data Treatment—Kinetic curves for each system investigated were analyzed by the previously reported method (1). The data treatment yielded values for the concentrations of unbound (D_f) and bound (D_b) species, for a number of different total small molecule concentrations (D_t). Results were displayed in the form of Scatchard plots where $\bar{\nu}/(D_f)$ ($\bar{\nu}$ = moles of small molecule bound per mole of protein) was plotted as a function of $\bar{\nu}$. Values of the binding parameters (n's and K's) were estimated by fitting the data, with the aid of a nonlinear regression computer program,[1] to the generalized binding expression given in Eq. 1.

$$\bar{\nu}_d = \sum_{i=1}^{i} \frac{n_i K_i (D_f)}{1 + K_i (D_f)} \qquad \text{(Eq. 1)}$$

Two classes of binding sites were assumed if the experimentally determined Scatchard plot exhibited curvature (3). In this report experimental data are indicated in the Scatchard plots by points while solid lines represent behavior predicted on the basis of computed n and K values.

[1] Revised Share Distribution No. 1428, S.U.N.Y.A.B. Library Subroutine-NLIN.

Table I—Summary of the Binding Constants for the Interaction of Various Small Molecules with BSA at pH 7–7.3 and 25°

Compound	Source of Constants	n_1[a]	n_2	K_1[b]	K_2
Phenol red	Experimental: ultrafiltration and dynamic dialysis	1	6	1.74×10^5	1.97×10^3
	Literature: ultrafiltration (15)	1	6	1.10×10^5	1.20×10^3
Methyl orange	Experimental: dynamic dialysis	22	—	2.08×10^3	—
	Literature: equil. dialysis (5)	22	—	2.27×10^3	—
	Literature: equil. dialysis (16)	16	—	3.80×10^3	—
Warfarin	Experimental: ultrafiltration and dynamic dialysis	1	6	6.24×10^6	2.61×10^3
Caffeine	Experimental: dynamic dialysis	1	—	1.02×10^3	—
	Literature: equil. dialysis-9° (7)	1	—	1.68×10^3	—
8-Nitrotheophylline	Experimental: dynamic dialysis	1	4	2.34×10^5	2.53×10^3
	Literature: equil. dialysis (pH 6.85 and 9°) (7)	1	2	2.64×10^5	1.27×10^3
8-Chlorotheophylline	Experimental: dynamic dialysis	1	4	2.16×10^5	1.47×10^3
	Literature: equil. dialysis (pH 6.85 and 9°) (7)	1	2	3.07×10^5	1.10×10^3
Salicylic acid	Experimental: dynamic dialysis	1	5	2.00×10^5	1.75×10^3

[a] n_i = the number of binding sites in the i'th class. [b] K_i = the intrinsic association constant for the i'th class of sites (l./M).

Ultrafiltration—Ultrafiltration was utilized to study the binding of warfarin by BSA and HSA and the procedure used was that previously described (2). These studies were performed with solutions containing warfarin in a concentration range of 0.032–0.380 mg./ml. in 0.04 M, pH 7.3, phosphate buffer. The initial protein concentration was 0.37% for the BSA studies, and 0.39 or 0.78% for the HSA studies. After centrifugation the concentration of small molecule in the filtrate and the total small molecule concentration of the solution remaining in the sacs was determined. From a knowledge of these concentrations and the total protein concentration in the system, the values for $\bar{\nu}/(D_f)$ and $\bar{\nu}$ were calculated. Preliminary experiments indicated that approximately 4% of the warfarin introduced into the system was bound to the membrane.

Equilibrium Dialysis Studies—The binding of warfarin by BSA was also studied by the technique of equilibrium dialysis. These experiments were conducted using 10-ml. capacity, Plexiglas dialysis cells. The warfarin concentrations employed in these studies ranged from 0.002–0.6 mg./ml., in 0.04 M, pH 7.3, phosphate buffer. The BSA concentration was 0.4%. Preliminary experiments indicated that about 3% of the warfarin was bound to the membrane separating the two compartments of the dialysis cell, and that equilibrium was attained in the cells within 12 hr. The systems were prepared by placing 5 ml. of warfarin solution on one side of the membrane, and 5 ml. of warfarin and protein solution on the other. The cells were agitated, on a platform shaker, for at least 14 hr. at room temperature, 25 ± 2°. After equilibration, both sides of the cell were sampled, and from a knowledge of the total warfarin in the system, the free warfarin concentration, the degree of membrane binding, and the total protein concentration, the values for $\bar{\nu}/(D_f)$ and $\bar{\nu}$ were calculated.

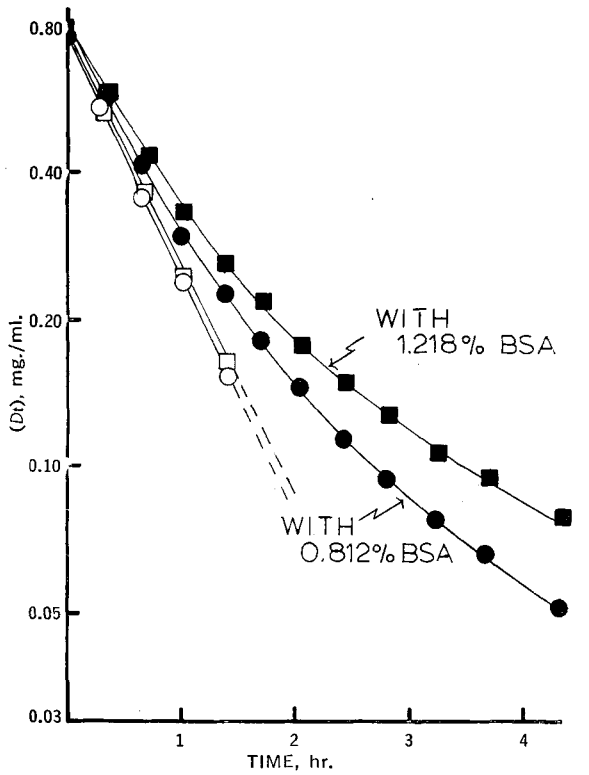

Figure 1—*The loss of 8-chlorotheophylline from inside a dialysis sac, in the presence and absence of BSA. The studies were run at pH 7.0 and 25°.*

Dynamic Dialysis Studies of Competitive Inhibition of Binding—The preliminary step in these studies was to determine if the presence of the competitor in the system exerted an effect on the intrinsic rate of dialysis of the small molecule under consideration. 8-Nitrotheophylline was utilized as the test species, and 8-chlorotheophylline or salicylic acid were employed as the competitors. A control run with 8-nitrotheophylline was first conducted, and then using the same membrane sac, the dialysis of 8-nitrotheophylline was followed in the presence of the competitor. Then an 8-nitrotheophylline and BSA system was dialyzed to obtain the kinetic and equilibria parameters operant in the absence of competitor. Finally, still using the same membrane, 8-nitrotheophylline, BSA, and competitor were dialyzed. Two approaches were used in the competitive studies. In the first, a known competitor concentration was incorporated inside the dialysis sac. The decline of both 8-nitrotheophylline and competitor concentration inside the sac was followed by determining the amounts of 8-nitrotheophylline and competitor appearing in the external solution. The second approach was such that the concentration of competitor remained essentially constant throughout the dialysis. Here a known competitor concentration was placed into the dialysis sac containing the 8-nitrotheophylline. In addition, the external buffer bathing solution was prepared to contain an equivalent concentration of competitor. Further, the samples which were withdrawn from the external solution were replaced with a solution which contained the same concentration of the competitor as originally present. In this manner the concentration of the competitor was held constant during the course of the dialysis. All studies involving 8-nitrotheophylline and/or competitor species were conducted at 25° in pH 7.0, 0.04 M, phosphate buffer.

Analytical Methods—The compounds studied were assayed spectrophotometrically with a Beckman DU spectrophotometer equipped with a Gilford power source and digital, absorbance read-out. Concentrations were determined from Beer's law plots constructed at appropriate wavelengths and pH values.

