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Measurement of Drug Displacement by Continuous Ultrafiltration

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Abstract □ Adaptation of the continuous ultrafiltration technique to allow precise measurement of the displacement of one ligand by another from binding sites on human serum albumin is described. The displacement of sodium urate and methyl orange by sodium salicylate is demonstrated with analysis of the number of binding sites and association constants for these reactions.

Keyphrases □ Drug displacement from human serum albumin—measurement of sodium urate and methyl orange displacement by sodium salicylate using continuous ultrafiltration □ Human serum albumin binding—displacement of sodium urate and methyl orange by sodium salicylate, measured using continuous ultrafiltration □ Binding of sodium urate and methyl orange to human serum albumin—displaced by sodium salicylate, binding measured by continuous ultrafiltration □ Ultrafiltration, continuous—measurement of sodium urate and methyl orange displacement from human serum albumin by sodium salicylate

Displacement of one substance by another from shared binding sites on the albumin molecule is increasingly being recognized as an important factor modifying expected physiological and pharmacological activity. The introduction of a continuous ultrafiltration technique (1) greatly facilitated the study of ligand–protein interactions, and the present study extends this simple ultrafiltration technique to allow a precise study of drug displacement over a wide range of ligand concentration. The displacement from human serum albumin (albumin) of the ligands, sodium urate and methyl orange, sodium *p*-[(*p'*-dimethylaminophenyl)azo]benzenesulfonic acid, by sodium salicylate was used as a model, but the technique is useful over a wide range of interactions. The only requirement is that an appropriate membrane be available to retain the macromolecule in the ultrafiltration chamber.

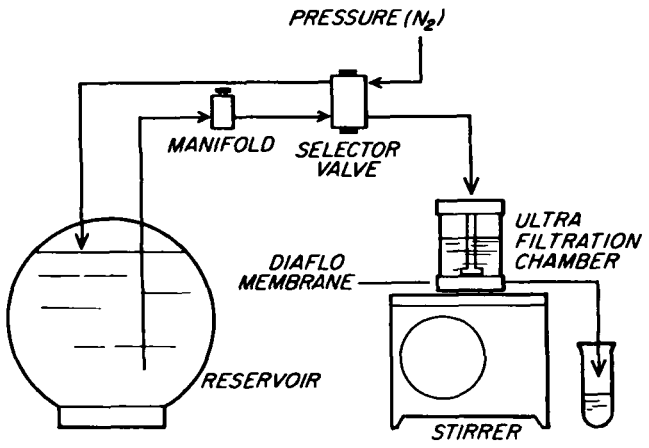

Figure 1—*Experimental setup, continuous filtration.*

EXPERIMENTAL

Crystallized human serum albumin[1] (electrophoretically pure, <0.05 m*M* of free fatty acids per m*M* of albumin) dried to constant weight was used in all studies. Methyl orange[2] was recrystallized from ethanol, dried to constant weight, and dissolved in phosphate buffer at pH 7.4. Sodium urate was made from uric acid triturated with 0.1 *N* NaOH and buffered with phosphate buffer to pH 7.4. The ionic strength of the final solutions was 0.16. Ultrafiltration was performed (Fig. 1) using an ultrafiltration chamber[3] connected through a concentration dialysis selector valve[4] to a nitrogen gas cylinder, which provided pressure for ultrafiltration, and to a 2.4-liter reservoir. A manifold in the line to the reservoir was used as a flow switch. During the experiment, since this was a closed system, the ultrafiltrate leaving the chamber was replaced by an identical volume of solution from the reservoir, with the chamber volume remaining constant. An ultrafiltration membrane[5] with a 10,000 molecular weight cut-off was used. All experiments were done at 22.5°. Methyl orange was

[1] Calbiochem.
[2] British Drug Houses.
[3] Amicon model 65.
[4] Amicon CDS-10.
[5] Diaflo PM10.

