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Kinetic Approach to Drug–Protein Binding

J. S. ROBERTSON[x] and B. W. MADSEN

Abstract □ Since estimates in the literature for the fundamental binding parameters of dicumarol to bovine serum albumin show wide disparity, these phenomena were reinvestigated, using a nonequilibrium partitioning technique. A three-phase system was used consisting of an aqueous drug–protein phase and a separate aqueous receiving phase, both in mutual contact with a third overlying immiscible organic phase. The phases were contained in a partitioned, temperature-controlled cylindrical glass cell which could be rotated along its horizontal axis to effect gentle yet thorough mixing and drug transfer. Free (unbound) drug diffused from the protein phase sequentially into the other phases where it was analyzed. This technique allowed estimates of free and bound drug to be made over a very wide range of drug–protein ratios, using a single drug–protein solution, in a short time. Data analysis using an iterative, nonlinear, least-squares computer program indicated that there were two classes of binding sites with the following characteristics; $n_1 = 2.3 \pm 0.15$, $K_1 = (1.8 \pm 0.23) \times 10^5$ M^{-1}; $n_2 = 14.0 \pm 0.71$, $K_2 = (3.0 \pm 0.36) \times 10^3$ M^{-1}. A computer-solved theoretical treatment of drug transfer within the cell showed good correlation with the experimental results. The rotating cell technique possesses a flexibility in adjustment of operational parameters which should suit it to the study of the binding characteristics of various compounds.

Keyphrases □ Dicumarol—reinvestigation of binding to bovine serum albumin, nonequilibrium partitioning technique, three-phase system □ Bovine serum albumin binding of dicumarol—reinvestigated using nonequilibrium partitioning technique, three-phase system □ Drug–protein binding—dicumarol and bovine serum albumin, kinetics determined using nonequilibrium partitioning technique □ Binding of dicumarol to bovine serum albumin—kinetics determined using nonequilibrium partitioning technique, three-phase system

Methods used to study drug–protein interactions can be grouped under four headings: subtractive, direct, electrostatic, and other (1). All methods have their advantages and disadvantages, and the selection of a particular approach depends mainly on the system under study and the aims of the experiment.

The major drawback of direct methods, where both free and bound drug concentrations are determined in the presence of protein, is that the protein can create assay problems. Therefore, most reports are based on the subtractive approach, in which free drug is separated from the protein prior to assay; by subtraction of free drug from the total quantity of drug added to the system, bound drug is obtained. Such methods include equilibrium dialysis, equilibrium partitioning, ultrafiltration, ultracentrifugation, and gel filtration. Of these, equilibrium dialysis is the most frequently used.

Equilibrium methods can involve considerable labor and cost of chemicals since they require the preparation of many drug–protein samples to cover an adequate range of binding ratios—each sample provides but one data point. There is also a time delay inherent in this approach, which may allow protein denaturation or microbial growth. Recently, there has been a growing interest in kinetic methods to circumvent some of these limitations.

Stein (2) introduced a dynamic dialysis method based on the use of an automated analyzer which reduced the experimental time from hours to minutes. He reported that the binding data of methyl orange to bovine serum albumin obtained by this technique were similar to those obtained (3) using equilibrium dialysis. While this work pioneered the kinetic approach, only one binding system was investigated over a very limited range. Although the technique eliminated prolonged waiting time, individual drug–protein solutions covering all the binding ratios still had to be prepared.

A flow dialysis technique was reported (4–6) which both reduced experimental time and enabled a number of data points to be obtained from a single drug–protein solution. The binding of a number of drugs was studied, and membrane effects were a disturbing factor.

Meyer and Guttman (7) reported preliminary work on a nonequilibrium dialytic method. They later published details of their studies on the various factors influencing the method, and they investigated the binding behavior of a number of compounds including phenol red, methyl orange, warfarin, caffeine, 8-nitrotheophylline, 8-chlorotheophylline, and salicylic acid (8, 9). In their technique, the drug and protein were placed together inside a membrane sac supported in drug-free buffer; unbound drug diffused into the buffer solution at a rate proportional to its concentration in the sac. The concentration of drug in the external buffer phase was kept at a low level by sampling at selected time intervals and replacement with fresh buffer. This method allowed rapid, easy generation of a large series of free and bound

drug concentrations, and it showed good correlation with literature reports using more standard techniques.

Farrell *et al.* (10) introduced a dynamic dialysis technique, with a mode of operation analogous to that of a hemodialysis unit. They claimed that their approach possessed the advantages (among others) of rapid evaluation of binding characteristics and ease of temperature adjustment to measure enthalpy and entropy effects. The method represents a considerable refinement over the initial approach of Stein (2), and their work included many more binding systems.

Blatt *et al.* (11) described a continuous diafiltration technique which was a blending of the better features inherent in the more orthodox methods of dialysis and ultrafiltration. Drug transfer across a semipermeable membrane was hastened by application of pressure, and the drug–protein solution volume was maintained constant with a reservoir assembly. This method was extended by sequentially assaying the eluate until equilibrium was reached (12).

