Dynamic Dialysis in Drug–Erythrocyte Interactions

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Abstract □ Application of dynamic dialysis to drug binding to erythrocytes was found to be more complex than previously described. Drug molecules located intracellularly in erythrocytes must diffuse through erythrocyte membranes before diffusing through the dialysis membrane to the external sink in dynamic dialysis. One must consider drug within the dialysis sack and within the erythrocyte and their corresponding volumes. When binding occurs in these internal compartments, different equations result for computing D_t, the total drug within the dialysis bag. With these equations and theoretically generated data, the results of "double dialysis" on the data produced in dynamic dialysis studies of drug–erythrocyte interactions were examined. The intracellular binding of drug by the erythrocyte to a single class of preexisting noninteracting sites could be misinterpreted as cooperative binding, or binding to at least two classes of sites, when the data of dynamic dialysis are treated in the Scatchard format.

Keyphrases □ Dialysis, dynamic—drug–erythrocyte interactions □ Erythrocytes—drug interactions, dynamic dialysis analysis

Dynamic dialysis (1) has been used to quantitate drug binding in whole blood and to suspended erythrocytes (2). However, due to the structure of the red blood cell, the technique of dynamic dialysis may not be quantitatively applicable to many drug–erythrocyte interactions.

BACKGROUND

The red blood cell's lipid-like membrane is perforated with positively charged aqueous channels of various diameters (3). Molecules may enter red blood cells by dissolving in the lipid phase or by passively diffusing through aqueous channels. The drug entry rate is governed primarily by the drug's lipid solubility, which is affected by drug ionization.

Drug may be bound (or adsorbed) by the erythrocyte membrane, but most drug taken up by the erythrocytes usually interacts with intracellular components, especially hemoglobin. Thus, to be bound, the drug must pass through the erythrocyte membrane and travel to the intracellular binding site(s). Drug transport is time dependent and is affected by the drug's physical properties (3). For example, a steady-state cell–medium ratio is obtained in less than 5 min for antipyrine and procainamide, 3 hr for serotonin, 7 hr for epinephrine, and >8 hr for norepinephrine. Drug may also exist in the intracellular fluid of the erythrocyte in a free form. Therefore, drug binding is more complicated in erythrocytes than in serum proteins such as albumin.

Cruze and Meyer (2), utilizing dynamic dialysis, reported that salicylate is bound by suspended erythrocytes and that the results obtained may be due to either cooperative binding or the presence of hemolysate. McArthur *et al.* (4), utilizing equilibrium dialysis, also reported salicylate to be bound by red blood cells. However, other studies (5–8) found that salicylate is not bound by red blood cells but is freely permeable to the erythrocyte membrane. Erythrocytes act as a salicylate reservoir, but the salicylate concentration inside the cell is equal to the free salicylate concentration in the plasma. These studies (5–8) suggested that salicylate within the red blood cell is in a free, diffusible form.

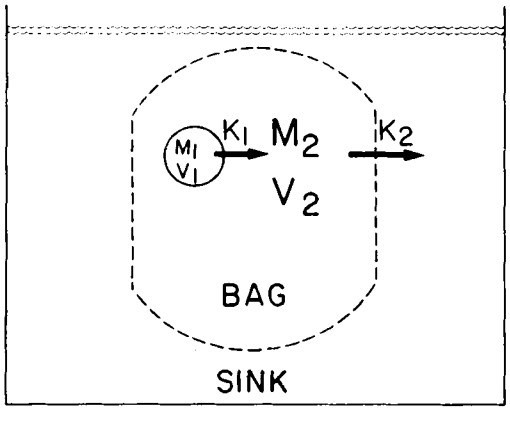

Figure 1—*Schematic representation of the double dialysis theory. For meaning of designations, see text.*