The concentration of 8-nitrotheophylline in the presence of BSA was determined spectrophotometrically at pH 11 *versus* an appropriate BSA blank. The absorbance due to protein was negligible in the visible region of the spectrum, and at pH 11 there were no spectral manifestations of interactions between the BSA and 8-nitrotheophylline.

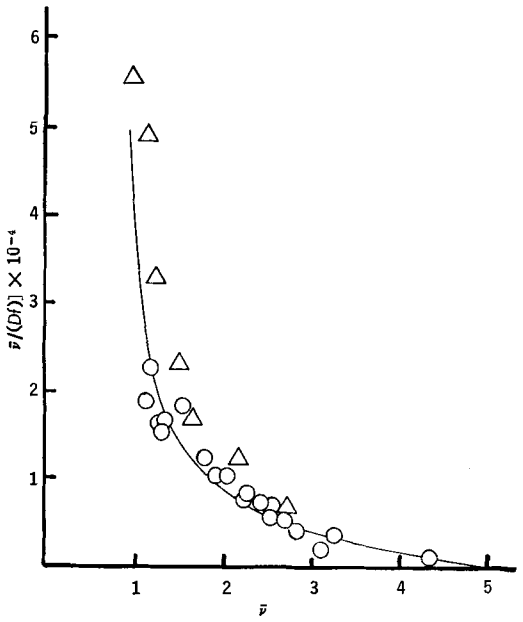

Figure 2—*The binding of 8-chlorotheophylline by BSA at pH 7.0 and 25°. Key: ○, 0.812 and 1.218% BSA, dynamic dialysis; △, literature values; (6), equilibrium dialysis at pH 6.85 and 9°.*

Because BSA interfered with the direct spectrophotometric determination of caffeine, warfarin, and 8-chlorotheophylline, extraction procedures were utilized. Two milliliters of sample containing caffeine and BSA were quantitatively extracted with 25 ml. of chloroform, and the organic layer was examined spectrophotometrically at 272 mμ *versus* an appropriate chloroform blank. Samples containing 8-chlorotheophylline and BSA were assayed for 8-chlorotheophylline by diluting 2 ml. of sample to 3 ml. with diluted phosphoric acid (1:5) and extracting with 25 ml. of a chloroform–isopropyl alcohol (19:1) solution. The organic phase was assayed spectrophotometrically at 276.5 mμ *versus* an appropriate solvent blank. Solutions containing both warfarin and BSA were assayed by the method of O'Reilly *et al.* (4). Centrifugation was utilized to break any emulsions formed during the extraction procedures.

Samples containing 8-nitrotheophylline in combination with 8-chlorotheophylline, caffeine, or salicylic acid were assayed for both components by a differential spectrophotometric assay conducted at 386 mμ, and 276.5, 272, or 302 mμ, respectively. Samples containing 8-nitrotheophylline, 8-chlorotheophylline, and BSA were adjusted to pH 11 and assayed for 8-nitrotheophylline at 386 mμ. The 8-chlorotheophylline concentration was determined by extracting an acidified sample and measuring the absorbance of the organic phase at 276.5 mμ. The latter absorbance was corrected for the small contribution of the 8-nitrotheophylline to the reading at this wavelength.

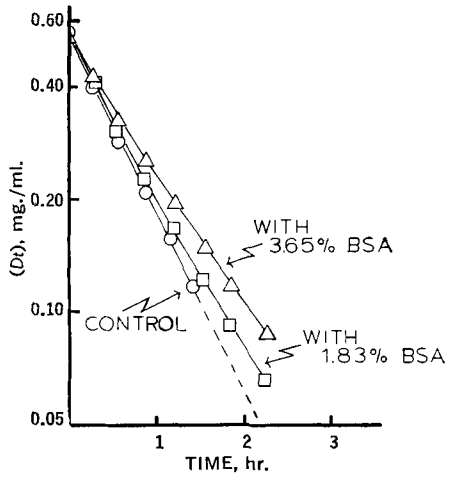

Figure 3—*The loss of caffeine from inside a dialysis sac, in the presence and absence of BSA. The studies were run at pH 7.0 and 25°.*

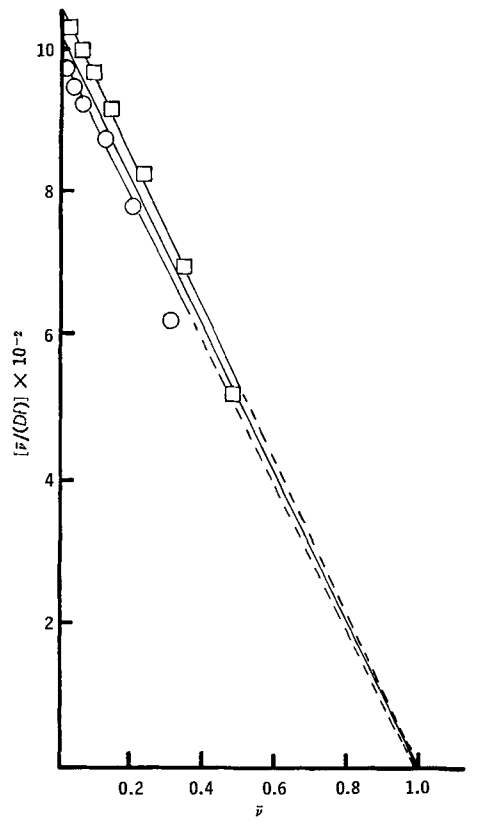

Figure 4—*The binding of caffeine by BSA at pH 7.0 and 25°, as depicted by a Scatchard plot. Key: ○, 1.83% BSA, □, 3.65% BSA.*

RESULTS AND DISCUSSION

Determination of the Binding Constants for the Interaction of Various Small Molecules with BSA—A previous report (1) described studies employing this technique with phenol red and methyl orange. For comparative purposes, binding parameters for these two compounds are presented in Table I, along with values for a number of other compounds which were investigated. It should be noted that parameters characterizing the binding of methyl orange (1, 5) were reported incorrectly and have been corrected in Table I.

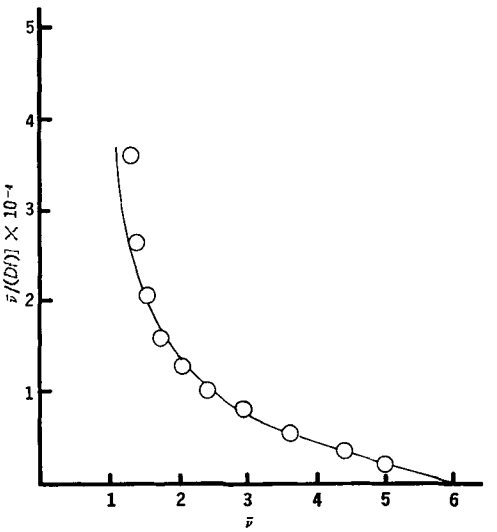

Figure 5—*The binding of salicylic acid by BSA at pH 7.0 and 25°.*

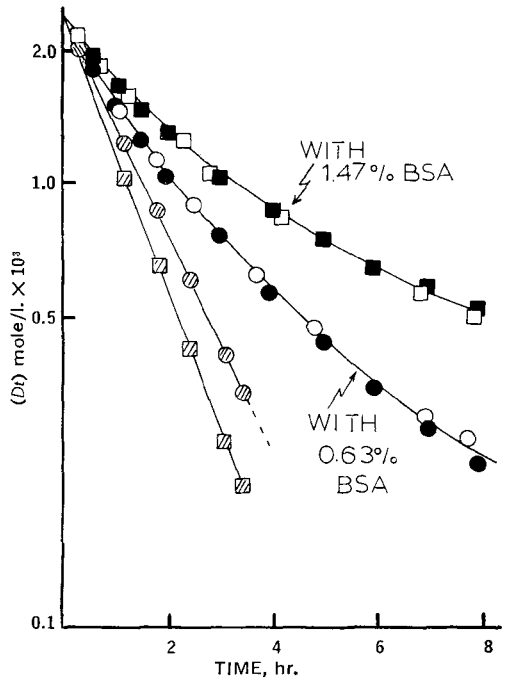

Figure 6—*The loss of warfarin from inside a dialysis sac, in the presence and absence of BSA. The studies were run at pH 7.3 and 25°. Key: ○, 0.63% control; □, 1.465% control; ●, 0.63% BSA, experimental; ■, 1.465% BSA, experimental; ○, 0.63% BSA, theoretical; □, 1.456% BSA, theoretical.*

Binding of 8-Chlorotheophylline—Figure 1 illustrates the results of dynamic dialysis experiments with 8-chlorotheophylline in the presence and absence of BSA at 25° and pH 7.0. Transformation of the data, in the manner described, yielded the Scatchard plot of Fig. 2. Data obtained by Eichman *et al.* (6) for this system at pH 6.85 and 9°, using equilibrium dialysis, is also shown. The small differences between Eichman's data and those of the present study can be attributed to a difference in the temperatures employed.