The work described in this report is a further application of a nonequilibrium method—*viz.*, kinetic partitioning, to investigate a drug whose binding properties have proven troublesome to characterize by the more standard techniques.

EXPERIMENTAL

Chemicals—Dicumarol[1] (I) recrystallized from cyclohexanone gave an uncorrected melting point of 286–289° (13). TLC, using silica gel H[2] with the solvent system acetone–ethyl acetate–petroleum ether (b.p. 60–80°)–water (100:100:33:3.5 v/v), showed a single spot of R_f 0.62 when viewed under UV light (of wavelength 254 nm) or when sprayed with phosphomolybdic acid reagent (14).

Bovine serum albumin[3] (II) was used as received. The molecular weight of II was assumed to be 69,000. 2-Octanol was redistilled before use (178–180° fraction collected). All other chemicals were of laboratory reagent grade.

UV Spectrophotometry—UV determinations were carried out using a spectrophotometer[4]. The organic phase absorbance was measured at 324 nm in a 4-cm cell, and the alkaline phase absorbance was measured at 315 nm in a 1-cm cell. Frequently, the spectrum of an alkaline phase sample was scanned from 200 to 450 nm as a precaution against false absorption.

From an increasing absorptivity and change in the spectrum, it appeared that some form of association of I was taking place in the organic phase; it became significant at concentrations greater than 20 μg/ml, and care was taken that this concentration was not exceeded during a kinetic run and assay. When background absorption of either phase was troublesome, a corrected absorbance ($A_{corr} = A_\lambda - A_{360}$) was used, where A_λ was the assay wavelength for the particular phase (15, 16).

Solutions of I and II were assayed spectrophotometrically for total I. Solutions were prepared with known amounts of I ranging from 0.595 to 8.33 × 10^{-5} *M* and a constant amount of II (2.38 × 10^{-5} *M*). A standard curve was then prepared by plotting the absorbances at 325 nm. While the presence of II affected the absorption of I, a Beer's plot was approximately linear in the 0–2 molar ratio (I/II) range. By diluting the sample from the rotating cell to a concentration of 2.38 × 10^{-5} *M* for II, the concentration of I was readily found by linear interpolation.

The interaction between I and II was further investigated by difference spectroscopy (17). Changes in UV absorption at various molar ratios of I (0.595–8.33 × 10^{-5} *M*) and II (2.38 × 10^{-5} *M*) were examined at 315 and 340 nm.

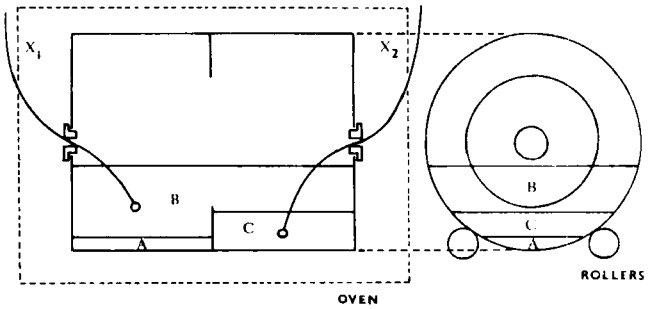


Figure 1—*Diagram of the rotating cell. Key:* A, *aqueous drug–protein phase;* B, *organic solvent phase;* C, *aqueous alkaline phase; and* X_1, X_2, *weighted nylon sampling tubes inserted into the cell* via *small holes in rubber stoppers. A motor-driven roller rotated the cell at 40 rpm; the thermostated oven temperature was maintained by an electric heating element and fan at each end.*

Computing—An analog computer[5] coupled to a recorder[6] was used to estimate rate constants. All extensive numerical calculations were performed on an automatic computer[7], using USASI FORTRAN IV[8] as the programming language.

Rotating-Cell Methods—A diagram of the rotating cell is shown in Fig. 1. The apparatus was previously described (18) except that an all-glass cell was used in the present investigation and the whole assembly was enclosed in an oven maintained at 37 ± 0.6°. All experiments using the cell were conducted at this temperature. The cell was designed with a central partition keeping separate the two aqueous phases, with the only connection between the two being *via* the overlying organic phase. The drug was initially placed in *A*, and the time course of transfer was followed by assaying phases *B* and *C*; the drug concentration in *A* was obtained by subtraction.