Dynamic dialysis (1) is based on the fact that ligand reversibly bound to protein within a dialysis bag is nondiffusible. The rate of loss of ligand from this compartment to an external sink of buffer is directly proportional to the concentration of unbound ligand. In the absence of protein, the movement of drug from the dialysis bag to the sink is first order and obeys:

$$-\frac{d[D_t]}{dt} = K[D_f] \qquad \text{(Eq. 1)}$$

where:

$[D_t]$ = total molar drug concentration within the dialysis bag at any time, t

K = first-order rate constant for the diffusion of drug out of the dialysis bag (hour^{-1})

$[D_f]$ = molar concentration of unbound drug within the dialysis bag at $[D_t]$

The rate constant, K, is found by dynamic dialysis in the absence of protein. In the presence of protein, the rate of loss of drug is no longer first order, and a plot of log $[D_t]$ *versus* t exhibits marked curvature. The instantaneous rate $-d[D_t]/dt$ at any particular value of $[D_t]$ may then be used, along with K, to determine $[D_f]$ using Eq. 1. In this way, a series of values of $[D_t]$ and their corresponding $[D_f]$ values may be obtained from a single dynamic dialysis experiment.

Since the drug movement across the erythrocyte membrane to its binding site(s) is not instantaneous, the incubation time of a drug–erythrocyte solution before addition of this solution to the dialysis sack may be critical in dynamic dialysis. This factor is not critical in dynamic dialysis studies of drug–albumin interactions since the actual chemical equilibrium of the binding reaction is normally reached within a fraction of a second (9, 10) and drug does not have to pass through a membrane to reach its binding sites.

A more serious potential problem is the effect of drug movement out of the erythrocyte and into the surrounding medium within the dialysis bag on the drug transport rate out of the dialysis bag into the external sink. In such a case, the drug movement out of the dialysis bag may be thought of as involving a double dialysis, *i.e.*, movement of drug out of the red blood cell to the surrounding medium followed by movement of drug through the dialysis membrane to the external sink.

Table I—Constants Used in the Double Dialysis Theory in the Presence of Intracellular Drug Binding

Figure	Curve	K_1, ml/hr	k, M^{-1}	$\frac{K_1}{K_2}$
5	K	0.375	1×10^4	0.25
	L	0.750	1×10^4	0.50
6	M	6	1×10^4	4.00
	N	150	1×10^4	100.00
	P	1,500	1×10^4	1,000.00
	Q	15,000	1×10^4	10,000.00
7	R	0.375	1×10^6	0.25
	S	0.750	1×10^6	0.50
	T	6	1×10^6	4.00
	U	150	1×10^6	100.00
	V	1,500	1×10^6	1,000.00
	W	15,000	1×10^6	10,000.00

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THEORETICAL

The double dialysis theory is represented schematically in Fig. 1. In this figure, the circle represents the erythrocytes of total volume V_1 that contain a mass of drug, M_1. Before dynamic dialysis, the drug within the red blood cells is in equilibrium with the mass of drug, M_2, outside the erythrocytes but within the dialysis bag. Drug mass M_2 is contained within volume V_2, which is equal to the total volume within the dialysis bag, V_t, minus V_1. The dotted line represents the dialysis membrane. K_2 (milliliter per hour) is the rate constant for movement of drug from V_2 to the sink, and K_1 (milliliters per hour) is the rate constant for the net movement of drug out of the erythrocyte during dynamic dialysis.

The simplest case of dynamic dialysis represented by Fig. 1 is when the drug is not bound by the erythrocyte and when, at $t = 0$, $(M_1/V_1) = (M_2/V_2)$. With the assumption that drug transport out of the red blood cell obeys Fick's law of diffusion (11), the equilibria are described by:

$$\frac{dM_1}{dt} = -K_1\left[\frac{M_1}{V_1} - \frac{M_2}{V_2}\right] \qquad \text{(Eq. 2)}$$

$$\frac{dM_2}{dt} = -\frac{K_2M_2}{V_2} + K_1\left[\frac{M_1}{V_1} - \frac{M_2}{V_2}\right] \qquad \text{(Eq. 3)}$$

where:

$$K_2 = \frac{D_eA_e}{h_e}$$

$$K_1 = \frac{D_mA_m}{h_m}$$

D_e = diffusion coefficient of the erythrocyte membrane
A_e = surface area of the erythrocyte membrane
h_e = effective thickness of the erythrocyte membrane
D_m = diffusion coefficient of the dialysis membrane
A_m = surface area of the dialysis membrane
h_m = effective thickness of the dialysis membrane