Binding of Caffeine—Caffeine has been shown to interact rather weakly with BSA (6, 7). It was, therefore, of interest to attempt to quantitate its binding behavior by the dynamic dialysis approach.

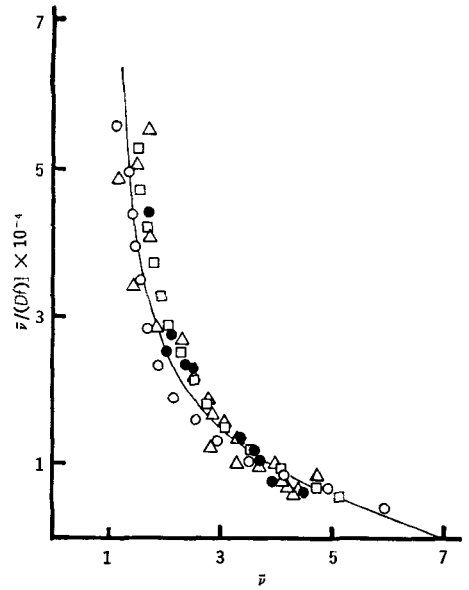

Figure 7—*The binding of warfarin by BSA and HSA at pH 7.3 and 25°. Key: △, BSA, ultrafiltration and equilibrium dialysis; ●, HSA, ultrafiltration; ○, 0.63% BSA, dynamic dialysis; □, 1.465% BSA, dynamic dialysis.*

Results obtained at 25° in 0.04 *M* pH 7 buffer are shown in Fig. 3. It can be seen that in spite of relatively high protein concentrations, the difference in kinetic behavior in the absence and presence of protein was small and reflects the weak nature of the interaction. Figure 4 shows the corresponding Scatchard plot. From a least-squares fit of the Scatchard plot, it was determined that the interaction between BSA and caffeine could be appropriately characterized by the parameters $n = 1$, and $K = 1.02 \times 10^3$ l./mole. These values are in reasonable agreement with the parameters reported by Eichman *et al.* (6) from equilibrium dialysis studies at pH 6.85 and 9°. The somewhat lower association constant obtained in the present study, which was conducted at a higher temperature than the work of Eichman *et al.* is consistent with a previous observation (8) of decreased xanthine binding with increased temperature.

It should be noted that the binding of a weakly interacting compound is difficult to quantitate accurately by any experimental technique due to magnification of experimental error by subsequent data treatment. The caffeine studies demonstrate that the dynamic dialysis method can be useful in characterizing even weak interactions.

Binding of Salicylic Acid—Figure 5 illustrates a Scatchard plot derived from the kinetics of dialysis of salicylic acid at pH 7.0 and 25° in the presence of 1.616% BSA and in the absence of BSA. The data were found to be consistent with the binding parameters given in Table I.

The binding observed in this study was somewhat stronger than that observed by Davison and Smith (9) in their studies conducted at 4° and pH 5.4, in acetate buffer. They reported binding constants of $n_1 = 0.37$, $n_2 = 3.5$, $K_1 = 3 \times 10^4$ l./mole, and $K_2 = 1 \times 10^2$ l./mole, and observed little difference in binding behavior between pH 5.4 and 7.0. They also reported that acetate can competitively inhibit salicylate binding and this might be the reason for the difference in strengths of binding between the two studies.

Binding of Warfarin—The binding of warfarin by albumin has been well studied and characterized (10) and thus, it was felt that warfarin would serve admirably as an additional compound with which to assess this new approach for quantitating binding behaviors. The results of the dynamic dialysis examination of warfarin binding are summarized in Fig. 6 which depicts the kinetics of dis-

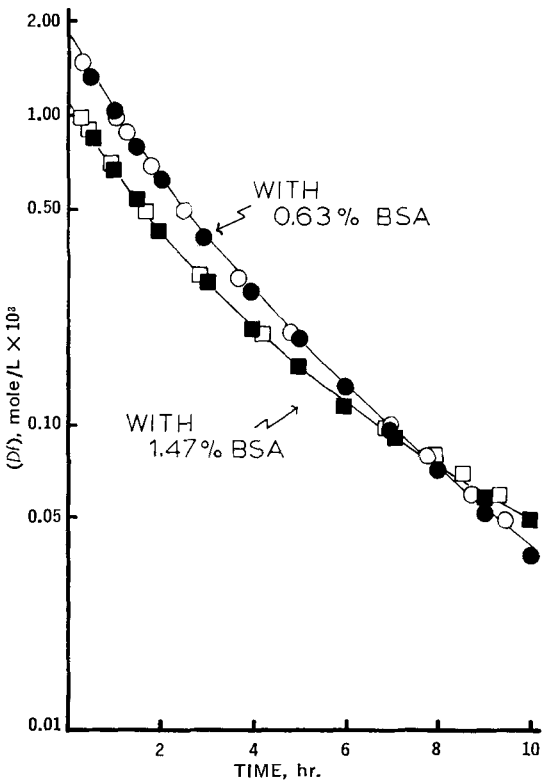

Figure 8—*The loss of free warfarin from inside a dialysis sac, in the presence of BSA, at pH 7.3 and 25°. Key: ●, ■, dynamic dialysis data; ○, □, theoretical data.*

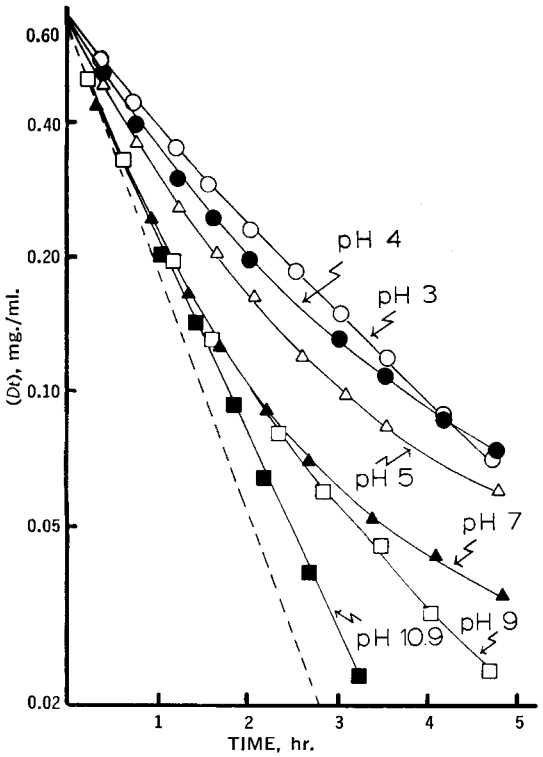

Figure 9—*The loss of 8-nitrotheophylline from inside a dialysis sac in the presence and absence of 0.75% BSA. The studies were conducted at a variety of pH's and 25°. Key: - - -, average control run.*