Phase *A* contained 0.05 *M* tromethamine adjusted to pH 7.4 (at 37°) and sufficient sodium chloride for an ionic strength of 0.15 to simulate physiological conditions. Phase *B* consisted of a mixture of cyclohexane–2-octanol (1:1 v/v). This ratio was based on the necessity for *B* to be immiscible, to be not too volatile, to be cheap, to present minimal problems in assay, and to ensure sufficient polarity for rapid transfer of I. Phase *C* was always 0.1 *N* NaOH, giving an alkaline to organic distribution ratio of 365:1. During an experiment, sample removal prevented any large accumulation of I in alkali, so that back-transfer from phase *C* to *B* could be considered negligible. The volumes of phases *A*, *B*, and *C* were 25, 360, and 125 ml, respectively. The choice of volumes for phases *A* and *B* was dictated by the wish to obtain a high *B* to *A* phase volume ratio. This ratio determined the net rate of transfer of I from phase *A* to *B* and thus the experimental time. The one all-glass rotating cell was used in all work to reduce variability from this source.

Preparation of Phases—Two liters of 0.1 *N* NaOH was shaken with 100 ml of organic solvent, sealed, and allowed to equilibrate in the oven (at 37°) overnight prior to a run.

Similarly with the buffer, 50 ml was shaken in a separating flask with 10 ml of organic solvent and allowed to equilibrate overnight. Presaturation of contiguous phases had an important bearing on the interphase rate constants. In addition, presaturation of the alkali phase was important from an assay viewpoint. 2-Octanol absorbs at λ = 280 nm and so interfered with the assay for I (at 315 nm). When alkali was presaturated with organic phase, the 2-octanol absorbance amounted to approximately 0.04 at 315 nm in 1-cm cells. By presaturating phases and using a blank, a correction was unnecessary.

Steinhardt and Reynolds (1) previously commented that the presence or absence of protein at the membrane interface in equi-

[1] Sigma Chemical Co., St. Louis, Mo.
[2] E. Merck, A-G, Darmstadt, Germany.
[3] Fraction V, lot 110C-0300, Sigma Chemical Co.
[4] Unicam SP800, Unicam Instruments Ltd., Cambridge, England.
[5] Solartron Electronic Group Ltd., Surrey, England.
[6] Model 2D2 X-Y, F. L. Mosley Co., Pasadena, Calif.
[7] 32K IBM 7040.
[8] A listing of the programs is available on request.

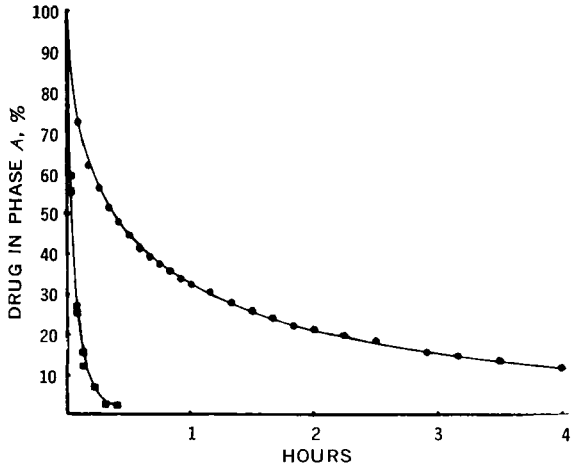


Figure 2—*Loss of dicumarol from the aqueous buffer phase with* (●) *and without* (■) *bovine serum albumin.*

librium dialysis could affect the transfer rate of free drug. This type of phenomenon was observed for the transfer of I between phases in the rotating cell. Preequilibration of phase *B* with 25 ml of protein solution depressed the two forward rate constants slightly, and this effect seemed to be independent of II concentration from 0.1 to 4% (w/v). Therefore, in cases where no protein was added to the aqueous phase, *e.g.*, for rate constant determination, the organic layer was shaken with the corresponding protein solution.

This organic layer was periodically checked to determine if extraction of any UV-absorbing substances (which might interfere with the assay) had occurred from the protein solutions. When the aqueous phase was at pH 7.4, no such substances were detected.

Inadequate cleaning of the cell reduced the rate of loss of drug from phase *A*, possibly due to an adsorption phenomenon. When the cell and all glassware were cleaned with chromic acid, this was not a problem. Prior to an experiment, adequate stock solutions of each phase were placed in the oven and time was allowed for temperature equilibration.

Preparation of the Cell—A volume of 125 ml of preequilibrated alkali was added to the right side of the cell; then 340 ml of organic solvent was gently added from the left side. The cell was plugged to prevent evaporation of any solvent, carefully placed on the rollers in the oven to avoid spillage, and rotated for 1 hr. The rotation of the glass cell was periodically checked and found to be constant at 40 rpm. Twenty-five milliliters of phase *C* was removed and replaced with fresh solvent. This sample served as a blank. The cell was then rotated for another hour to ensure complete temperature equilibration of the cell and contents.

Compound I, in separate amounts of 2.1, 6.07, 11.24, and 22.0 mg, was weighed directly into a standard 25-ml flask to prevent loss. The dissolution of crystalline I at physiological pH was very time consuming and it was better first to add a few drops of 1 *N* NaOH for this step. To avoid any slight pH change in the buffer, the appropriate quantity of 0.2 *M* HCl was added later. If protein was to be present, an amount sufficient to produce 1% (w/v) was inserted and the contents were made up to volume with buffer. The pH of the final solution was checked frequently at this stage and was 7.8 at room temperature (20°). The buffer capacity was adequate to maintain constancy with up to 1% (w/v) protein. This solution was then given a minimum of 1 hr to heat to 37°.