Table I—Procedures to Establish Binding of Ligand *A* to Protein *P*, and Extent of Displacement by Competing Ligand *B*

Procedure	Ultrafiltration Chamber	Reservoir	Plot of (Total *A*) *versus* (Free *A*)	Interpretation
1	Buffer only	$(A)^a$	$dF/dT^b = 1$	No binding of *A* to membrane, no reflection of *A* at membrane
2	(B)	(A), $(B)^a$	$dF/dT = 1$	No effect of *B* on the ultrafiltration of *A*
3	(P)	(A)	$dF/dT < 1$	Binding of *A* by *P*
			$dF/dT = 1$	No binding of *A* by *P*
4	(P)	(B)	Ultrafiltration until (B) in ultrafiltrate equals (B) in reservoir	Establishes the equilibrium: $(P) + (B) \rightleftharpoons (PB)$
At the end of Step 4, the chamber now containing (P) and (PB) in equilibrium with (B) is connected to a new reservoir:				
5	(P), (PB), (B)	(A), (B)	$dF/dT > dF/dT$ of Step 3	Displacement of *A* by *B*
			$dF/dT = dF/dT$ of Step 3	No displacement of *A* by *B*

[a] (A) and (B) represent constant concentrations of the primary ligand *A* and competing ligand *B*, respectively. [b] $dF/dT = d(\text{free } A)/d(\text{total } A)$.

measured spectrophotometrically in the acid phase at 510 nm. Urate was measured using the method of Liddle *et al.* (2). Sodium salicylate[6] dissolved in pH 7.4 phosphate buffer, ionic strength of 0.16, was made as a standard solution of 0.25 and 0.5 m*M*.

Binding of Primary Ligand—The following ultrafiltration procedure was used to study the binding of the primary ligand, ligand *A* (in this case, sodium urate or methyl orange), to albumin. A known concentration of ligand *A* in buffer was placed in the reservoir (Fig. 1). The same reservoir solution *(R)* was then used in two separate continuous ultrafiltration experiments. In the first (Procedure 1, Table I), only buffer was in the ultrafiltration chamber; in the second (Procedure 3, Table I), an albumin solution *(P)* was in the chamber. Procedure 1 examines whether there is binding of sodium urate or methyl orange to the membrane or reflection of these ligands by the membrane. Correction for such effects may then be made in the analysis of Procedure 3 where binding of the ligand is examined.

Having filled the tubing and selector valves with the reservoir solution, the flow was stopped by the manifold switch and the ultrafiltration chamber connected by selector switch to the gas cylinder, allowing equal pressurization of the whole system. The selector valve was then changed to connect the reservoir to the chamber, the manifold switch was simultaneously opened, and ultrafiltration commenced. Ultrafiltration was carried out at 0.703 kg/cm² (10 lb/sq in.) pressure; the ultrafiltrate, which formed at the rate of 10–15 ml/hr, was collected in 2–4-ml volumes in previously dried and weighed tubes, the volume collected (v_i) being determined by reweighing (to four decimal places) and converting to volume, allowing for temperature effects on volume.

In essence, a series of separate ultrafiltration experiments at constant *(P)* but increasing concentration of ligand *A* were performed in sequence. However, in each collection period, the concentration of total, free, and bound *A* could be precisely calculated or measured. Since *(R)*, *(P)*, and the collection volumes (v_i) were accurately determined, the only limit on accuracy in this technique is ligand analysis.

Calculation of Primary Ligand-Albumin Interaction—Let v_i = volume in liters of the *i*th ultrafiltrate collected, V = volume of solvent in chamber in liters, *(R)* = ligand *A* concentration in the reservoir (moles per liter), and $(M)_i$ = ligand *A* concentration in the *i*th collection (moles per liter). Since this is a closed system, the volume of solution entering the chamber in the *i*th period exactly equals the volume leaving (v_i). Therefore:

$$\text{moles into chamber} = v_i(R) \quad \text{(Eq. 1)}$$

$$\text{moles out of chamber} = v_i(M)_i \quad \text{(Eq. 2)}$$

$$\text{moles retained} = v_i(R) - v_i(M)_i \quad \text{(Eq. 3)}$$

At the end of the *i*th period, the:

$$\text{increase in total concentration in chamber during } i\text{th period} = \frac{1}{V}[v_i(R) - v_i(M)_i] \quad \text{(Eq. 4)}$$

At the end of the *n*th period:

$$\text{total moles/liter in chamber} = \frac{1}{V}\sum_{i=1}^{n}[v_i(R) - v_i(M)_i] \quad \text{(Eq. 5)}$$