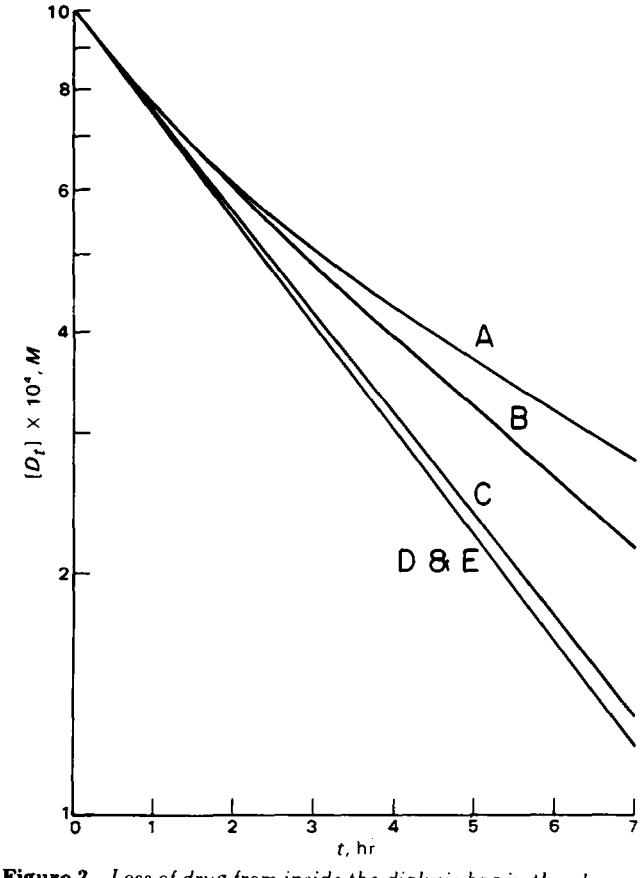

Figure 2—*Loss of drug from inside the dialysis bag in the absence of drug binding by erythrocyte. The value of* K_2 *(milliliters per hour) for each curve is: A, 0.375; B, 0.750; C, 6; and D, 150. Curve E represents drug loss in the absence of protein.*

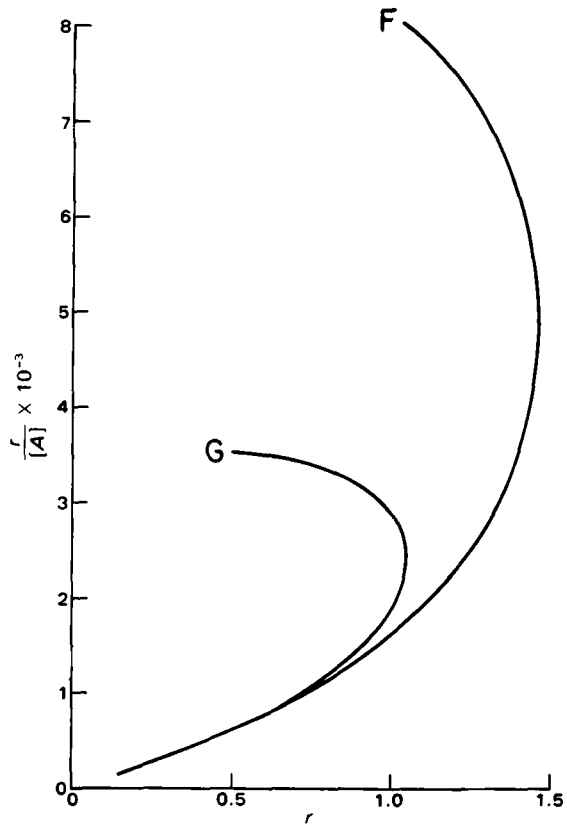

Figure 3—*Scatchard plots resulting from curves A and B of Fig. 2 using* $K = 0.3\ hr^{-1}$.

