Novel Method for Studying Protein Binding

Sir:

The binding of drugs to proteins has been the subject of numerous investigations and many experimental techniques have been described for determining the fraction of total drug concentration which is unbound and/or bound in a protein-small molecule system (1, 2). Equilibrium dialysis and ultrafiltration are widely used methods and have general applicability in protein binding studies. Both methods are, however, somewhat tedious, require a considerable number of individual measurements to define binding behavior, and are rather inconvenient to use over a wide range of temperatures. In addition, equilibrium dialytic studies require a rather long equilibration time and the ultrafiltration approach can suffer from the approximations necessary to assess the changing protein concentration during the course of filtration.

We wish to report details of a rather simple method for studying the protein binding of small molecules which is based on the determination of the rate of dialysis of a small molecule from a protein-containing compartment. The method differs from other dynamic dialytic approaches suggested, for example, by Stein (3) and Agren and Elofsson (4) in that the time course of disappearance of small molecule from a protein compartment is followed for extended periods of time. In our system, the relative concentrations of bound and unbound small molecule change continuously and, as a result, data which are obtained from a single kinetic run permit characterization of binding behavior over a wide range of small molecule concentrations. The method is based on the fact that nondiffusible protein-small molecule complexes are reversibly formed in the protein compartment and that the rate of loss of small molecule from that compartment is directly proportional to the concentration of unbound small molecule, provided that care is taken to insure that sink conditions are maintained for the diffusing species, *i.e.* that back diffusion into the protein compartment is insignificant.

The experimental system consisted of a jacketed beaker (internal height = 9 cm., internal diameter = 6.5 cm.) in combination with a constant-temperature water bath and circulator which provided excellent temperature stability. A large three-hole rubber stopper was obtained to fit the beaker. One hole accommodated a short piece of glass tubing (i.d. = 1 cm.). Two-hundred milliliters of buffer solution was placed in the beaker. Hydrated cellophane dialysis tubing [width = 2.47 cm. (0.984 in.)] was knotted at one end to form a bag (length = 7 cm.) and was attached with a rubber band to the glass tubing of the stopper. Seven milliliters of small molecule or small molecule-protein solution was placed in the bag. The stopper with the attached bag was fitted on the beaker. The solution external to the bag was stirred with a magnetic stirrer. Care was taken to insure that there was sufficient clearance between the bag and the bottom of the beaker to allow free motion of the stirring bar. The solution within the bag was stirred with a twisted glass rod which passed through the glass tubing and was connected to a variable speed stirring motor. All experiments were conducted at constant temperature, pH, liquid volume, bag size, and stirring rate. One-hundred milliliters of the solution external to the bag was periodically removed through one hole of the stopper and immediately replaced with 100 ml. of fresh buffer which was introduced through the other hole. In this manner the concentration of small molecule in the external solution was always maintained at a level sufficiently lower than that in the protein compartment to approximate sink conditions. The concentration of small molecule in the removed sample was determined spectrophotometrically and the concentration of small molecule in the protein compartment was calculated from a knowledge of the initial concentration and the total amount of small molecule which had appeared in the external solution.

Typical results are shown in the form of semi-log plots in Fig. 1 and depict experiments in which phenol red was employed as the small molecule and bovine serum albumin (BSA) was the macromolecule. In the absence of protein, the dialytic behavior obeyed the expected rate law:

$$\frac{-d(Dt)}{dT} = K(Df) \qquad \text{(Eq. 1)}$$

where $[-d(Dt)/dT]$ = rate of loss of small molecule from the dialysis sac. K = first-order rate constant which characterizes the diffusion

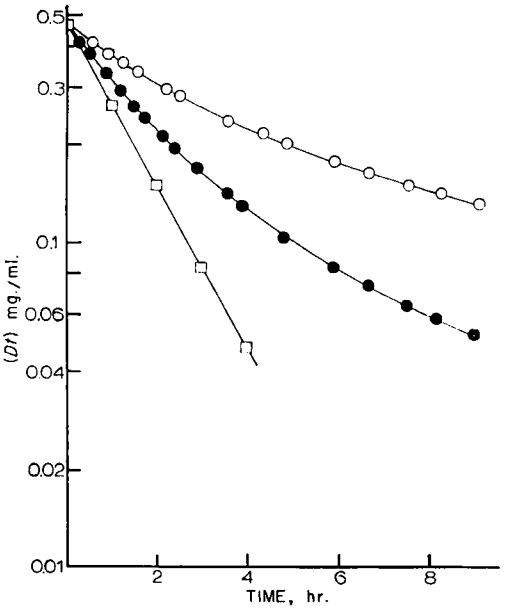

Fig. 1—The loss of phenol red from inside a dialysis bag, in the presence and absence of BSA. The studies were run at pH 7.3 and 25°. Key: ○, 1.442% BSA; ●, 0.618% BSA; □, 0% BSA.

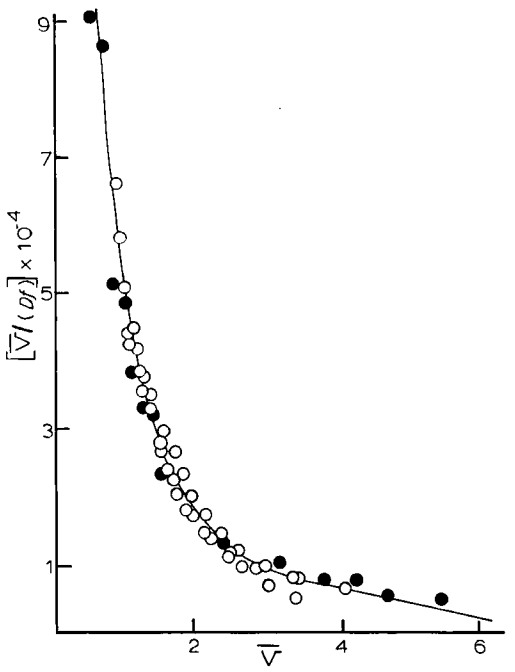

Fig. 2—The binding of phenol red by BSA at pH 7.3 and 25°. Key: ●, ultrafiltration data; ○, kinetic data.

process and which incorporates the area and thickness of the membrane. (Df) = concentration of unbound small molecule in the dialysis sac.