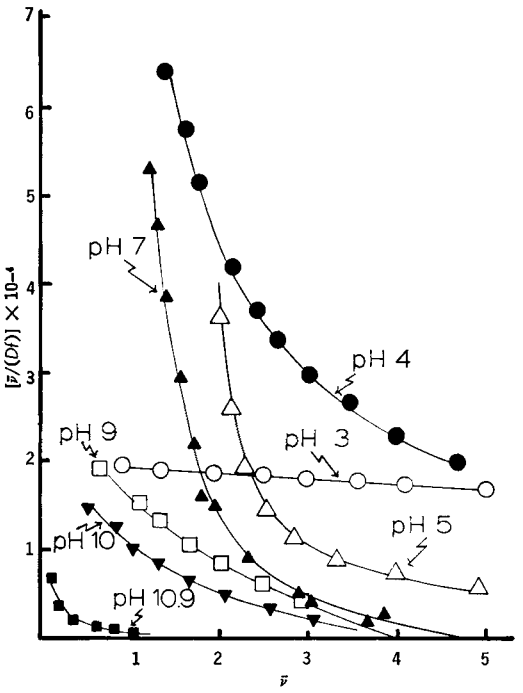

Figure 10—*The binding of 8-nitrotheophylline by BSA at various pH's and 25°.*

appearance of warfarin from a 0.630% solution of BSA and from a 1.465% solution of BSA, as well as for corresponding control systems. The difference between control runs is significant and reflects the fact that a different membrane was used for the two systems. The control runs were repeated and found to be entirely reproducible.

The Scatchard plot is shown in Fig. 7. It was disturbing to compare this plot with that presented by O'Reilly (10) who studied, by equilibrium dialysis, the interaction of warfarin with human serum albumin (HSA). O'Reilly's data indicated a much weaker degree of interaction and the marked lack of agreement prompted further studies on the binding of warfarin. To assess whether or not HSA possessed a significantly different affinity for warfarin than did BSA, the binding of warfarin by the two proteins was investigated using ultrafiltration. The results are also shown in Fig. 7 and demonstrate that the nature and extent of binding were the same for both proteins. The interaction was also investigated by equilibrium dialysis and the results are presented in Fig. 7. It is obvious that the three different experimental methods provided data which were in excellent agreement and, therefore, indicated that neither the source of the albumin nor the experimental approach could explain the lack of agreement with the results of O'Reilly. In view of this, O'Reilly's data were examined more critically to reveal two computational errors. First, a correction for sac binding was made which was based on the total concentration of warfarin rather than on the concentration of free warfarin in the system. This, however, resulted in only a minor error since the sac binding was only of the order of 3.6%. A more serious computational error was involved in the calculation of $\bar{\nu}$. When a correction was made the $\bar{\nu}$ values increased and were quite consistent with those of the present study. The combined data of Fig. 7 were used to generate the n and K values presented in Table I.

Theoretical Treatment Describing the Escape of a Small Molecule from a Protein-Containing Compartment—It has been demonstrated that kinetic data obtained in the dialysis of a small molecule in the absence and presence of protein can be treated to gain estimates of (D_f) and (D_b) and subsequently n's and K's. The validity of the approach has been confirmed by the close agreement of binding parameters, estimated by using this method, with those determined by classical methods. It did seem desirable, however, as a further check, to compare experimentally observed kinetic behavior with that theoretically expected on the basis of initial concentration conditions and binding parameters. Theoretical expressions for such a system have been considered, in a pharmacokinetic context, by Kruger-Thiemer (11) who derived equations which describe the rates of loss of unbound and total small molecule capable of being bound from a protein compartment. His derivations were restricted to situations where binding occurred to one class of sites on the protein but can be readily modified to account for the existence of two classes of sites. Such a modification results in Eq. 2 which describes dynamic dialysis behavior, *i.e.*, the rate of disappearance of total concentration of small molecule (D_t) from a protein compartment as a function of an apparent first-order elimination constant, K_e, the concentration of unbound small molecule (D_f), the total concentration of protein (P_t), the number of binding sites

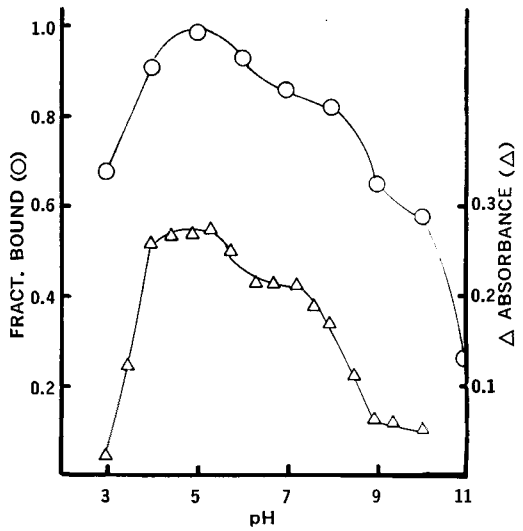

Figure 11—*The influence of pH on the binding of 8-nitrotheophylline at 25°. Key: △, delta absorbance values determined from spectrophotometric studies; ○, fraction bound values calculated from dynamic dialysis studies.*

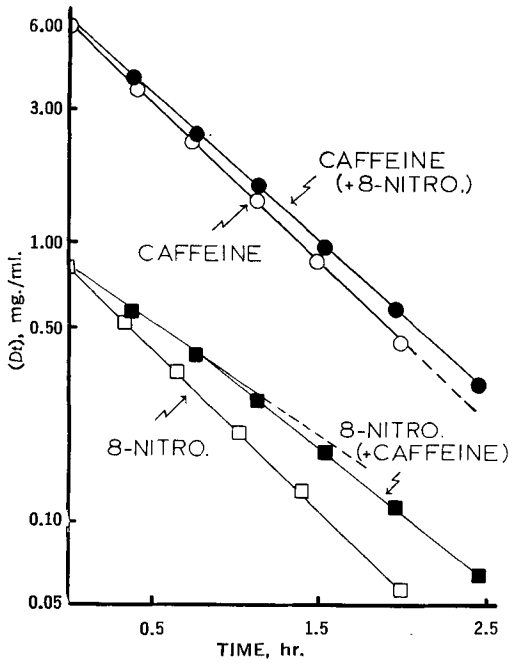

Figure 12—*The loss of 8-nitrotheophylline in the presence and absence of caffeine, and the loss of caffeine in the presence and absence of 8-nitrotheophylline, from inside a dialysis sac. The studies were conducted at pH 7.0 and 25°.*

in each of two classes, n_1 and n_2, and the corresponding dissociation constants, k_1 and k_2.

$$\frac{-d(D_t)}{dt} = \frac{K_e(D_t)}{1 + [n_1(P_t)]/[(D_f) + k_1] + [n_2(P_t)]/[(D_f) + k_2]} \quad \text{(Eq. 2)}$$

Equation 3 gives the rate of loss of free drug (D_f) from the protein compartment.

$$\frac{-d(D_f)}{dt} = \frac{K_e(D_f)}{1 + [n_1k_1(P_t)]/[(D_f + k_1)^2] + [n_2k_2(P_t)]/[(D_f + k_2)^2]} \quad \text{(Eq. 3)}$$

Finally, Eq. 4 gives the relationship between the total and unbound drug concentration.

$$(D_t) = (D_f)\left[1 + \frac{n_1(P_t)}{(D_f) + k_1} + \frac{n_2(P_t)}{(D_f) + k_2}\right] \quad \text{(Eq. 4)}$$

An expression for (D_t) as a function of time cannot be obtained directly by integration of Eq. 2 and, therefore, a more circuitous approach must be utilized to generate theoretical kinetic data. First, the value for the initial free drug concentration (D_f^0) in the protein compartment is calculated as follows: Eq. 4 may be written in terms of initial total (D_t^0) and initial unbound (D_f^0) drug concentrations. The resulting equation may then be rearranged to yield a cubic expression in (D_f^0):

$$(D_f^0)^3 + (D_f^0)^2[k_1 + k_2 + (P_t)(n_1 + n_2) - (D_t^0)] + (D_f^0)[k_1k_2 + (P_t)(n_1k_2 + n_2k_1) - (D_t^0)(k_1 + k_2)] - (D_t^0)(k_1k_2) = 0 \quad \text{(Eq. 5)}$$

