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Use of Membrane-Covered Rotating-Disk Electrode to Study Binding of Drugs to Macromolecules: Competitive Binding of 2-(4'-Hydroxybenzeneazo)benzoic Acid and α-(4-Chlorophenoxy)-α-methylpropionic Acid to Serum Albumins

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Abstract □ A new technique is introduced to study the binding of drugs to macromolecules. The method utilizes rotating-disk polarography in which a semipermeable membrane covers the surface of the electrode. The membrane permits passage of an electroactive drug but restricts permeability of the macromolecule and of bound drug. The current generated at the electrode at steady state is directly proportional to the concentration of free drug in bulk solution, from which a calculation of the amount of drug bound may be made. The methodology used provides a complete characterization of binding within about 1 hr. and is, therefore, faster than conventional techniques presently used. Another advantage of this technique is that relatively small amounts of the compounds being studied are required. The method is compared with equilibrium dialysis and ultrafiltration techniques by studying the binding of 2-(4'-hydroxybenzeneazo)benzoic acid (I) to serum albumin and is found to give precisely the same results. α-(4-Chlorophenoxy)-α-methylpropionic acid (II), in previous spectrophotometric studies, showed an anomalous effect on the binding of I to rat serum albumin. At low concentrations, there was an indication that II may increase the binding of I to rat serum albumin. This phenomenon was reinvestigated using the new electrochemical method; it was found that II competitively inhibits the binding of I at all concentration levels of the inhibitor. The conclusion is reached that II affects the nature of the I binding site rather than the number of molecules bound.

Keyphrases □ 2-(4'-Hydroxybenzeneazo)benzoic acid binding to serum albumins—presence, absence of α-(4-chlorophenoxy)-α-methylpropionic acid, rotating-disk polarography method, compared to conventional techniques □ α-(4-Chlorophenoxy)-α-methylpropionic acid—competitive binding with 2-(4'-hydroxybenzeneazo)benzoic acid to serum albumin, rotating-disk polarography method □ Polarography, rotating disk—used in binding studies, compared to equilibrium dialysis and ultrafiltration techniques □ Rotating-disk polarography—used in binding studies, compared to equilibrium dialysis and ultrafiltration techniques □ Serum protein binding, competitive—2-(4'-hydroxybenzeneazo)benzoic acid and α-(4-chlorophenoxy)-α-methylpropionic acid, rotating-disk polarography method □ Binding of drugs to macromolecules—studied using rotating-disk polarography, compared to equilibrium dialysis and ultrafiltration techniques

Classical methods employed to detect and determine the binding characteristics of small molecules to serum albumin have been discussed by several authors (1–3). Techniques such as equilibrium dialysis, ultracentrifugation, light absorption and rotation, and ultrafiltration have been used. The methods conventionally used require long periods of time to collect analyzable data and/or relatively large amounts of reactants, some of which can be costly. Nonequilibrium dialysis techniques (4–7) are generally faster and use less materials than conventional methods but can require cumbersome data analysis. The method of Meyer and Guttman (5), for example, takes about 6 hr. to collect data and requires the use of computer curve-fitting techniques to estimate the number of molecules bound to albumin.

To overcome some of these disadvantages, we applied a technology, developed in these laboratories (8), that was previously used to study mass transport. The technique employed to study mass transport across membranes utilized rotating-disk polarography where a membrane sheet is placed on the surface of a carbon or mercury-coated platinum tip electrode. If the membrane used is semipermeable, allowing only small electrochemically active molecules to penetrate, a system is obtained that is analogous to the thermodynamically well-defined equilibrium dialysis technique. There is,

however, one notable difference. The steady-state current obtained at the electrode at a constant applied potential is directly proportional to the concentration of electrolyzable material in the bulk solution. Since only small molecules can penetrate the membrane, it is possible to measure in a very short time (from 18 to 30 sec.) the concentration of unbound drug in a solution containing a macromolecule with which it interacts. By successively increasing the total amount of small drug molecule in the bulk solution, enough data can be obtained in 1 hr. (up to 16 points in the present study) to characterize completely the binding of the drug. The membrane also functions to protect the surface of the electrode from contamination by protein (9).