In the presence of protein, it is seen that the semilog plots were not linear but exhibited marked curvature. The curvature reflects the fact that as the concentration of small molecule in the protein compartment decreased, the fraction of small molecule which was bound increased. The concentration of unbound small molecule (Df) in the protein compartment at any total drug concentration (Dt) can be calculated from Eq. 1 with a knowledge of K and the instantaneous rate $[-d(Dt)/dT]$ at that particular (Dt). The value for K was obtained from the slope of the semilog plot of (Dt) *versus* time in the absence of macromolecule. The instantaneous rate at a value of (Dt) in the presence of macromolecule can be estimated graphically from a plot of (Dt) *versus* time. We, however, found it more convenient and accurate to fit data from plots of (Dt) *versus* time to an empirical equation with the aid of a digital computer (a six-parameter triexponential equation has been found to yield excellent fits with all systems studied thus far). The differential of the empirical equation was then calculated to obtain instantaneous rates at various values of (Dt) from which (Df) values were obtained. These two values permitted computation of the concentration of bound small molecule. The binding data obtained in this manner were used to construct the Scatchard plot of Fig. 2 where $(\bar{v}/Df)$ *versus* $\bar{v}$ was plotted ($\bar{v}$ = moles of bound small molecule per mole of protein). The open circles represent data points obtained from the dynamic dialysis experiments and the closed circles those obtained from ultrafiltration studies.

Table I—Summary of Binding Constants

Method	n_1[a]	n_2	k_1[b]	k_2
		Phenol Red		
Experimental				
Ultrafiltration and kinetic	1	6	1.74×10^5	1.97×10^3
Literature (5)				
Ultrafiltration	1	6	1.1×10^5	1.2×10^3
		Methyl Orange		
Experimental				
Kinetic	22	—	4.8×10^4	—
Literature (6-7)				
Equilibrium dialysis	22	—	4.4×10^4	—

[a] n_i = number of binding sites in the i'th class; [b] k_i = association constant for the i'th class.

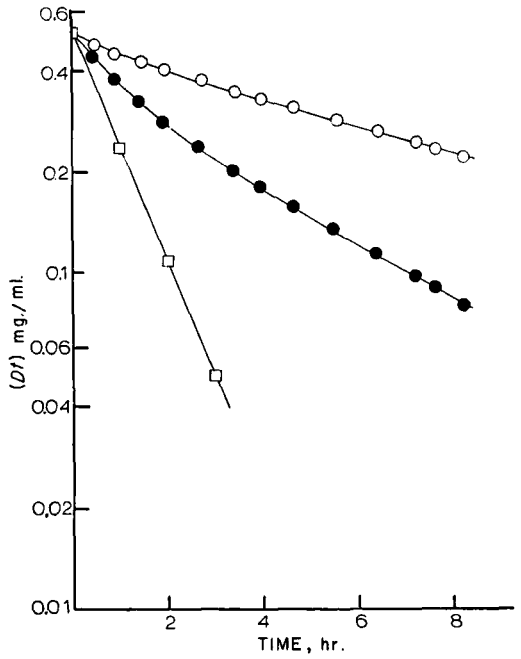

Fig. 3—The loss of methyl orange from inside a dialysis bag, in the presence and absence of BSA. The studies were run at pH 7.3 and 25°. Key: ○, 1.235% BSA; ●, 0.620% BSA; □, 0% BSA.

There is close agreement between data obtained by the two different techniques. The Scatchard plot was mathematically analyzed in terms of the fundamental binding parameters (n = number of sites in a class, k = intrinsic association constant characterizing the binding to a site) and was found to be consistent with an interaction involving two classes of sites on the protein molecule. Values for n and k are given in Table I where literature values are also shown. Figure 3 shows the dialytic behavior of methyl orange in the absence and presence of BSA. These data were analyzed in a similar manner. The binding parameters are shown in Table I and are seen to be in excellent agreement with values previously reported.

These preliminary results show the promise of this approach for studying protein binding. The relative ease and rapidity of the method, the minimum amount of sample preparation required, the convenient means of temperature control, and the economical utilization of protein are definite advantages of this approach. These factors become especially important when the supply of protein is limited or when a variety of experimental conditions such as pH or temperature are to be studied.

Studies are currently in progress to define the influences of viscosity, binding by the dialysis membrane, stirring rate, bag size, liquid volume, temperature, and other variables on rates of dialysis and the effects that these variables might have in applying this kinetic approach to the study of protein-small molecule interactions.

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Keyphrases

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Diffusion—small molecules from protein containing compartment

Apparent Discrepancy Between Theory and Experimental Data for Dissolution from the Rotating Disk Under Stirred and Unstirred Conditions

Sir:

A recent article published by Gibaldi *et al.* (1), regarding the dissolution mechanism observed using a rotating disk, did not reach the same conclusions reported in our recent article (2). Their study indicated that the diffusion layer model was operative at 100 r.p.m., but could not reach a definite conclusion regarding the mechanism operative under static conditions. It is the purpose of this communication to show that their data can be used to confirm our conclusions, that the rotating disk model rather than the diffusion layer model is operative under stirred conditions and that the Danckwerts' model is operative under static conditions.