It may be noted that when one class of binding sites is involved, a quadratic expression in (D_f^0) results. By integration of Eq. 3 between the limits of $t = 0$ to t, and $(D_f) = (D_f^0)$ to (D_f), an expression for time may be obtained:

$$t = \frac{1}{K_e}\left\{\ln\frac{(D_f^0)}{(D_f)} + \frac{n_1(P_t)}{k_1}\left[\ln\frac{(D_f^0)(k_1 + D_f)}{(D_f)(k_1 + D_f^0)}\right] + \frac{n_2(P_t)}{k_2}\ln\left[\frac{(D_f^0)(k_2 + D_f)}{(D_f)(k_2 + D_f^0)}\right] - (P_t)(D_f^\circ - D_f) \times \left[\frac{n_1}{(k_1 + D_f^0)(k_1 + D_f)} + \frac{n_2}{(k_2 + D_f^0)(k_2 + D_f)}\right]\right\} \quad \text{(Eq. 6)}$$

Thus, for values of n_1, n_2, k_1, k_2, (D_t^0), and (P_t), a value of (D_f^0) may be calculated from Eq. 5. Then assuming various values for (D_f), the time, t, at which the assumed (D_f) concentrations will be present in the protein compartment can be calculated from Eq. 6. Finally, using Eq. 4, the values for (D_t), corresponding to the times which were determined from Eq. 6, are calculated. These computations were carried out with the aid of a Fortran IV computer program and a CDC 6400 computer.

Comparisons between dialytic behaviors which were theoretically expected and experimentally determined for warfarin are shown in Figs. 6 and 8. Theoretical points were obtained using the outlined treatment with (D_t^0) and (P_t) values selected to correspond to the experimental system. The values of n and K used were those tabu-

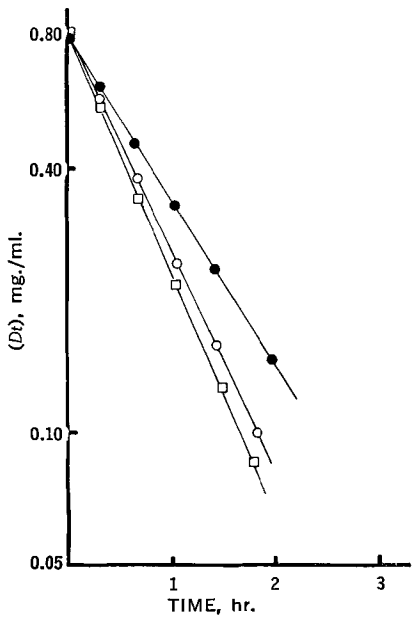

Figure 13—*The loss of 8-nitrotheophylline from inside a dialysis sac, in the presence and absence of 8-chlorotheophylline. The studies were conducted at pH 7.0 and 25°. Key: □, control; ○, with 0.75 mg./ml. 8-chlorotheophylline; ●, with 1.74 mg./ml. 8-chlorotheophylline.*

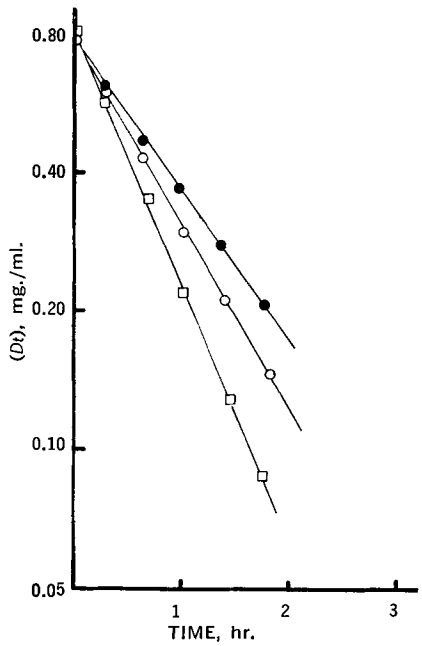

Figure 14—*The loss of 8-nitrotheophylline from inside a dialysis sac, in the presence and absence of salicylate. The studies were conducted at pH 7.0 and 25°. Key: □, control; ○, with 9.8 mg./ml. salicylic acid; ●, with 22.6 mg./ml. salicylic acid.*

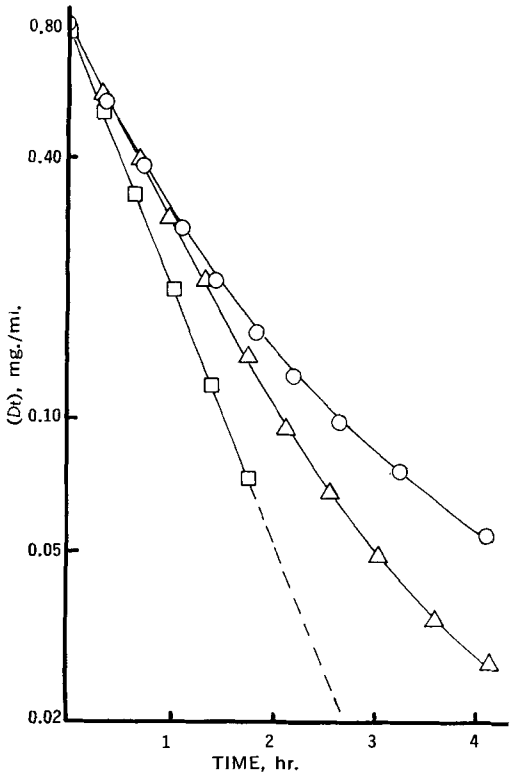

Figure 15—*The loss of 8-nitrotheophylline from inside a dialysis sac, in the presence and absence of BSA, and BSA and 8-chlorotheophylline. The studies were conducted at pH 7.0 and 25°. Key: □, control; ○, with 0.818% BSA; △, with 0.818% BSA and an initial 8-chlorotheophylline concentration of 1.6 mg./ml.*

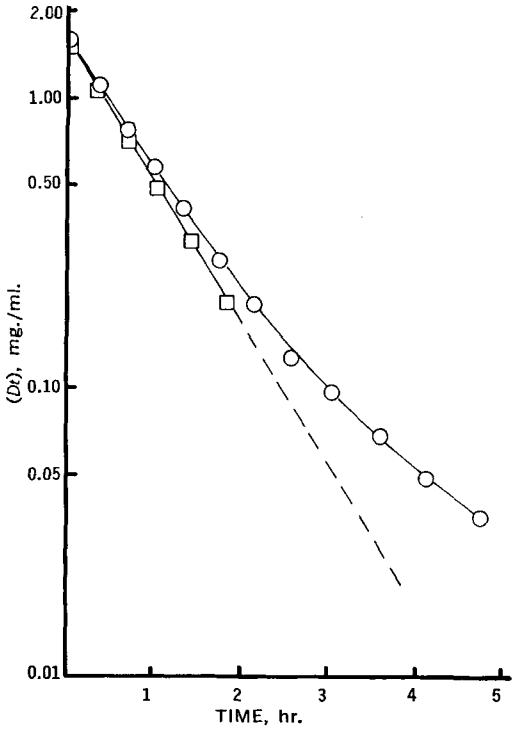

Figure 16—*The loss of 8-chlorotheophylline from inside a dialysis sac, in the presence and absence of BSA and 8-nitrotheophylline. These data were obtained in the same experimental run as was illustrated in Fig. 15. Key: □, control; ○, with 0.818% BSA and 0.82 mg./ml. 8-nitrotheophylline.*

lated in Table I. The K_e values employed corresponded to the experimentally determined apparent permeability constants for the membranes. The close agreements between experimental results and theoretical predictions illustrate the applicability of the equations and supports the validity of this experimental approach for studying binding behavior.