At $t = 0$, let $M_2 = (M_2)_0$ and $M_1 = (M_1)_0$. Equations 2 and 3 may be integrated to yield:

$$M_1 = \frac{(B - Aa)e^{-at} - (B - Ab)e^{-bt}}{b - a} \qquad \text{(Eq. 4)}$$

$$M_2 = \frac{(B' - A'a)e^{-at} - (B' - A'b)e^{-bt}}{b - a} \qquad \text{(Eq. 5)}$$

where:

$$a = \tfrac{1}{2}\left[\frac{K_1 + K_2}{V_2} + \frac{K_1}{V_1} + \sqrt{\left(\frac{K_1 + K_2}{V_2} + \frac{K_1}{V_1}\right)^2 - \frac{4K_1K_2}{V_1V_2}}\right]$$

$$b = \tfrac{1}{2}\left[\frac{K_1 + K_2}{V_2} + \frac{K_1}{V_1} - \sqrt{\left(\frac{K_1 + K_2}{V_2} + \frac{K_1}{V_1}\right)^2 - \frac{4K_1K_2}{V_1V_2}}\right]$$

$$A = (M_1)_0$$

$$B = (M_1)_0\left(\frac{K_1 + K_2}{V_2}\right) + \frac{(M_2)_0K_1}{V_2}$$

$$A' = (M_2)_0$$

$$B' = \frac{K_1}{V_1}[(M_1)_0 + (M_2)_0]$$

The total mass of drug within the dialysis bag, M_t, at any time t is equal to $M_1 + M_2$. Therefore, upon addition of Eqs. 4 and 5, Eq. 6 is obtained:

$$M_t = \frac{e^{-at}[B + B' - a(A + A')] - e^{-bt}[B + B' - b(A + A')]}{b - a} \qquad \text{(Eq. 6)}$$

or, in terms of concentration:

$$[D_t] = \frac{e^{-at}[B + B' - a(A + A')] - e^{-bt}[B + B' - b(A + A')]}{V_t(b - a)} \qquad \text{(Eq. 7)}$$

where $[D_t]$ is the total drug concentration within the dialysis bag. Equation 7 may be used to generate theoretical data that would result

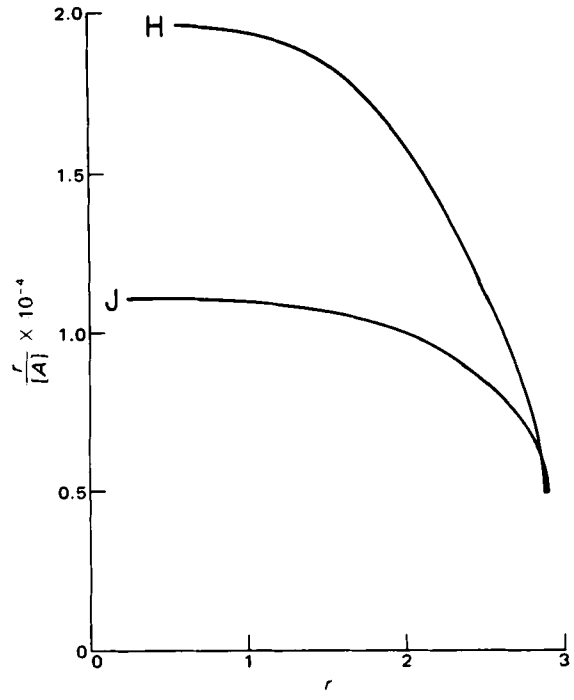

Figure 4—*Scatchard plots resulting from curves A and B of Fig. 2 using corrected* K *of 0.516 hr*$^{-1}$.

from the situation represented by Fig. 1 in the absence of drug binding by erythrocytes.