This paper describes the binding of 2-(4'-hydroxy-benzeneazo)benzoic acid (I) to various serum albumins in the presence and absence of a competitive inhibitor α-(4-chlorophenoxy)-α-methylpropionic acid (II). In addition to showing that the new methodology gives the same binding results as those obtained by conventional equilibrium dialysis and ultrafiltration techniques, it provides further insight into the effect that II has on the interaction of I to rat serum albumin.

In an earlier work carried out in this laboratory (10), the competitive binding of I and II to several different serum albumins was studied using equilibrium dialysis and extrinsic rotatory dispersion. A unique behavior was observed for rat serum albumin; using spectrophotometry, low concentrations of II appeared to enhance the binding of I to the albumin. This observation and concomitant extrinsic rotatory dispersion studies suggested that II was causing a small molecular perturbation in rat serum albumin. The results could be interpreted in a number of ways. Either II liberates additional binding sites for interaction with I, or II affects the nature of the interaction of I without increasing the number of I molecules bound. This study was undertaken to determine which of these possible mechanisms was taking place.

EXPERIMENTAL

Materials—Bovine serum albumin[1] (Fraction V, fatty acid poor) and rat serum albumin[2] (Fraction V) were used as obtained without further purification. Two brands[3] of 2-(4'-hydroxybenzeneazo)-benzoic acid (I) were used; both showed similar electrochemical and binding properties. α-(4-Chlorophenoxy)-α-methylpropionic acid (II) was prepared according to a method previously described (11). All solutions used were buffered at pH 7.4 using 0.1 M sodium phosphate.

Dialysis tubing[4] with an average pore radius of 24 Å and a dry thickness of 0.0041 cm. (0.0016 in.) was used. Disks of 0.9 cm. (0.37 in.) diameter were cut and washed several times with boiling double-distilled water and stored in a preserving solution containing 0.3% benzoic acid and 10% glycerin. One day before use, the disk was washed with distilled water and soaked overnight in refrigerated phosphate buffer. The disk was positioned on the surface of the electrode, as described in an earlier study (8), immediately prior to collection of data.

Binding by Electrochemical Method—The apparatus and techniques used in these studies were the same as those described previously (8). A small modification in procedure was made to cope
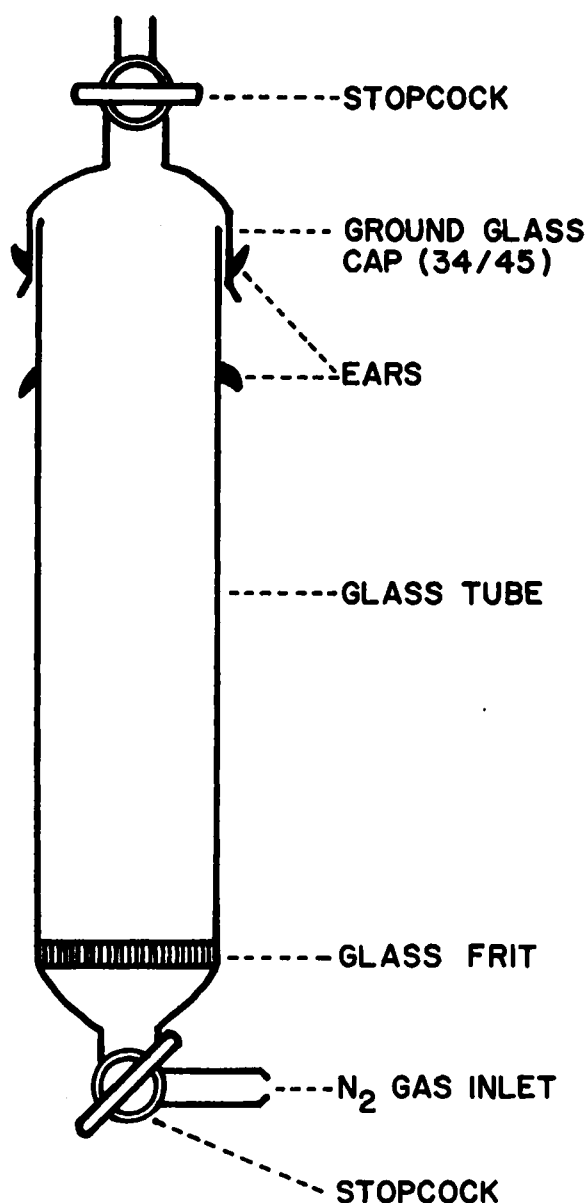
[1] Nutritional Biochemical Corp., Cleveland, Ohio.
[2] Pentex, Kankakee, Ill.
[3] Eastman, Rochester, N. Y., and Nutritional Biochemical Corp.
[4] Type 1 7/8 ss, Union Carbide Corp., Chicago, Ill.

