Direct and Indirect Determination of Binding Constants of Drug–Protein Complexes with Microparticles

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Abstract □ Albumin can be immobilized in microparticles of polyacrylamide in such a way that its ligand-binding properties are retained. With radiolabeled salicylic acid, warfarin, and tryptophan, the same characteristics are obtained for binding to albumin in the microparticles as in free solution. The particles can be used conveniently to determine association constants and the number of binding sites directly. The association constant of a competitive displacer can be determined indirectly as well, as shown with diazepam–salicylic acid and tryptophan–salicylic acid.

Keyphrases □ Binding, protein—salicylic acid, warfarin, and tryptophan to albumin in free solution and in microparticles, direct and indirect determination of constants □ Protein binding—salicylic acid, warfarin, and tryptophan to albumin in free solution and in microparticles, direct and indirect determination of constants □ Microparticles, polyacrylamide—containing albumin, binding of salicylic acid, warfarin, and tryptophan compared to binding in free solution, direct and indirect determination of constants

Considerable efforts have been devoted to the quantitative characterization of ligand–macromolecule complexes, especially between drugs and serum albumin. In the absence of better methods, the time-consuming and laborious equilibrium dialysis technique is still most commonly used and is considered to give correct data when properly applied. Some modifications (1, 2) were tried to speed up equilibrium. Ultrafiltration is a rapid technique, but it generally gives too high a degree of binding because of the increased local concentration of protein just over the membrane (3).

Gel filtration also has been used in ligand binding studies (4), but unspecific interactions with the gels, together with difficulties in correctly calculating the binding constants thermodynamically, have precluded its general application. Circular dichroism measurements (5) can, in some cases, be used to determine binding constants accurately in homogeneous solutions without any interfering membranes or matrixes. However, the limited availability of spectropolarimeters and the costs involved limit the applicability.

It recently was reported (6–8) that immunoglobulins, albumin, and enzymes retain their biological properties when immobilized in microparticles of highly cross-linked polyacrylamide. The proteins are partly entrapped in the network of the polymer and partly fixed in the thick threads forming the network. The relatively large pores of the polyacrylamide allow the equilibrium between the interior of the particles and the environment to be attained rapidly.

The present paper demonstrates that the quantitative relationships for the binding of some drugs to albumin in microparticles and in true solution (as in equilibrium dialysis) are the same. Moreover, it is shown how albumin in microparticles can be used conveniently for both direct and indirect determinations of binding constants.

EXPERIMENTAL

Materials—The purity of the human serum albumin[1] was more than 98% according to the manufacturer's specifications.

^{14}C-Salicylic acid (52 mCi/mmole), ^{14}C-warfarin (23.5 mCi/mmole), and ^{14}C-tryptophan (52 mCi/mmole) were used[2]. The radiochemical purity was checked by TLC and was >98%. The unlabeled drugs were used as received. Other chemicals were analytical grade.

Radioactivity was measured by liquid scintillation counting.

Microparticles of polyacrylamide containing human serum albumin, and with a diameter primarily between 1 and 3 μm, were prepared as described by Ekman and Sjöholm (7). An appropriate amount of human serum albumin was dissolved in a solution of acrylamide (6% w/v) and *N,N'*-methylenebisacrylamide (2% w/v) in 0.1 *M* KCl and 0.005 *M* phosphate buffer, pH 7.4. In general, a concentration of 150 mg of albumin/ml was used.

After the addition of the catalyst system consisting of *N,N,N',N'*-tetramethylethylenediamine and ammonium peroxydisulfate, the water phase (5 ml) was immediately homogenized in 200 ml of an organic phase[3] [toluene–chloroform (4:1)]. The polymerization started immediately and was completed in 5 min. The microparticles were isolated by centrifugation and washed several times with buffer. The amount of human serum albumin incorporated in the particles was determined by amino acid analysis after hydrolysis in 6 *M* HCl at 105° for 20 hr.