The two nylon sampling tubes connected to the exterior of the oven were then inserted in the cell *via* the narrow holes in the stoppers. Subsequently, all additions and withdrawals were made *via* these tubes to minimize heat loss. Absorption of drug and protein by the nylon tubes was tested for and found to be unimportant under the prevailing conditions of agitation.

Commencement of Transfer—When ready, the rotation was stopped, drug solution was pipeted in, and the standard flask and pipet were washed with a final 20 ml of organic solvent required to make up the 360-ml organic phase. Then the rotor was switched on and timing was begun. This injection phase was dead time and was done as quickly as possible, commonly taking about 1 min.

If phase *A* contained I alone, typical sampling times were 2, 5, 9, 14, 20, and 25 min; if protein was present, usually 30–40 samples were taken at frequent intervals for up to 6 hr. The sequence followed was removal first of organic phase and then of alkali and then replacement in the reverse order, usually taking about 2 min. The sample volumes were 10 ml for phase *B* and 25 ml for phase *C*. The interval between replacement of fresh solvent and the withdrawal of the next sample, if greater than 1–2 min, was adequate to ensure homogeneity of the phases.

At the end of a run, the total content of the cell (510 ml) was measured to guard against cumulative error in sampling and replacement and the possibility that a sample had not been replaced. The pH of the drug–protein phase was checked to ascertain whether accidental spillage of alkali into the buffer phase could have occurred. This solution was then assayed spectrophotometrically for total I.

The possibility of denaturation of the protein having occurred due to intimate contact with the organic phase was examined by testing for changes in the UV spectrum and in viscosity (1), using a viscometer[9] (19). No changes were observed.

In a further experiment, a solution containing I (10.7 mg) and II was incubated at 37° for 11 hr prior to a transfer experiment.

The pKa_2 of I was estimated by a solubility method (20). A partitioning experiment was designed between the buffer and organic phases at 37° in the pH range 6.54–8.62. Both phases were assayed spectrophotometrically, and the concentration data were then rearranged according to an equation given by Martin (21):

$$\frac{K_a + [H_3O^+]}{C_w} = \frac{K + 1}{C}[H_3O^+] + \frac{K_a}{C} \qquad \text{(Eq. 1)}$$

where K_a = the dissociation constant, C_w = concentration of I in the aqueous phase, C = sum of the concentrations of I in each phase, and K = true distribution coefficient.

RESULTS AND DISCUSSION

The loss of I from phase *A*, both with and without II, is shown in Fig. 2. A model of the transfer process in the absence of protein is shown in Scheme I. The kinetics of such a scheme can be described by the following differential equations:

$$dA/dt = -k_5A + k_6B \qquad \text{(Eq. 2)}$$

$$dB/dt = k_5A - k_6B - k_7B \qquad \text{(Eq. 3)}$$

$$dC/dt = k_7B \qquad \text{(Eq. 4)}$$

Evaluation of the rate constants by analog computer gave the means and standard deviations as: $k_5 = 0.324 \pm 0.009$ min^{-1}, $k_6 = 0.027 \pm 0.003$ min^{-1}, and $k_7 = 0.054 \pm 0.001$ min^{-1}.

$$\boxed{A} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} \boxed{B} \xrightarrow{k_7} \boxed{C}$$

Scheme I

Calculations with Protein Present—From the initial weighing and the terminal assay for I in the buffer phase of the rotating cell, the original (A_0) and final (A_{final}) values for total drug in phase *A* were known. Between these two values, there was a whole series of values (A_i) which could be calculated as follows.

At the first sampling interval, the amount of drug in phase *A* was simply the original minus the quantities in phases *B* and *C*:

$$A_1 = A_0 - (B_1 + C_1) \qquad \text{(Eq. 5)}$$

Estimation of A_2 was similar, except that allowance had to be made for drug lost from the system by sampling:

$$A_2 = A_0 - \left(\frac{B_1}{36} + \frac{C_1}{5}\right) - (B_2 + C_2) \qquad \text{(Eq. 6)}$$

In general, if R_i equaled the total drug remaining in the cell at

[9] Ostwald.