Figure 1—*Deaeration column for concentrated drug solutions or protein-containing solutions.*

with the tendency of protein and concentrated drug solutions to foam.

Stock solutions of I, II, and albumin were made separately in pH 7.4 phosphate buffer. Deaeration was done in a specially fabricated apparatus (Fig. 1) to avoid subsequent foaming in the electrolysis cell. Adequate deaeration was accomplished in 5 min. Fifteen milliliters of deaerated protein solution was delivered with the use of a syringe to the right-hand side of the polarographic cell; a nitrogen atmosphere was maintained above the solution. The cell holder, containing the dialysis membrane, was assembled with the carbon electrode tip in place as previously described (8). The membrane electrode assembly was mounted on the electrode body and rotated at 1200 r.p.m. This rotation speed was used since it did not produce significant foaming.

A potential of −1.0 v. was applied to the working electrode, and the current was measured[5]. With no I added, the current represents background. Using a calibrated syringe, 1.0 ml. of a concentrated I solution was injected rapidly into the working compartment. After a short induction period, the current increased with time, eventually reaching a steady-state value. Depending on the concentration of I,

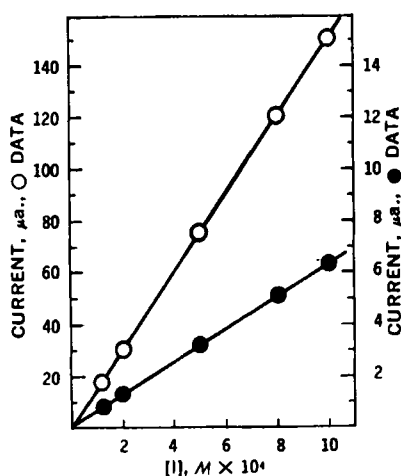[5] The current was recorded on a calibrated Varian model HG-14 recorder.

Figure 2—*Relation between steady-state diffusion current and concentration of I in bulk solution. Key: O, bare electrode, slope = 150×10^3 μa. M^{-1}; and ●, dialysis membrane-covered electrode, slope = 6.3×10^3 μa. M^{-1}.*

the time needed to reach steady state was 18–30 sec. After the steady-state current was attained, another 0.40 ml. of concentrated solution was injected, thereby increasing the bulk concentration of I and giving a new steady-state current. Additional 0.40-ml. portions of the concentrated solution may be added in a similar manner, giving rise to new steady-state currents. In studying the competitive binding of I and II, 1.0 ml. of a concentrated I solution was added to the protein solution and the steady-state current was recorded. Compound II was then added in consecutively increasing amounts to the I–protein solution, and the change in steady-state current after each addition was recorded. The concentration of all reactants was corrected for the small volume change following the addition of the consecutively added concentrated solutions. The amount of I removed from bulk solution due to reduction at the electrode is negligible. All studies were made at a constant temperature of 30°.