Drug–Protein Binding with Microparticles—The experiments were carried out at room temperature (23°). Microparticles containing albumin were suspended in a 0.03 *M* phosphate buffer and 0.1 *M* NaCl, pH 7.4. The concentration of albumin in the suspension was 0.8 mg/ml (1.2×10^{-5} *M*). Aliquots of 1 ml were pipetted into plastic centrifuge tubes. Different amounts of the drug studied, also containing the ^{14}C-labeled compound, were dissolved in the same buffer and added (in 100 μl) to the tubes containing the microparticle suspension. Earlier studies (7) showed that equilibrium is obtained rapidly; the samples were generally centrifuged within 5–15 min for 20 min at 3000×*g*.

After centrifugation, the concentration of the free drug, [*D*], in the system was determined by measuring the radioactivity in 0.1-ml aliquots taken in duplicate from the supernate. The concentration of the bound drug, [*DP*], was calculated by subtracting the free drug from the total concentration of drug added.

Unspecific binding to the polyacrylamide gel of the respective ligands was tested in the same way with microparticles not containing proteins.

In the interaction studies, the displacing drug, which was not radioactively labeled, was added to the microparticle suspension so that a constant molar ratio between displacer and albumin was obtained in all samples in a series. After mixing and centrifuging, the radioactivity was determined as previously described.

Equilibrium Dialysis—The protein binding also was determined by equilibrium dialysis at 23° as described earlier (9). The albumin concentration was determined from the absorption at 278 nm ($A^{1\%}_{1\,cm}$ = 5.80).

Evaluation of Data—The binding data obtained with the different ligands were analyzed according to Scatchard (10). The following equation:

$$\frac{r}{[D]} = n \cdot K_a - r \cdot K_a \qquad \text{(Eq. 1)}$$

was used, where r is the moles of bound drug per mole of albumin, n is the number of binding sites, K_a is the association constant for the

[1] Batch 30299, KABI AB, Stockholm, Sweden.
[2] Radiochemical Centre, Amersham, England.
[3] An Ultraturrax TP 18-10 was used as the homogenizer, and the detergent was Pluronic F68, Wyandotte Chemicals Corp., Wyandotte, Mich.

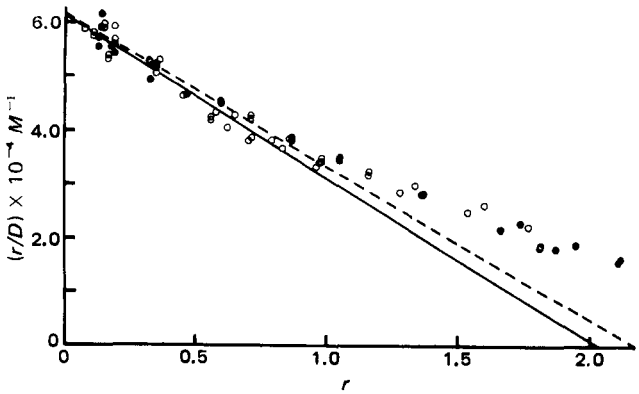

Figure 1—*Scatchard plot showing salicylic acid binding to human serum albumin in free solution determined by equilibrium dialysis (●) and to albumin in microparticles (○). The lines (--- and —, respectively) are the regression lines calculated from the experimental points with r < 1.0. Albumin (1.2 × 10⁻⁵ M) was dissolved or, when present in microparticles, suspended in 1.0 M NaCl with 0.03 M phosphate, pH 7.4; the temperature was 23°.*

drug–albumin complex, and [D] is the concentration of free drug. The data also were evaluated by the double-reciprocal plot:

$$\frac{1}{r} = \frac{1}{nK_a} \cdot \frac{1}{[D]} + \frac{1}{n} \qquad \text{(Eq. 2)}$$

When the binding of a ligand to a particular site on the macromolecule is inhibited by a competitive inhibitor, the following relation exists (11) between the association constant, K_{app}, determined in the presence of the inhibitor, I, and the true association constant, K_a, obtained in the absence of inhibitor:

$$K_{app} = \frac{K_a}{1 + [I]K_i} \qquad \text{(Eq. 3)}$$

where $[I]$ is the concentration of the free displacing drug and K_i is the association constant for the protein–displacer complex. From this equation, the association constant for the displacing drug, K_i, can be calculated if K_a and $[I]$ are known.