The drug may be bound by the intracellular components of the erythrocyte. Reversible binding of a ligand by a macromolecule is described by (12):

$$r = \sum_{i=1}^{m} \frac{n_i k_i [A]}{1 + k_i [A]} \qquad \text{(Eq. 8)}$$

where:

- r = mole of ligand bound per total mole of macromolecule in the system
- n_i = number of binding sites in the ith class of sites
- k_i = intrinsic association constant for the binding of drug to the ith class of sites
- $[A]$ = molar concentration of unbound ligand

This equation holds for a macromolecule that only contains noninteracting classes of preexisting binding sites. In the present case of drug binding to intracellular components of erythrocytes, Eq. 8 may be restated as:

$$\frac{M_b}{P} = \sum_{i=1}^{m} \frac{n_i k_i \frac{M_1}{V_1}}{1 + k_i \frac{M_1}{V_1}} \qquad \text{(Eq. 9)}$$

where:

- M_b = moles of drug bound by intracellular components of erythrocytes
- P = moles of macromolecule in the system
- M_1 = moles of drug within the erythrocyte in the free, diffusible form

Equation 9 may be rearranged to obtain:

$$M_b = \sum_{i=1}^{m} \frac{n_i k_i M_1 P}{V_1 + k_i M_1} \qquad \text{(Eq. 10)}$$

When drug is bound by the intracellular components of the red blood cells, the total mass of drug within the dialysis bag, M_t, at any time t is equal to $M_1 + M_2 + M_b$. Therefore, using Eq. 6:

$$M_t = \frac{e^{-at}[B + B' - a(A + A')] - e^{-bt}[B + B' - b(A + A')]}{b - a} + M_b \qquad \text{(Eq. 11)}$$

which may be stated as the following by use of Eqs. 4 and 10:

$$M_t = \frac{e^{-at}[B + B' - a(A + A')] - e^{-bt}[B + B' - b(A + A')]}{b - a} + \sum_{i=1}^{m} \frac{n_i k_i P[(B - Aa)e^{-at} - (B - bA)e^{-bt}]}{V_1(b - a) + k_i[(B - Aa)e^{-at} - (B - bA)e^{-bt}]} \qquad \text{(Eq. 12)}$$

or, in terms of concentration:

$$[D_t] = \frac{e^{-at}[B + B' - a(A + A')] - e^{-bt}[B + B' - b(A + A')]}{V_t(b - a)} + \sum_{i=1}^{m} \frac{n_i k_i P[(B - Aa)e^{-at} - (B - bA)e^{-bt}]}{V_1 V_t(b - a) + V_t k_i[(B - Aa)e^{-at} - (B - bA)e^{-bt}]} \qquad \text{(Eq. 13)}$$

Equation 13 may be used to generate theoretical data that would result from the situation represented by Fig. 1 in the presence of intracellular drug binding by erythrocytes.

To utilize Eq. 1 to find $[D_f]$ (or $[A]$ as defined herein) at the corresponding value of $[D_t]$, the instantaneous slope $d[D_t]/dt$ must be found. In the theoretical work, this instantaneous slope is found by differentiation of the applicable equation used to generate the data. Thus, for the data of Eq. 7:

$$\frac{d[D_t]}{dt} = \frac{-a[B + B' - a(A + A')]e^{-at} + b[B + B' - b(A + A')]e^{-bt}}{V_t(b - a)} \qquad \text{(Eq. 14)}$$

and for the data of Eq. 13:

$$\frac{d[D_t]}{dt} = \frac{-a[B + B' - a(A + A')]e^{-at} + b[B + B' - b(A + A')]e^{-bt}}{V_t(b - a)} + \sum_{i=1}^{m} \frac{n_i k_i P V_1 V_t (b - a)[b(B - Ab)e^{-bt} - a(B - Aa)e^{-at}]}{[V_1 V_t(b - a) + V_t k_i[(B - Aa)e^{-at} - (B - Ab)e^{-bt}]]^2} \qquad \text{(Eq. 15)}$$