During the *n*th period, the:

$$\text{ligand } A \text{ concentration in ultrafiltrate collected} = \text{moles/liter } \textit{free} \text{ in chamber} \quad \text{(Eq. 6a)}$$

$$= (M)_n \quad \text{(Eq. 6b)}$$

$$= \frac{1}{V}\sum_{i=1}^{n}[v_i(R) - v_i(M)_i] - (M)_n \quad \text{(Eq. 7)}$$

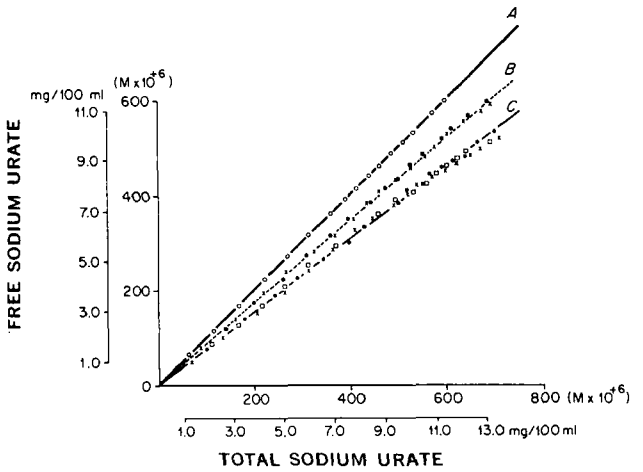

Figure 2—*Binding of sodium urate to albumin and its displacement by sodium salicylate. Key:* A, *buffer only in chamber (Procedure 1, Table I);* B, *5 g/100 ml albumin and 0.5 mM salicylate (Procedures 4 and 5); and* C, *5 g/100 ml albumin in chamber (Procedure 3). The conditions were: temperature, 22.5°; ionic strength, 0.16; and pH, 7.4.*

[6] Eastman Organic Chemicals.

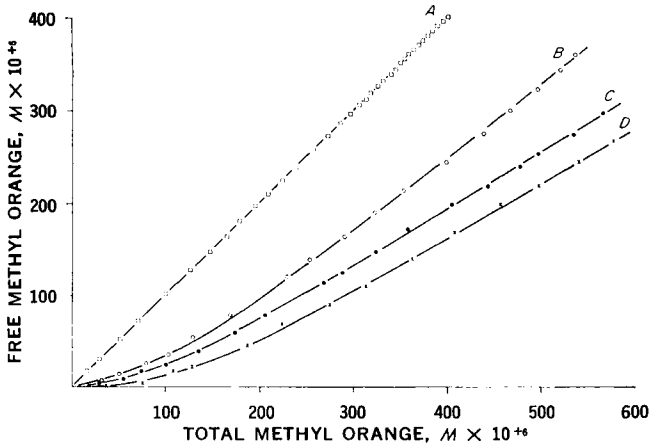

Figure 3—*Binding of methyl orange to albumin. Key:* A, *buffer only in chamber (Procedure 1, Table I);* B, *0.2 g/100 ml albumin and 2.5 mM salicylate (Procedures 4 and 5);* C, *0.2 g/100 ml albumin and 0.5 mM salicylate (Procedures 4 and 5); and* D, *0.2 g/100 ml albumin in chamber (Procedure 3). The conditions were: temperature, 22.5°; ionic strength, 0.16; and pH, 7.4.*

A plot may then be constructed of (total *A*) *versus* (free *A*).

Displacement of Primary Ligand *A* by Displacing Ligand *B*—It is necessary first to establish an equilibrium between ligand *B* and the protein *(P)* solution (Procedure 4, Table I). This is accomplished by connecting a reservoir containing only ligand *B* to the ultrafiltration chamber containing the protein solution *(P)*. Ultrafiltration is then carried out until the reaction *(B)* + *(P)* ⇄ *(PB)* reaches equilibrium. It is essential that at least three times the chamber volume be passed from the reservoir through the chamber. The protein and the *PB* complex are then in equilibrium with ligand *B* at the concentration of *B* in the reservoir. A simple test of this is that the *(B)* in the ultrafiltrate and the *(B)* in the reservoir are equal.

The reservoir and tubing are then filled with the ligand *A* solution but now also containing ligand *B* at the same concentration as was just used to equilibrate the protein. The ultrafiltration process is then carried out as described in the section *Binding of Primary Ligand,* and calculations of (total *A*), (free *A*), and (bound *A*) now reflect binding under a specified *free* concentration of ligand *B*. The total ligand *B* concentration to which this free level corresponds may be determined by measurement of (*B*) in the chamber, and (bound *B*) is obtained by subtraction of *(B)* in the reservoir or ultrafiltrate from (total *B*).