In the plots, the data points were fitted to straight lines by linear regression analyses using the values on the x-axis as the independent variable and the values on the y-axis as the dependent variable.

RESULTS

Protein Binding—The unspecific binding to the polyacrylamide gel is generally low, and no such binding was detected for salicylic acid, warfarin, and tryptophan. The characteristics for the binding to human serum albumin in microparticles, as displayed in the Scatchard plots, are shown for salicylic acid, warfarin, and tryptophan in Figs. 1–3, respectively. The binding of the drugs to albumin incorporated in the microparticles is the same, within the experimental errors, as the binding to albumin in free solution as determined by equilibrium dialysis. The straight lines were calculated by linear regression and gave the values of nK_a (intercept on the y-axis), n (intercept on the x-axis), and K_a (Table I).

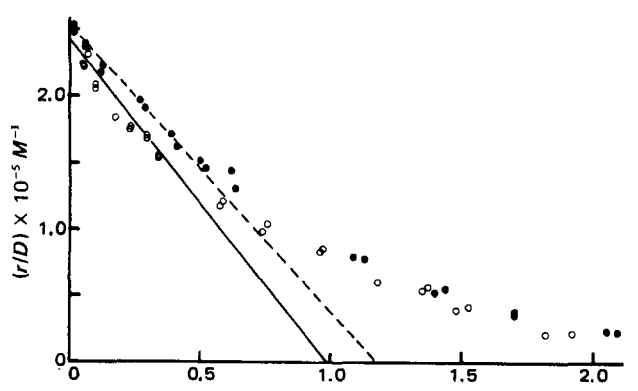

Figure 2—*Scatchard plot showing warfarin binding to human serum albumin in free solution determined by equilibrium dialysis (●) and to albumin in microparticles (○). The lines (--- and —, respectively) are the regression lines calculated from the experimental points with r < 0.6. The experimental conditions were the same as in Fig. 1.*

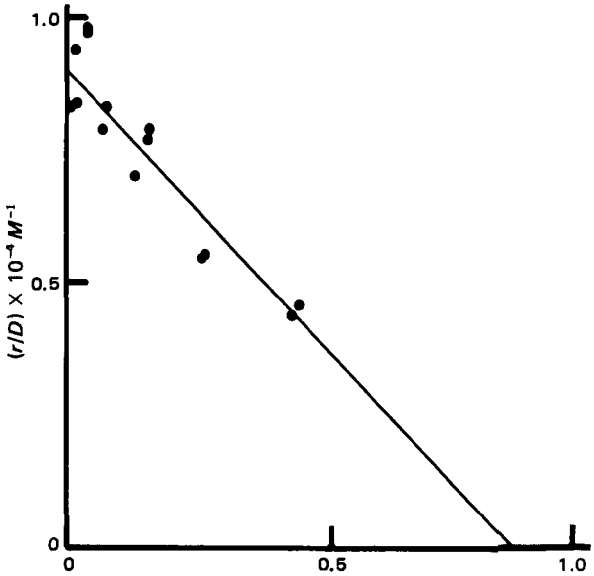

Figure 3—*Scatchard plot showing L-tryptophan binding to human serum albumin in microparticles (●). The line drawn is the regression line. The experimental conditions were the same as in Fig. 1.*

Inhibition of Binding—The changes in the binding of salicylic acid to albumin in microparticles brought about by adding competing ligands are shown in Figs. 4 and 5. These double-reciprocal plots show that both diazepam and tryptophan inhibit ^{14}C-salicylic acid binding.