Kinetic Determination of the pH-Binding Profile for the Interaction of 8-Nitrotheophylline with BSA—The influence of pH on the binding of 8-nitrotheophylline by BSA, as determined by spectrophotometric investigations, has been previously reported (12). It seemed appropriate, in view of the interesting pH effects observed, to study more quantitatively the effect of pH on the interaction. In addition, it was thought that such a study would serve to evaluate further the dynamic dialysis method and demonstrate its applicability and convenience. From kinetic data obtained with the dynamic dialysis procedure, it was possible to generate Scatchard plots characterizing the interaction at each pH. Thus, binding behavior over a wide range of pH could be conveniently obtained in a relatively short period of time. Accumulation of such data by conventional techniques of equilibrium dialysis or ultrafiltration would require considerably more effort and a much greater expenditure of time.

The results of these studies are summarized in Fig. 9 where the kinetic behavior of 8-nitrotheophylline in the presence of BSA under different conditions of pH is shown. An average control run is also depicted. Data for pH 6, 8, and 10 were also obtained, but were omitted to reduce the complexity of the figure. From examination of the data of Fig. 9, it would appear that as the pH was increased from pH 3 to 10.9, the degree of interaction between the 8-nitrotheophylline and the BSA steadily decreased. The data were further treated by computer analysis, in the usual manner, to obtain the values for $\bar{\nu}$ and $\bar{\nu}/(D_f)$. In addition, the fraction of 8-nitrotheophylline bound to the BSA at each total 8-nitrotheophylline concentration was determined. The resulting Scatchard plots are shown in Fig. 10. Data for studies conducted at pH 6 and 8, which were similar to that obtained for pH 7, have been omitted to permit the figure to be more readily interpreted. This figure dramatically demonstrates differences in binding behavior as the pH was varied. At pH 3 the interaction was somewhat unique. The apparent linearity of the plot indicates the participation of one class of binding sites, containing a relatively large number of sites. The Scatchard plots representing other pH values are curved, indicating that at these pH's more than one class of sites were involved. The unusual behavior apparent at pH 3.0 is likely related to a pH dependent alteration of the protein configuration. Here the interaction is relatively weak as apparent from the shallow slope of the Scatchard plot. Thus the high degree of binding, suggested by the kinetic data

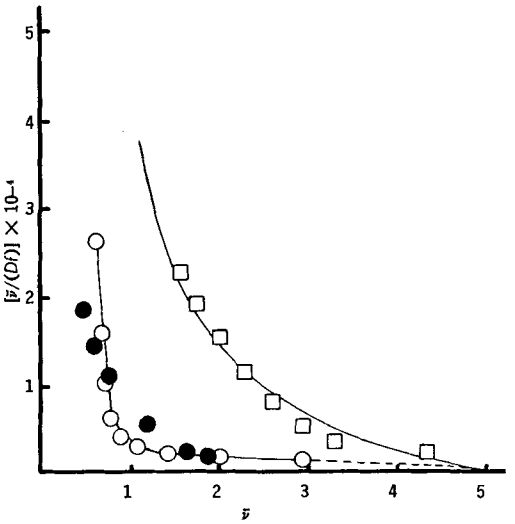

Figure 17—*The binding of 8-nitrotheophylline by BSA in the presence and absence of a variable 8-chlorotheophylline concentration. The studies were conducted at pH 7.0 and 25°. Key: □, 8-nitrotheophylline and 0.818% BSA; ○, 8-nitrotheophylline, 0.818% BSA and 8-chlorotheophylline; ●, theoretical data for the binding of 8-nitrotheophylline in the presence of 8-chlorotheophylline.*

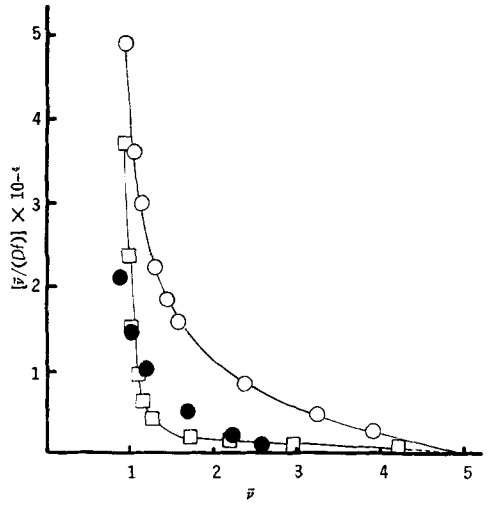

Figure 18—*The binding of 8-chlorotheophylline by BSA in the presence and absence of 8-nitrotheophylline. The studies were conducted at pH 7.0 and 25°. Key: ○, 8-chlorotheophylline and 0.818% BSA; □, 8-chlorotheophylline, 0.818% BSA and 8-nitrotheophylline; ●, theoretical data for the binding of 8-chlorotheophylline by BSA in the presence of 8-nitrotheophylline.*

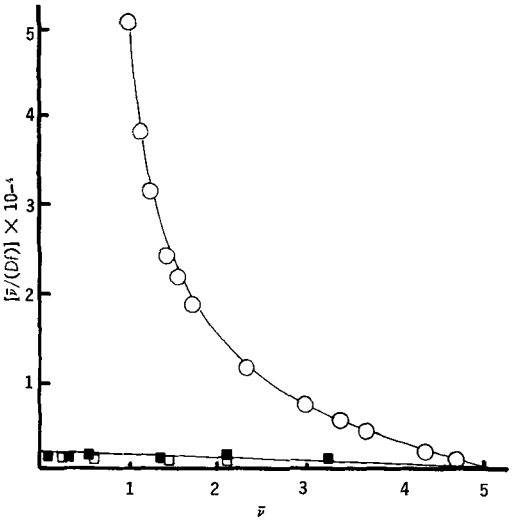

Figure 20—*The binding of 8-nitrotheophylline by BSA in the presence and absence of a constant 8-chlorotheophylline concentration of 0.76 mg./ml. Key: ○, 8-nitrotheophylline and BSA; ■, 8-nitrotheophylline, BSA, and 0.76 mg./ml. 8-chlorotheophylline; □, theoretical data for the binding of 8-nitrotheophylline in the presence of 0.76 mg./ml. 8-chlorotheophylline.*

obtained at pH 3, was a reflection of the involvement of a relatively large number of binding sites rather than a high intrinsic affinity. The pH 4 Scatchard plot also suggests the participation of a large number of binding sites with the added involvement of a second class of sites with a significantly higher affinity. Two or more classes of sites were apparently involved throughout the remainder of the pH range studied. An increase in pH from 4 to 6 resulted in a decrease in the total number of sites available for binding and an increase in the strength of association. As the pH was increased from 7 to 10.9 the extent of interaction diminished, probably as a result of changes in the degree of ionization of participating groups on the protein and/or pH-induced conformation changes (13). In order to compare more readily the results of this study to those obtained from the spectrophotometric studies previously reported (12), a plot of fraction bound *versus* pH was constructed and is shown in Fig. 11. The fraction bound, which is plotted, was that obtained at a total 8-nitrotheophylline concentration of 1.4×10^{-4} mole/l. in the presence of 0.75% BSA. The pH profile previously reported, for the interaction between 1.42×10^{-4} *M* 8-nitrotheophylline and 0.4% BSA, is illustrated for comparative purposes. There is good qualitative agreement between the two methods. However, at the extremes of pH, *i.e.*, at pH 3 and 10.9, the two techniques yielded significantly different results. The spectrophotometric studies indicated a relatively small degree of interaction at these pH extremes, whereas the dynamic dialysis method detected appreciable binding, with about 68% bound at pH 3 and 23% bound at pH 10.9. Some of this difference can, of course, be attributed to the higher BSA concentration used in the dynamic dialysis systems. However, the data may also reflect a shortcoming of the spectrophotometric method in that if interaction does not alter spectral properties of the small molecule, then the interaction cannot be detected from spectral studies. It is important to note that both studies yielded pH profiles having the same general shape and both studies, therefore, indicated that three or more classes of sites on BSA are involved in the binding of 8-nitrotheophylline, as previously discussed (12).