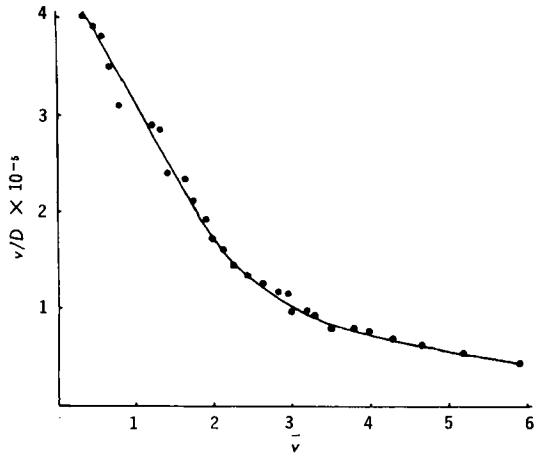


Figure 3—*Scatchard plot of I–II interaction obtained from a single experiment. The small scatter of the points is indicative of the precision of the technique.*

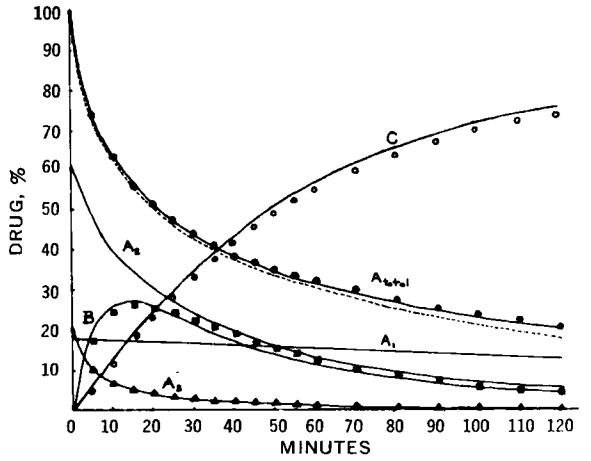


Figure 4—*Comparison of observed and predicted drug levels changing with time for each compartment shown in Scheme II. The solid curves were obtained by solution of the differential Eqs. 17–20, while the points are experimenal. The dotted line is the predicted* A_{total} *obtained by altering* k_7 *(Scheme II) by +15%.*

any time, then since $R_0 = A_0$:

$$R_{i+1} = R_i - \left(\frac{B_i}{36} + \frac{C_i}{5}\right) \qquad \text{(Eq. 7)}$$

and:

$$A_i = R_i - (B_i + C_i) \qquad \text{(Eq. 8)}$$

The series of values (A_i) so calculated represented the total amount of drug (free plus bound) in A at each of the sampling times (t_i) based on the original weight A_0.

From Eq. 7, it was evident that the error in R_i was cumulative. If the error in R_2 was $\pm S_2$, comprised of errors in timing, assay, and model assumptions, the error in R_3 was augmented by that in R_2 and so on. Hence, the probable error in A_i also increased in the series, and the calculated amount at any time A_i could have varied by $\pm\Sigma|S_i|$, with the largest error occurring in A_{final}. Furthermore, since A_i decreased with time, the absolute error increased and the relative error increased even more. To minimize this effect, the values for A_i were calculated on the basis of the $A_{final,\ assay}$, which, being obtained directly, was subject only to assay error. The largest correction required was when A_0 = 22 mg and samples were taken at 37 time intervals. In this case, $A_{final,\ predicted}$ = 0.78 mg and $A_{final,assay}$ = 0.64 mg, which gave a relative error of 22%. When $A_{final,\ assay}$ was used to determine A_0, then $A_{0,\ predicted}$ = 21.86 mg, which meant a maximum probable error of 0.6% for the A_i series.

In the presence of protein, the total amount of drug in phase A (A_{total}) was made up of both bound (A_{bound}) and free (A_{free}) species.

Rearranging Eq. 2 gave Eq. 9:

$$A_{free} = \frac{k_6 B - dA_{total}/dt}{k_5} \qquad \text{(Eq. 9)}$$

The value of the expression dA_{total}/dt at each sampling time was obtained from Eq. 10 for closely spaced intervals (Fig. 2):

$$\left(\frac{dA_{total}}{dt}\right)_{i+1} \cong \frac{(A_{i+2} - A_i)}{(t_{i+2} - t_i)} \qquad \text{(Eq. 10)}$$

Substitution in Eq. 9 then gave a series of A_{free} values for the various sampling times, and the bound drug was found by subtraction:

$$A_{i(bound)} = A_{i(total)} - A_{i(free)} \qquad \text{(Eq. 11)}$$

$A_{i(free)}$ and $A_{i(bound)}$ were converted to the molar quantities D_f, $\bar{v}$, and $\bar{v}/D_f$ to allow construction of a Scatchard plot, where D_f = molar free drug concentration and $\bar{v}$ = ratio of moles of drug bound per total moles of protein. From such a Scatchard plot (Fig. 3), estimates for the binding constants were obtained to initiate the nonlinear regression computer program.

The expression used to calculate $\bar{v}$ was:

$$\bar{v} = \sum_i^N \frac{n_i K_i D_f}{1 + K_i D_f} \qquad \text{(Eq. 12)}$$

where N = number of classes of binding sites, n_i = number of binding sites in the ith class, and K_i = association constant for the ith class.