Diazepam is known to bind to one primary site on human serum albumin (12, 13); this site is evidently one of the two sites to which salicylic acid binds. The two lines have the same intercept, giving $n = 2$; *i.e.*, the binding of salicylic acid is competitively inhibited. From the slopes of the two lines, K_a and K_{app} can be calculated; K_i, the association constant characterizing diazepam binding to albumin, may subsequently be calculated from Eq. 3. Thereby, in Eq. 3, the total concentration of the inhibitor may, as an approximation, be substituted for the value of the free inhibitor concentration, $[I]$. The value of K_i is given in Table I and approaches the value $1.8 \times 10^5\ M^{-1}$ obtained with circular dichroism (13). The albumin used in this investigation was not treated with charcoal, which is usually done to eliminate the strongly bound fatty acids.

Figure 5 shows that salicylic acid binding successively decreased with increasing tryptophan concentration. Tryptophan is probably bound to more than one site on human serum albumin, even if the Scatchard plot in Fig. 3 indicates only one site. Therefore, the approximation that $[I] = [I]_{total}$ is more uncertain, which introduces a larger error in the calculation of K_i.

DISCUSSION

Immobilization of biologically active macromolecules, *e.g.*, proteins, by covalent binding to solid matrixes generally decreases or changes the activity. However, in the microparticles of highly cross-linked polyacrylamide (in this work, T – C = 8–25%)[4], the proteins are only physically entrapped in the network as well as in the threads, and the activity of the immobilized proteins is retained (6, 7).

In the present case, the binding affinity between albumin and several ligands was the same in the microparticles as in free solution. The obvious consequence of this observation is that microparticles with immobilized albumin (or other proteins) might be used to characterize the quantitative relationship between protein and ligand.

The present work showed that the association constant for drug

[4] The gels prepared are characterized according to the nomenclature suggested by Hjertén (15). The first numeral, T, denotes the total amount of monomer (g/100 ml of solvent), and the second numeral, C, denotes the amount of *N,N*-methylenebisacrylamide expressed as the percentage (weight/weight) of the total amount of monomers.

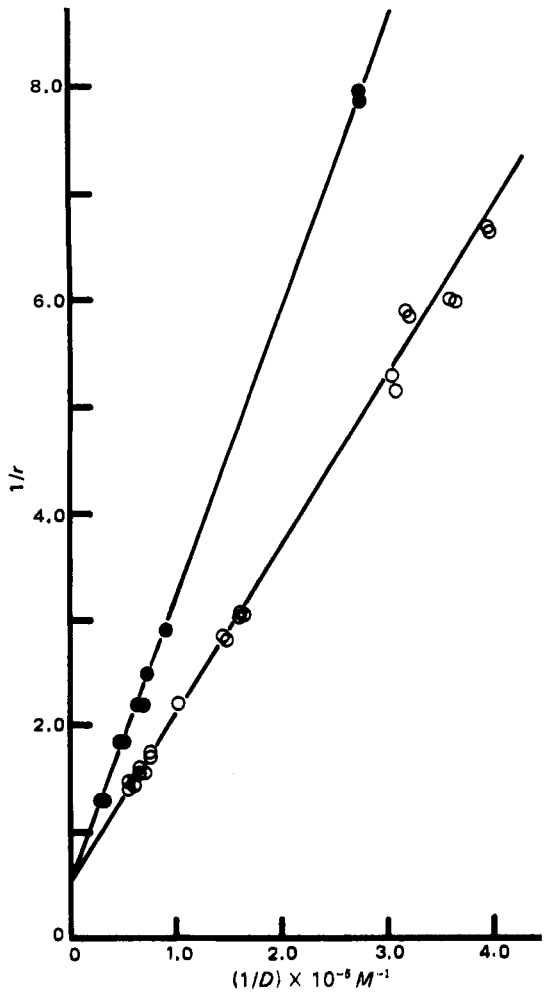

Figure 4—*Double-reciprocal plot showing salicylic acid binding to human serum albumin in microparticles in the absence (○) and presence (●) of 1.5 × 10⁻⁵ M diazepam. The experimental conditions were the same as in Fig. 1.*