Since a competing ligand could conceivably have an effect on the ultrafiltration of the primary ligand, it is necessary in practice also to perform Procedure 2 (Table I) where the ultrafiltration of *A* in the presence of *B* is examined. A plot of (total *A*) *versus* (free *A*) from Procedure 2 should be identical to a similar plot of Procedure 1.

RESULTS

In the first series of experiments performed, ligand *A* was sodium urate and ligand *B* was sodium salicylate. Figure 2 clearly demonstrates the line of identity of free sodium urate *versus* total sodium urate (Procedure 1, Table I), indicating that there was no binding of sodium urate to the membrane and no sieving or reflection of sodium urate by the membrane. This line was identical in the experiment (Procedure 2) in which sodium urate plus salicylate was ultrafiltered. Sodium salicylate, therefore, had no effect on the process of ultrafiltering the sodium urate. With 5.0 g/100 ml albumin in the chamber (Procedure 3), the free sodium urate concentration *(F)* no longer equaled the total urate *(T)*, ($dF/dT < 1$), indicating binding of urate. After equilibrating 5.0 g/100 ml albumin with 0.5 m*M* sodium salicylate (Procedure 4) and exposing the albumin to 0.5 m*M* salicylate during the ultrafiltration with sodium urate in the reservoir (Procedure 5), less urate was bound to the albumin at all levels of total sodium urate than when salicylate was not present. This indicated displacement of sodium urate by salicylate.

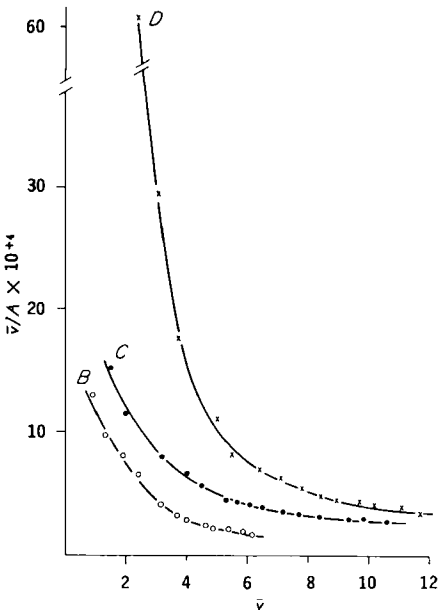

Figure 4—*Scatchard plot of methyl orange–albumin interaction, where* $\bar{v}$ = *moles bound per mole albumin, and* A = *free methyl orange. Points correspond to experimental points of Fig. 3. Key:* B, *without salicylate;* C, *0.5 mM salicylate; and* D, *2.5 mM salicylate. Line was computed from* n *and* k *values of Table II and formula in text (Eq. 8).*

Comparable experiments with methyl orange were performed, and Fig. 3 illustrates the line of identity of (total) *versus* (free) methyl orange, the binding of methyl orange to 0.2 g/100 ml albumin, and the displacing effect of 0.5 and 2.5 m*M* sodium salicylate.

DISCUSSION

The continuous ultrafiltration technique (1) allows, in a single ultrafiltration experiment, a precise investigation of ligand–macromolecule interaction over a wide range of ligand concentration. From one or two experiments, a Scatchard plot may then be constructed, analysis of which gives the number of binding sites *(n)* and their respective association constants *(k)*. From the latter, the free energy of reaction is derived so that precise information as to the molecular interaction is obtained. The value of the Scatchard plot in analyzing the factors responsible for decreased binding was recently discussed (3).

Part of a Scatchard plot derived from a single methyl orange–albumin experiment, together with those plots determined in the presence of 0.5 and 2.5 m*M* salicylate, is given in Fig. 4. These

Table II—Number of Binding Sites (*n*) and Association Constants (*k*) of Methyl Orange–Albumin and Sodium Urate–Albumin Interaction at 22.5°, Ionic Strength 0.16, and pH 7.4