A least-squares approach, similar to that of Fletcher and Spector (22), was adopted to obtain best fit, regressing $\bar{v}$ onto D_f and minimizing the sum of squares of the deviations R, where:

$$R = \sum_i^n \omega_i(\bar{v}_{calc} - \bar{v}_{obs})^2 \qquad \text{(Eq. 13)}$$

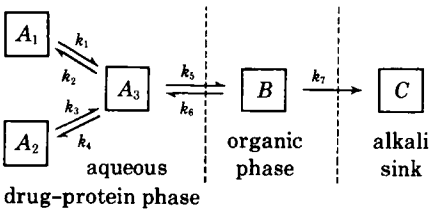
and n = number of data points, ω_i = weighting term for the ith data point, $\bar{v}_{calc}$ = drug bound per total moles of protein calculated by Eq. 12 from observed values of D_f, and $\bar{v}_{obs}$ = observed drug bound per total moles of protein. After preliminary trials, ω_i was assigned a value of unity and the function was minimized[10].

The results of these computations on pooled data (130 data points) from four experiments indicated that there were two classes of binding sites for I, with n_1 = 2.3 ± 0.15[11], K_1 = (1.8 ± 0.23) × 10^5 M^{-1}, n_2 = 14.0 ± 0.71, and K_2 = (3.0 ± 0.36) × 10^3 M^{-1}. Due to the relatively small difference between the values of n_1K_1 and n_2K_2, a minimum of 20–30 samples for each experiment was found necessary to allow estimation of the parameters with an acceptable standard deviation. For comparison, a previous report (24) of these parameters, obtained using equilibrium dialysis, gave n_1 = 3.8 ± 0.6 and K_1 = (7 ± 1) × 10^5 M^{-1}, the values being estimated by eye; it was assumed that contribution from the weaker class of sites would not interfere with estimates of the first class.

Theoretical Treatment—From the foregoing, Scheme II was taken to represent the transfer of I within the rotating cell when II was present. To generate theoretical data on the distribution of drug with time, initial levels for each compartment shown in Scheme II had to be calculated. The amount of drug originally placed in the buffer phase (11.24 mg = $A_1 + A_2 + A_3$) was known, and Eq. 12 relates A_1 to A_3 for i = 1 and A_2 to A_3 for i = 2. These three equations were solved iteratively giving values of 2.441 × 10^{-4}, 8.214 × 10^{-4}, and 2.745 × 10^{-4} M for A_1, A_2, and A_3, respectively. Phases B and C were zero.

[10] Using FUNMIN, a library subprogram, Basser Computing Centre, Sydney University, which uses the Simplex method of Nelder and Mead (23).

[11] Best estimate ± standard deviation.



Scheme II—Representation of drug transfer in the cell. Compartment A_1 *represents drug bound on the first class of sites on II, and* A_2 *represents drug bound on the weaker second class. Both of these compartments and the organic phase* (B) *equilibrate with compartment* A_3*, which is the free drug in the buffer phase.*

Differential equations describing drug transfer in Scheme II were derived by first considering the equilibration of Compartments A_1 and A_3. From Eq. 12 for one class of sites:

$$\frac{A_1}{P_t} = \frac{n_1 K_1 A_3}{1 + K_1 A_3} \qquad \text{(Eq. 14)}$$

where P_t = molar protein concentration.

Rearranging Eq. 14:

$$A_1 = K_1 A_3 (n_1 P_t - A_1) \qquad \text{(Eq. 15)}$$

therefore:

$$\frac{A_1}{A_3(n_1 P_t - A_1)} = K_1 = \frac{k_2}{k_1} \qquad \text{(Eq. 16)}$$

so the forward-transfer rate was given by $k_1 A_1$ and the back-transfer rate was given by $k_2 A_3 (n_1 P_t - A_1)$. At equilibrium, these two rates would be equal, as implied by Eq. 16. For nonequilibrium cases, the net rate of change was found from the difference in these rates:

$$\frac{dA_1}{dt} = k_2 A_3 (n_1 P_t - A_1) - k_1 A_1 \qquad \text{(Eq. 17)}$$

The other equations were derived similarly and are:

$$\frac{dA_2}{dt} = k_4 A_3 (n_2 P_t - A_2) - k_3 A_2 \qquad \text{(Eq. 18)}$$

$$\frac{dA_3}{dt} = k_1 A_1 + k_3 A_2 + k_6 B - k_5 A_3 - k_2 A_3 (n_1 P_t - A_1) - k_4 A_3 (n_2 P_t - A_2) \qquad \text{(Eq. 19)}$$

$$\frac{dB}{dt} = k_5 A_3 - k_6 B - k_7 B \qquad \text{(Eq. 20)}$$

A problem arose in assigning values to k_1 and k_3. Individual rate constants for drug-protein interactions are not known with any accuracy. Froese *et al.* (25) reported a dissociation rate constant of about 1 sec^{-1} for an analogous system. A digital computer solution for the system shown in Scheme II, with k_1 and k_2 having such large absolute values as 1 sec^{-1} and 1×10^6 sec^{-1}, respectively, would have been very slow, costly, and associated with a large degree of error. Provided the assumed value of k_1 did not make it the rate-limiting step, its absolute value rapidly became of diminishing importance in regard to total transfer. Accordingly a value of 0.01 sec^{-1} was assumed for k_1 and k_3 as a computing compromise. When this value was doubled, the maximum deviation between predicted levels for the different compartments at the various experimental times shown was less than 2%.