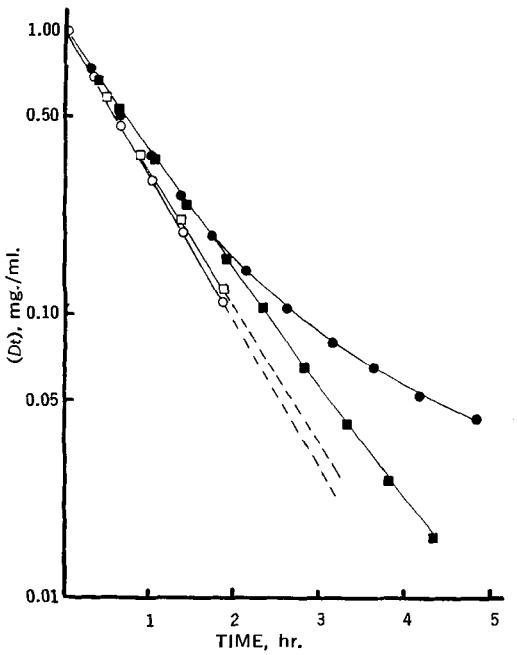

Figure 19—*The loss of 8-nitrotheophylline from inside a dialysis sac, in the presence and absence of BSA, and BSA and a constant 8-chlorotheophylline concentration. The studies were conducted at pH 7.0 and 25°. Key: ○, 8-nitrotheophylline control; □, 8-nitrotheophylline control in the presence of 0.76 mg./ml. 8-chlorotheophylline; ●, 8-nitrotheophylline and 0.778% BSA; ■, 8-nitrotheophylline, 0.778% BSA and 0.76 mg./ml. 8-chlorotheophylline.*

The binding of 8-nitrotheophylline by BSA, at pH 7.0, was further evaluated to determine the binding parameters characterizing this interaction. The data appeared to be adequately described by an interaction involving two classes of binding sites, where $n_1 = 1$, $n_2 = 4$, $K_1 = 2.34 \times 10^5$ l./mole, and $K_2 = 2.53 \times 10^3$ l./mole.

Competitive Inhibition of Protein Binding—Inhibition of protein-small molecule interactions due to the presence of a species capable of competing for binding sites is a well recognized phenomenon and has recently been reviewed (14). Equation 7 describing competitive binding may be easily derived from mass law considerations and defines the dependency of binding of a species "*D*," in the presence of a competitor "*C*," on the unbound "*D*" concentration (D_f), and unbound competitor concentration (C_f), the number of binding sites on the protein (n) which interact with the drug and the competitor, and the corresponding intrinsic association constants for "*D*" and "*C*" (K_d and K_c).

$$\bar{\nu}_d = \frac{n_1 K_{d1}(D_f)}{1 + K_{d1}(D_f) + K_{c1}(C_f)} + \frac{n_2 K_{d2}(D_f)}{1 + K_{d2}(D_f) + K_{c2}(C_f)} \quad \text{(Eq. 7)}$$

Here, $\bar{\nu}_d$ = moles of Species "D" bound per total mole of protein. This equation may be rearranged in the form of a Scatchard equation, as given in Eq. 8.

$$\frac{\bar{\nu}_d}{(D_f)} = \frac{\Sigma nK_d + (D_f)K_{d1}K_{d2}\,\Sigma n + (C_f)(n_1K_{c2}K_{d1} + n_2K_{c1}K_{d2})}{1 + (C_f)[\Sigma K_c + K_{c1}K_{c2}(C_f)]} - \bar{\nu}_d\left[\frac{\Sigma K_d + K_{d1}K_{d2}(D_f) + (C_f)(K_{c2}K_{d1} + K_{c1}K_{d2})}{1 + (C_f)[\Sigma K_c + K_{c1}K_{c2}(C_f)]}\right] \quad \text{(Eq. 8)}$$

It may be shown that while Eq. 8 is derived in terms of $\bar{\nu}_d$, a completely analogous equation can similarly be derived in terms of $\bar{\nu}_c$ (moles of Species "C" bound per mole of protein).

An expression describing the rate of loss of small molecule "D" from a protein compartment containing a competitor "C," can be derived from the consideration that the rate of loss of "D" is proportional to the concentration of unbound species (D_f):

$$-\frac{d(D_t)}{dt} = K_e(D_f) \quad \text{(Eq. 9)}$$

From mass balance:

$$(D_t) = (D_b) + (D_f), \quad \text{(Eq. 10)}$$

where (D_b) is the concentration of "D" which is bound. Further, in the presence of competitor, it can be shown that,

$$(D_b) = \frac{n_1K_{d1}(D_f)(P_t)}{1 + K_{d1}(D_f) + K_{c1}(C_f)} + \frac{n_2K_{d2}(D_f)(P_t)}{1 + K_{d2}(D_f) + K_{c2}(C_f)} \quad \text{(Eq. 11)}$$

Combining Eqs. 10 and 11 and solving for (D_f) an expression for (D_f) is obtained which may then be substituted into Eq. 9 to yield:

$$\frac{-d(D_t)}{dt} = \frac{K_e(D_t)}{1 + [n_1K_{d1}(P_t)]/[1 + K_{d1}(D_f) + K_{c1}(C_f)] + [n_2K_{d2}(P_t)]/[1 + K_{d2}(D_f) + K_{c2}(C_f)]} \quad \text{(Eq. 12)}$$

The intuitive expectation that the presence of a competitor will result in a faster decline of (D_t) is verified by inspection of Eq. 12, which predicts that the rate of loss of (D_t) will increase with increasing (C_f). Further, the equation indicates that as (D_f) and (C_f) approach zero, the slope of a semilog plot of (D_t) *versus* time will approach a constant value which is a function of K_e, n, (P_t), and the association constants (K_d, K_c) for the given system. Finally, Eq. 12 shows that if the values of K_{d1} (D_f), and K_{d2} (D_f) are significantly smaller than the values of K_{c1} (C_f), and K_{c2} (C_f), then the slope of a semilog plot of (D_t) *versus* time will be constant, if (C_f) is constant.

A series of studies were conducted to evaluate dynamic dialysis as a tool for studying the phenomenon of competitive inhibition of protein binding. The studies were carried out by using 8-chlorotheophylline and salicylic acid as the small molecules "C" competing with 8-nitrotheophylline "D" for binding sites on the BSA molecule.

As a prerequisite to these studies it was necessary to determine if the presence of "C" exerted an effect on the rate of dialysis of "D," in the absence of BSA. Thus, a control run with 8-nitrotheophylline was first conducted, and then using the same membrane sac, the dialysis of 8-nitrotheophylline was followed in the presence of competitor, with the competitive concentration either maintained constant or allowed to decline. The loss of both "D" and "C," from inside the sac, was followed in the latter case.

The results of such studies where caffeine was employed as the inhibitor are shown in Fig. 12. Here the initial caffeine concentration was approximately eight times that of 8-nitrotheophylline. Only a slight reduction in the permeation rate of the caffeine caused by the presence of 8-nitrotheophylline was noted. However, the dialytic rate of 8-nitrotheophylline was significantly reduced by the presence of caffeine. In addition, the semilog plot for such a system was not linear. A reasonable explanation for the observed effect is that complex formation occurred between 8-nitrotheophylline and caffeine and that the complex diffused through the membrane at a slower rate than did 8-nitrotheophylline alone.

The effect of a constant 8-chlorotheophylline concentration on the dialysis of 8-nitrotheophylline was also measured and the results are shown in Fig. 13. It is apparent that the presence of 8-chlorotheophylline resulted in a decrease in the rate of loss of 8-nitrotheophylline.