Binding by Equilibrium Dialysis and Ultrafiltration—The procedure used to determine drug binding by equilibrium dialysis studies was identical to the one reported earlier (10). Ultrafiltration measurements were made using a 10-ml. ultrafiltration cell[6] and a membrane[7]. Ten milliliters of drug–protein solution was placed in the cell, and 1 ml. of filtrate was collected. The filtrate was analyzed spectrophotometrically for free I. The absorbance for I at pH 7.4 is 17,682 1. M^{-1}.
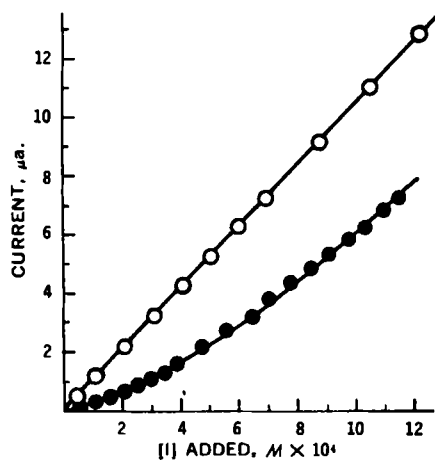

Figure 3—*Effect of bovine serum albumin on the steady-state current of I at various initial bulk concentrations. Key: O, no serum albumin added; and ●, in the presence of 1×10^{-4} M serum albumin.*
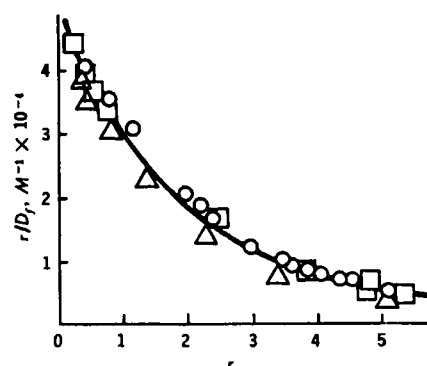
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[6] Amicon model 12.
[7] PM10 Diaflo, Amicon Corp.

Figure 4—*Scatchard treatment of the binding of I to bovine serum albumin; r is the number of I molecules bound per mole of albumin, and D_f is the concentration of I free in solution. Key: O, electrochemical method; □, ultrafiltration technique; and △, equilibrium dialysis method.*

RESULTS AND DISCUSSION

Use of Rotating-Disk Polarography to Study Protein Binding—At pH 7.4 and 30°, the azo group of I is electrochemically reduced at the carbon electrode, producing two reduction waves with half-wave potentials of -0.40 and -0.60 v. *versus* the standard calomel electrode, respectively. One electron is consumed at each wave. Compound II is electrochemically inactive in this potential range and does not interfere with the reduction of I. The maximum difference in viscosity between I, albumin, and I–albumin mixtures is about 4%. Thus, any viscosity effect on the electrochemical properties of I is negligible.

At an applied constant potential of -1.0 v. (the plateau or diffusion-controlled potential), the steady-state current at a bare electrode due to the reduction of I is a linear function of the concentration of I (Fig. 2). The slope of this linear relation is 1.5×10^5 μa. M^{-1}. A linear relation between current and concentration also was observed for the membrane-covered electrode (Fig. 2). However, owing to the porosity of the membrane which decreases the effective area of the electrode and increases the diffusional path length, the slope of the line is reduced. The ratio of the slope without membrane to slope with membrane in Fig. 2 is 0.042. Even with this reduction in sensitivity, reliable data are obtained.

When serum albumin is present, the current generated at steady state is reduced due to the binding of I to the macromolecule (Fig. 3). Since the steady-state current is proportional to the concentration of free drug, the difference in current in the presence and absence of protein at a particular total concentration of I added is proportional to the drug bound. By using data as presented in Fig. 3, it is possible to calculate the moles of I bound per mole of albumin using a molecular weight of 67,000 for the albumin. Treating the data thus collected by the Scatchard method (12) shows that more
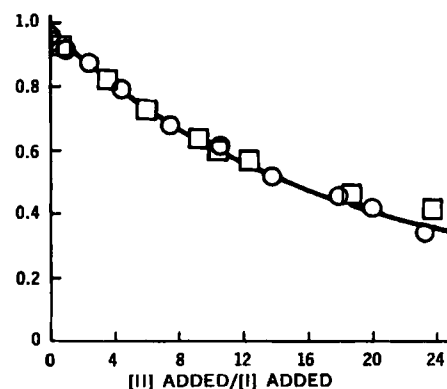

Figure 5—*Relation between the moles of I bound per mole of rat serum albumin r, with varying ratios of II:I added to bulk solution. The rat serum albumin and I concentrations are held constant at 9.4×10^{-5} and 1×10^{-4} M, respectively. The concentration of added II varies from 0 to 2.38×10^{-3} M.*

than one binding site is involved in the binding of I to bovine serum albumin (Fig. 4). The binding results obtained using the electrochemical method compare very well with those obtained using equilibrium dialysis and ultrafiltration (Fig. 4). However, results utilizing the rotating-disk technique can be obtained in a much shorter time and with considerably smaller amounts of materials.