The values of k_2 and k_4 were calculated from the derived association constants, while the values of k_5, k_6, and k_7 were known. The system of Eqs. 17–20 was solved by computer[12]. The results are shown in Fig. 4. The dotted line in this figure was also computer generated from the equations but with an "error" of +15% in the value of k_7, the rate-limiting step. The resulting deviation from the experimental data was an indication that the proposed model was realistic, being sensitive to an error of this magnitude.

[12] Using DHPCG, IBM System/360 Scientific Subroutine Package. This *subroutine required* rate constants and initial compartmental values for input and computed the levels in each compartment at appropriate time intervals.

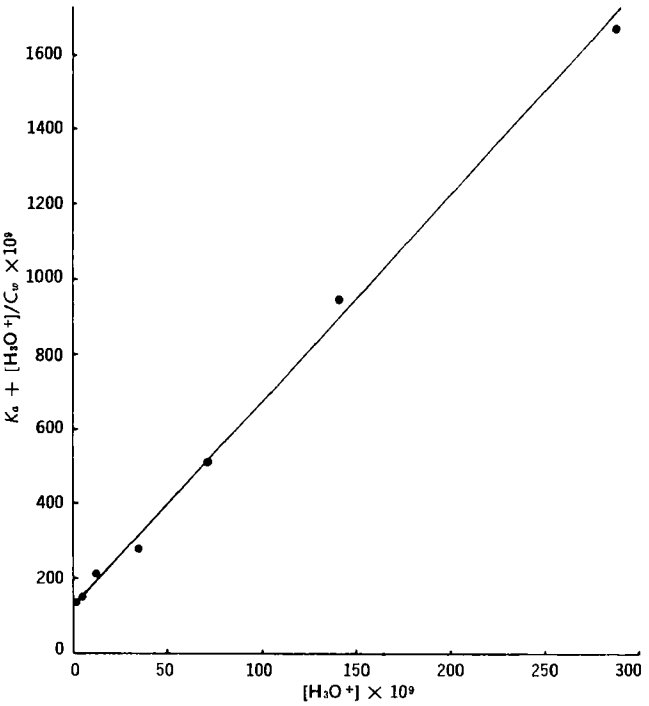


Figure 5—*Partitioning of I between the organic and buffer phases at 37° in the pH range 6.54–8.62.*

Chignell and Starkweather (26) reported that prolonged incubation together increased the affinity of human serum albumin for I, particularly in the strongly bound sites. This phenomenon was investigated for the I-II system, but no obvious difference in binding behavior was observed. In this report (26) the data for the control (*i.e.*, no incubation) were taken from a literature source.

The solubility method for determination of pKa was selected because it was felt that data obtained in solvent mixtures such as 95% ethanol (27) and dimethylformamide-water (28) should not be extrapolated to purely aqueous systems (20, 29). The apparent pKa_2 of I at 37° in the described buffer was estimated to be 6.9. The very low solubility of the neutral molecule [0.5 mg/liter (28)] made estimation of pKa_1 vague, but it would seem to be at least 1.5 units below this figure. Therefore, at pH 7.4 most of I existed as the diionized salt with somewhat less in the monoionized form and a very low concentration of nonionized species. To account for the observed high transfer rate of I from buffer to organic phase, it seemed necessary to assume that the species in the organic phase was the monoionized form. This hypothesis was supported by the two-phase partitioning experiment (Fig. 5) with K_a in Eq. 1 given a value of 6.9. On the basis of this graph, it would not be necessary to postulate any species other than the diionized and monoionized forms for transfer between phases *A* and *B*.

A marked change in spectral properties at a molar ratio of 1.5 (I/II) was noted in the 340-nm region but not at 315 nm (Fig. 6) as reported previously (17). Whatever the reasons for this change, it ought not to be assumed to reflect a change in binding strength (1).

The rotating cell technique would seem a suitable method for the study of drug-protein binding. In common with other dynamic dialysis methods, it has the advantages over equilibrium techniques of a saving in time and materials since only one drug-protein solution need be prepared to produce data for the whole range of binding ratios. There is no need to make estimates or assumptions regarding drug-membrane binding, because the "membrane" is assayed. Also, the properties of the organic phase can be more accurately controlled than can those of dialysis tubing. Compared with other kinetic techniques, the apparatus is relatively simple, with the advantage of readily adjustable pa-