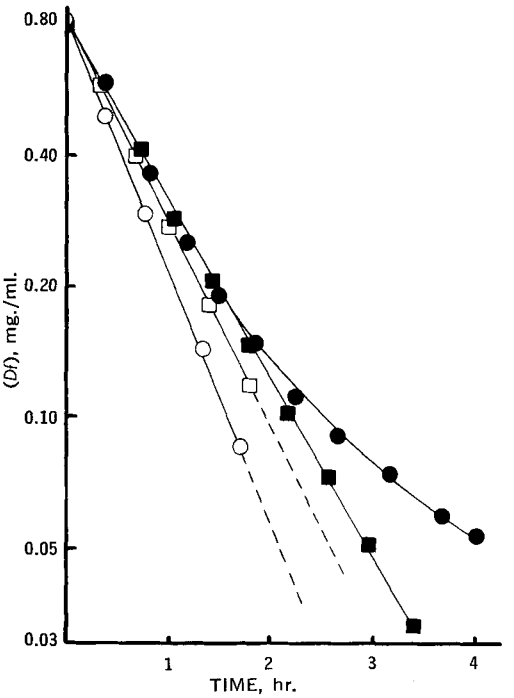

Figure 21—*The loss of 8-nitrotheophylline from inside a dialysis sac, in the presence and absence of BSA, and BSA and 9.8 mg./ml. salicylic acid. The studies were conducted at pH 7.0 and 25°. Key: ○, 8-nitrotheophylline control; □, 8-nitrotheophylline control in the presence of 9.8 mg./ml. salicylic acid; ●, 8-nitrotheophylline and 0.802% BSA; ■, 8-nitrotheophylline, 0.802% BSA, and 9.8 mg./ml. salicylic acid.*

Finally, the influence of the presence of salicylate on the dialytic behavior of 8-nitrotheophylline was studied using a constant salicylate concentration. The results are illustrated in Fig. 14 and demonstrate the previously observed effect; the permeation rate of the 8-nitrotheophylline was decreased in the presence of salicylate ion.

This unexpected effect is currently being rigorously investigated. Preliminary results indicate that complex formation is indeed

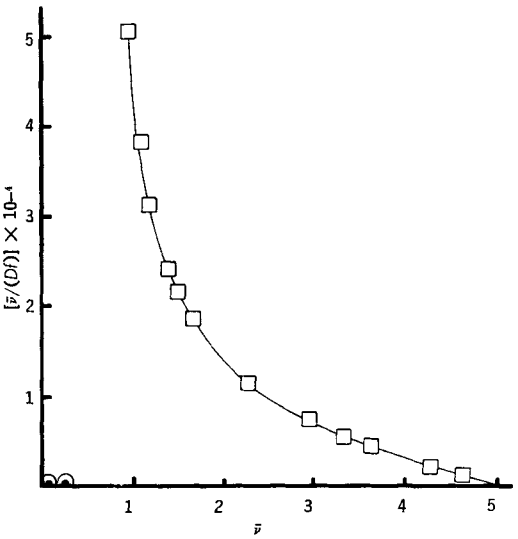

Figure 22—*The binding of 8-nitrotheophylline by BSA in the presence and absence of a constant salicylic acid concentration of 9.8 mg./ml. The studies were conducted at pH 7.0 and 25°. Key: □, 8-nitrotheophylline and BSA; ○, 8-nitrotheophylline, BSA and 9.8 mg./ml. salicylic acid; ●, theoretical data for the binding of 8-nitrotheophylline by BSA in the presence of 9.8 mg./mg. salicylic acid.*

responsible for the observed behavior. Studies are in progress to evaluate the possibility that dynamic dialysis might be of utility in investigating and quantitating small molecule-small molecule interactions as well as small molecule-macromolecule binding.

Relative to protein binding studies, these results emphasize the importance of considering small molecule-small molecule interactions before attempting to study the phenomenon of competitive inhibition. Thus, if the degree of interaction between "*D*" and "*C*" is moderately large, results of competitive binding studies might well be uninterpretable since one does not know *a priori* whether inhibition is due to competition for binding sites or the inability of "*D:C*" complexes to be bound by the protein. In the present studies, for example, the interaction between 8-nitrotheophylline and caffeine was sufficiently large to preclude a meaningful study of the inhibition of 8-nitrotheophylline binding by caffeine. The ability of 8-chlorotheophylline and salicylate to inhibit the binding of 8-nitrotheophylline could, however, be studied since in the former case relatively low concentrations could be employed and in the latter, the apparent degree of complexation was relatively low.

The binding of 8-nitrotheophylline by BSA in the presence of a variable concentration of 8-chlorotheophylline was studied at pH 7.0 and 25° and the results are depicted by Fig. 15. Figure 16 summarizes results which characterize the dialysis of 8-chlorotheophylline from the competitive system. It should be noted that preliminary experiments demonstrated that 8-chlorotheophylline, in the concentration used in this study, did not significantly affect the intrinsic rate of 8-nitrotheophylline dialysis. Figure 15 clearly illustrates the displacing effect caused by competition and which resulted in an increase in the rate of loss of 8-nitrotheophylline from the protein compartment. The data of Figs. 15 and 16 were analyzed in the manner previously described to obtain the concentrations of unbound 8-nitrotheophylline and 8-chlorotheophylline at various times during the course of the dialysis. Then, knowing unbound and total concentrations, the bound concentrations could be calculated. This permitted subsequent computations of $\bar{\nu}_c/(C_f)$, $\bar{\nu}_d/(D_f)$, $\bar{\nu}_c$, and $\bar{\nu}_d$. The results of these computations are illustrated in Figs. 17 and 18 in the form of Scatchard plots. The competitive effect is clearly evident. In addition the plots suggest that both of the theophylline derivatives were bound to the same sites on the protein since the ordinate intercept, which is equal to Σn, appears to be the same for all systems.

Also depicted in Figs. 17 and 18 are points obtained by independent considerations of competitive binding. Here, Eq. 8 was utilized to solve for the concentration of bound species by using n and K values determined from independent studies and experimental values of (D_f) and (C_f). The concentration of protein used corresponded to that in the experimental systems. Thus, theoretical points for a Scatchard plot were generated and are represented by the closed circles in the figures. The agreement of theory and experiment is reasonable, particularly in view of the fact that small variations in the values of the four association constants can have rather marked influences on the shape and position of a theoretically generated plot.

Figure 19 compares results obtained in the dialysis of various 8-nitrotheophylline systems with and without a constant concentration of 8-chlorotheophylline. The constancy of the latter was ensured by formulating internal solution, external solution, and replacement solution to contain the same concentration of 8-chlorotheophylline. It is apparent from the figure that the rate of dialysis of 8-nitrotheophylline was markedly increased by incorporation of 8-chlorotheophylline in the system. In addition, it can be observed that for the competitive study, the semilog plot appeared essentially constant after about 1 hr. which is indicative of the binding of a constant fraction of the 8-nitrotheophylline. Figure 20 illustrates the Scatchard plot corresponding to this system. The permeability constant utilized in analyzing the kinetic data to obtain the Scatchard plot was obtained from the study of 8-nitrotheophylline dialysis in the presence of 0.763 mg./ml. of 8-chlorotheophylline. The competitive effect is dramatically reflected by this figure. Theoretical points are also shown in Fig. 20. These were calculated in the manner previously described using the assumption that here (C_f) could be approximated by the analytical concentration of competitor in the system. The agreement between theoretical and experimental data is excellent and serves to confirm the utility of the dynamic dialysis method in studying competitive binding.

The results of similar studies, employing salicylate as a competing species, are depicted in Figs. 21 and 22. These similarly demonstrate the kinetic manifestations of competitive inhibition and the ability to quantitate competitive binding behavior by the dynamic dialysis approach.

In summary, this investigation has demonstrated the utility of the dynamic dialysis approach by application to a variety of protein-binding systems. The method was shown to be applicable to the quantitation of binding covering a spectrum of binding strengths. In addition it was demonstrated that competitive inhibition of binding can be readily detected and quantitatively evaluated by the technique. Some idea of the relative rapidity and convenience of the method was evidenced by the ability to comprehensively study the influence of pH, over a wide range, on the interaction of 8-nitrotheophylline with BSA. Although the present study was limited to protein-containing systems, there is no reason why the method could not be used in studies of binding by other natural macromolecules, synthetic polymers, adsorbents, and micelle-forming materials.

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