Competive Binding between II and I—The observation made by Witiak *et al.* (10), indicating an unusual binding of I to rat serum albumin in the presence of low concentrations of II, was reinvestigated using both this electrochemical method and ultrafiltration. The spectrophotometric results in the Witiak *et al.* study could be interpreted in one of two ways: (*a*) II at low concentrations actually increases the number of molecules bound to rat serum albumin, or (*b*) II does not change the number of sites available for interaction but, rather, changes the nature of the binding. Use of the electrochemical method permitted direct measurement of the amount of I free in solution. The early studies (10) were based on indirect spectrophotometric measurement. Ultrafiltration also provided direct measurement of free I.

In the electrochemical studies, the concentrations of I and rat serum albumin were twice those used by Witiak *et al.* (10), but the ratio of I to rat serum albumin was the same (1.07). Hence, the binding character also should be the same. Increasing the II concentration from 0 to 23.8 × 10⁻⁴ M produced the results shown in Fig. 5. The concentration of II used falls into the range where anomalous effects previously were observed spectrophotometrically. As shown in Fig. 5, identical results were obtained using the ultrafiltration technique. It is clear from this figure that II replaces I from its binding sites even at low concentrations. Therefore, it is apparent that II increases the ε⁴⁷⁷ for bound I at lower concentrations (10), and this increase is not a reflection of increased binding of I to rat serum albumin.

The reasons for this increase in ε⁴⁷⁷ may, as previously suggested, be due to a small molecular perturbation in rat serum albumin, which changes the I-rat serum albumin extinction coefficient, or to an interaction of bound I with closely bound II.

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Model Transport Studies Utilizing Lecithin Spherules III: Transport of Taurocholic Acid-[cholic-³H(G)] in Buffered D-Glucose Solutions

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Abstract ☐ The transport of taurocholic acid-[cholic-³H(G)] in liposome dispersions prepared from lecithin–dicetyl phosphate in buffered glucose solutions at different pH's was studied. The transport experiments were carried out using previously developed techniques in which the release of the solute is studied from dispersions contained in the dialysis bags. The experimental results of the direct-release and the uptake-release cases were analyzed by the monosize, multiconcentric layer Model 1 which was developed, evaluated, and used recently for D-glucose transport studies. Uniformly good fits of the experimental data with the model were observed, and the permeability coefficients and the aqueous–lipid partition coefficients for taurocholate transport in liposome dispersions were calculated. The pH dependence of the permeability

coefficient indicated that the unionized form of taurocholic acid was being preferentially transported at low pH and that the taurocholate ion was the main species involved at high pH.

Keyphrases ☐ Permeability coefficients, taurocholic acid, radiolabeled—liposome dispersions ☐ D-Glucose solution—transport of taurocholic acid-[cholic-³H(G)], lecithin spherule dispersions, permeability coefficients, pH effect ☐ Taurocholic acid, radiolabeled—transport in D-glucose solution, lecithin spherule dispersions, permeability coefficients, pH effect ☐ Lecithin spherules—model transport studies, taurocholic acid-[cholic-³H(G)] in D-glucose solution, permeability coefficients ☐ Transport studies, model using lecithin spherules—taurocholic acid-[cholic-³H(G)] in D-glucose solution, permeability coefficients, pH effect

In previous investigations (1, 2), quantitative methods were developed and used in the determination of permeability coefficients for D-glucose and 3-*O*-methyl-D-glucose in complex aqueous liposome dispersions. The release of the solutes from dispersions contained in a dialysis bag was studied as a function of time using