binding can conveniently be performed directly. The constants can be determined by the Scatchard plot, especially when the number of binding sites involved, n, is of interest. In addition, the double-reciprocal plot can be used to study the effects of inhibitors. The association constant for the binding of the inhibitor to the specific site can be estimated from the changed slopes of the lines obtained with increasing inhibitor concentration. Certainly, the inhibitor may bind to other sites as well, so some uncertainty will exist as to the approximation that the free inhibitor concentration approaches the total. In this case, information may be obtained from determinations at varying inhibitor concentrations. Consistent values of K_i indicate that the approximation is correct.

The use of microparticles with immobilized protein simplifies the determination of association constants considerably. The procedure is rapid and simple, and the values will be precise when radiolabeled ligands are used or when sensitive methods are available for the determination of the free ligand in the supernate obtained after centrifugation. In these

Table I—Binding Constants for the Binding to Human Serum Albumin

Compound	Microparticles K_a, $M^{-1} \times 10^{-4}$	n	Microparticles K_i, $M^{-1} \times 10^{-4}$	Other Methods K_a, $M^{-1} \times 10^{-4}$	Other Methods n
Salicylic acid	3.0	2.0	—	2.8	2.2[a]
Warfarin	24.0	1.0	—	21.0	1.2[a]
Tryptophan	1.1	0.8	0.9	1.1	0.9[b]
Diazepam	—	—	11.0	18.0	1.0[c]

[a] Equilibrium dialysis (this work). [b] Gel filtration (14). [c] Circular dichroism (13).

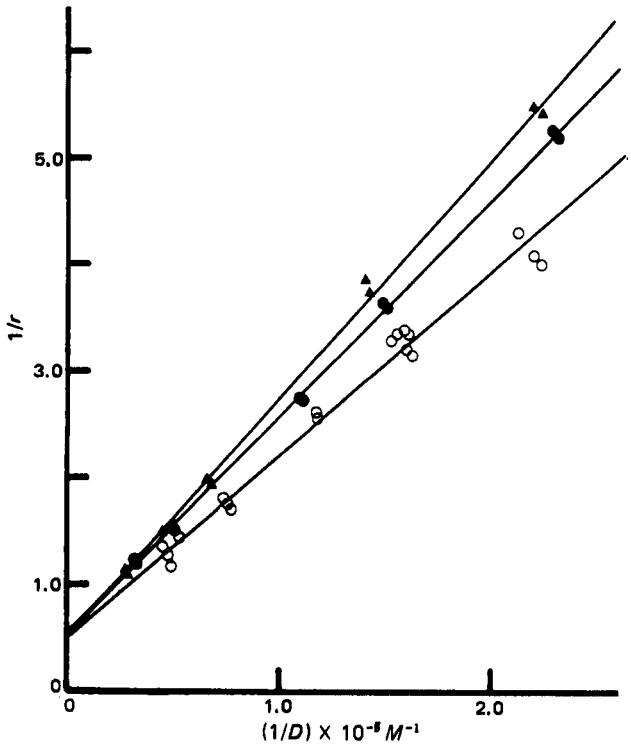

Figure 5—*Double-reciprocal plot of salicylic acid binding to human serum albumin in microparticles, alone (○), in the presence of 5.8 × 10⁻⁵ M tryptophan (●), and in the presence of 9.6 × 10⁻⁵ M tryptophan (▲). The experimental conditions were the same as in Fig. 1.*

cases, low concentrations of protein and ligand can be used, which means that the degree of binding will be low. Such conditions are favorable for the determination of the primary binding constant, which most often represents the physiologically significant binding.

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