Interaction	n_1	k_1	n_2	k_2
Methyl orange–albumin	3.5	37×10^4	13.5	3.7×10^3
Methyl orange–albumin + 0.5 m*M* sodium salicylate	2.5	12.4×10^4	14	2.8×10^3
Methyl orange–albumin + 2.5 m*M* sodium salicylate	2.0	6.8×10^4	10	2.5×10^3
Sodium urate–albumin	1.0	4.5×10^2	—	—
Sodium urate–albumin + 0.5 m*M* sodium salicylate	1.0	2.1×10^2	—	—

curves were interpreted in terms of two sets of binding sites, and the derived n and k values are listed in Table II. The points on Fig. 4 are experimental; the lines are drawn from the equation:

$$\frac{\bar{v}}{A} = \frac{n_1 k_1}{1 + k_1 A} + \frac{n_2 k_2}{1 + k_2 A} \qquad \text{(Eq. 8)}$$

using the derived n and k values. It is clear that the addition of salicylate at a free concentration of 0.5 mM affects the first set of binding sites; at the higher concentrations of free salicylate (2.5 mM), methyl orange is displaced from both the first and second sets of binding sites. The urate albumin interaction was analyzed previously (4), and the effect of 0.5 mM sodium salicylate on the derived n and k values is shown in Table II.

After determining n and k values, it is possible to predict fractional binding at any concentration of protein and ligand. The fraction bound (β) is given by:

$$\beta = \frac{1}{1 + \frac{1}{(P)nk} + \frac{(A)}{n(P)}} \qquad \text{(Eq. 9)}$$

(P) and (A) being protein and free ligand concentrations, respectively.

Preequilibration of the protein with the competing ligand (ligand B) (Procedure 4, Table I) is necessary, because useful data on displacement may only be obtained when the binding of the primary ligand is studied in the presence of a constant concentration of the competing ligand.

A full discussion of the analyses that allow the association constant of the displacing ligand (ligand B), in this case of salicylate–albumin interaction, to be determined was made by Edsall and Wyman (5).

This method is ideally suited to the *in vitro* determination of drug–protein interaction and drug displacement. It is fast, and analysis of a few experiments yields as much information on the molecular interactions involved as many conventional ultrafiltration, equilibrium dialysis, or gel filtration experiments. Adequate analysis of the data allows a precise prediction of free drug concentration to be made in the presence of varying concentrations of displacing compounds. The results, if indicating displacement, may also alert one clinically to potentiation of action and/or to toxicity when the therapeutic regimen requires the patient to take more than one drug.

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2-Nitrophenylhydrazine: A Selective Reagent for Colorimetric Determination of Carboxylic Acid Anhydrides and Chlorides

JAMES W. MUNSON

Abstract □ Carboxylic acid anhydrides and chlorides react with 2-nitrophenylhydrazine to form the corresponding hydrazides. These hydrazides in aqueous basic solution give a blue color which can be utilized for colorimetric analysis. Both acid anhydrides and acid chlorides undergo this reaction in water, while only the acid chlorides react readily in acetonitrile. The use of acetonitrile as the solvent allows the analysis of acid chlorides in the presence of acid anhydrides. A variety of other carboxylic acid derivatives and carbonyl compounds gave no interference. Suggested analytical procedures for acid anhydrides and acid chlorides are given along with analytical data.

Keyphrases □ 2-Nitrophenylhydrazine—colorimetric reagent for determination of carboxylic acid anhydrides and chlorides □ Carboxylic acid anhydrides and chlorides—colorimetric determination using 2-nitrophenylhydrazine □ Colorimetry—determination, carboxylic acid anhydrides and chlorides using 2-nitrophenylhydrazine

The analysis of carboxylic acid anhydrides and carboxylic acid chlorides has generally been accomplished by one of three methods: direct titration, condensation with a nucleophile and subsequent back-titration of excess nucleophile, or condensation with a nucleophile and subsequent spectrophotometric determination of the product.

Carboxylic acid anhydrides have been titrated directly with sodium methoxide in a nonaqueous medium (1). Carboxylic acid chlorides are readily hydrolyzed to the parent carboxylic acid with the addition of water. The parent acid can be then titrated directly with a standard base. However, any mineral acid present, such as hydrochloric acid, will also be titrated and lead to higher values (2). Acid anhydrides are also hydrolyzed by water, but the reaction is slower than that of acid chlorides.

Anhydrides have been determined by condensation with excess aniline to form the anilide. The unreacted aniline is determined *via* titration with a standard acid (3). An interference encountered in this procedure is the formation of anilides from carboxylic acids which may be present. Other nucleophiles such