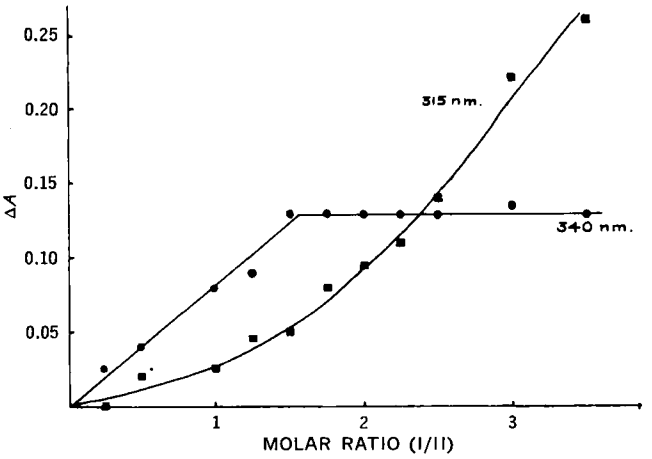


Figure 6—*Differential absorption of the I–II interaction, with the sharp discontinuity at a molar ratio of 1.5.*

rameters. The polarity of the organic phase can be altered to give the desired drug transfer rate, as can the phase volume ratios, protein concentration, and sink conditions. The latter parameter can be altered to minimize back transfer of drug instead of assuming that keeping the external phase drug concentration below 10% of the internal free drug concentration achieves this objective (8). The organic phase has a further advantage in allowing substances to pass on the basis of polarity rather than of dialytic permeation. The latter process, unlike the former, permits passage of water-soluble UV-absorbing substances present in proteins, which can interfere with a UV assay process.

REFERENCES

(1) J. Steinhardt and J. A. Reynolds, "Multiple Equilibria in Proteins," Academic, New York, N.Y., 1969, chap. 3.

(2) H. H. Stein, *Anal. Biochem.*, **13**, 305(1965).

(3) I. M. Klotz, in "The Proteins," H. Neurath and K. Bailey, Eds., Academic, New York, N.Y., 1953, chap. 8.

(4) A. Agren and R. Elofsson, *Acta Pharm. Suecica*, **4**, 281(1967).

(5) A. Agren, *ibid.*, **5**, 37(1968).

(6) A. Agren, R. Elofsson, U. Meresaar, and S. O. Nilssen, *ibid.*, **7**, 105(1970).

(7) M. C. Meyer and D. E. Guttman, *J. Pharm. Sci.*, **57**, 1627(1968).

(8) *Ibid.*, **59**, 33(1970).

(9) *Ibid.*, **59**, 39(1970).

(10) P. C. Farrell, R. P. Popovich, and A. L. Babb, *J. Pharm. Sci.*, **60**, 1471(1971).

(11) W. F. Blatt, S. M. Robinson, and H. J. Bixler, *Anal. Biochem.*, **26**, 151(1968).

(12) J. S. Crawford, R. L. Jones, J. M. Thompson, and W. D. E. Wells, *Brit. J. Pharmacol.*, **44**, 80(1972).

(13) K. P. Link, *Fed. Proc.*, **4**, 176(1945).

(14) D. Kritchevsky and M. R. Kirk, *Arch. Biochem. Biophys.*, **35**, 346(1952).

(15) R. Nagashima, G. Levy, and E. J. Sarcione, *J. Pharm. Sci.*, **57**, 1881(1968).

(16) P. G. Welling, K. P. Lee, U. Khanna, and J. G. Wagner, *ibid.*, **59**, 1621(1970).

(17) J. H. Perrin and P. Idsvoog, *ibid.*, **60**, 602(1971).

(18) J. S. Robertson and O. Bode, *J. Pharm. Pharmacol.*, **22**, 423(1970).

(19) J. A. Reynolds, S. Herbert, H. Polet, and J. Steinhardt, *Biochemistry*, **6**, 937(1967).

(20) A. Albert and E. P. Sergeant, "Ionization Constants of Acids and Bases," Wiley, New York, N.Y., 1962.

(21) A. N. Martin, "Physical Pharmacy," 1st ed., Kimpton, London, England, 1960, p. 383.

(22) J. E. Fletcher and A. Spector, *Comp. Biomed. Res.*, **2**, 164(1968).

(23) J. A. Nelder and R. Mead, *Computer J.*, **7**, 308(1965).

(24) D. Rudman, T. J. Bixler, II, and A. E. Del Rio, *J. Pharmacol. Exp. Ther.*, **176**, 261(1971).

(25) A. Froese, A. H. Sehon, and M. Eigen, *Can. J. Chem.*, **40**, 1786(1962).

(26) C. F. Chignell and D. K. Starkweather, *Mol. Pharmacol.*, **7**, 229(1971).

(27) J. J. Burns, S. Wexler, and B. B. Brodie, *J. Amer. Chem. Soc.*, **75**, 2345(1953).

(28) M. J. Cho, A. G. Mitchell, and M. Pernarowski, *J. Pharm. Sci.*, **60**, 196(1971).

(29) L. Benet and J. Goyan, *ibid.*, **56**, 665(1967).

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