THE MODEL

By using Eq. 7 and by choosing values for its constants, the effect of K_1 on the drug diffusion rate out of the bag could be examined theoretically. In this simple model, the total volume within the dialysis bag was 5 ml. The erythrocyte concentration used was the same concentration normally found in blood (2). Assumption of a 46% hematocrit with 9% extracellular fluid in the packed red blood cell mass (3) gives:

$$V_1 = 5 \text{ ml}[46\% - (9\%)(46\%)] = 2.093 \text{ ml} \qquad \text{(Eq. 16)}$$

$$V_2 = V_t - V_1 = 2.907 \text{ ml} \qquad \text{(Eq. 17)}$$

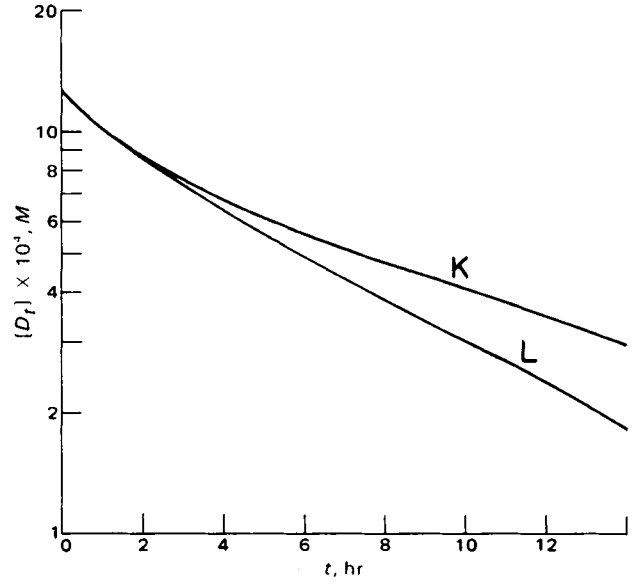

Figure 5—*Loss of drug from inside the dialysis bag in the presence of drug binding by intracellular components of the erythrocyte. See Table I for constants used in generation of individual curves.*

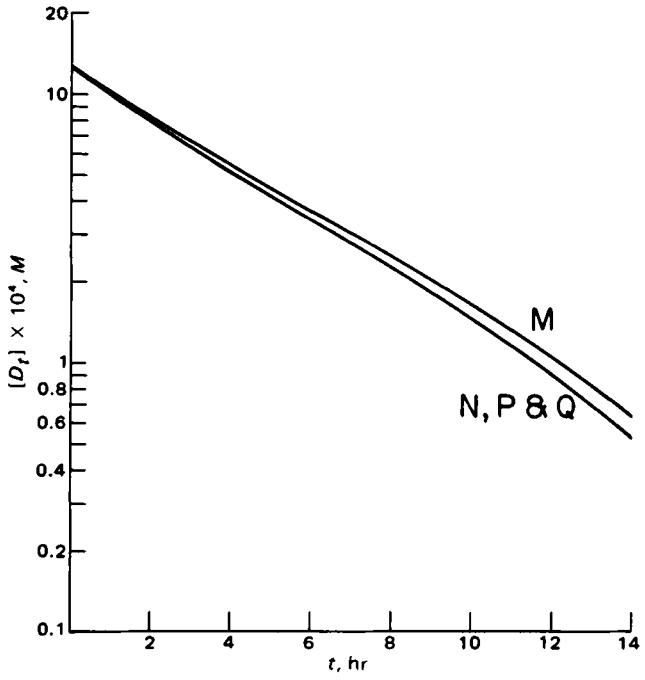

Figure 6—*Loss of drug from inside the dialysis bag in the presence of drug binding by intracellular components of the erythrocyte. See Table I for constants used in generation of individual curves.*

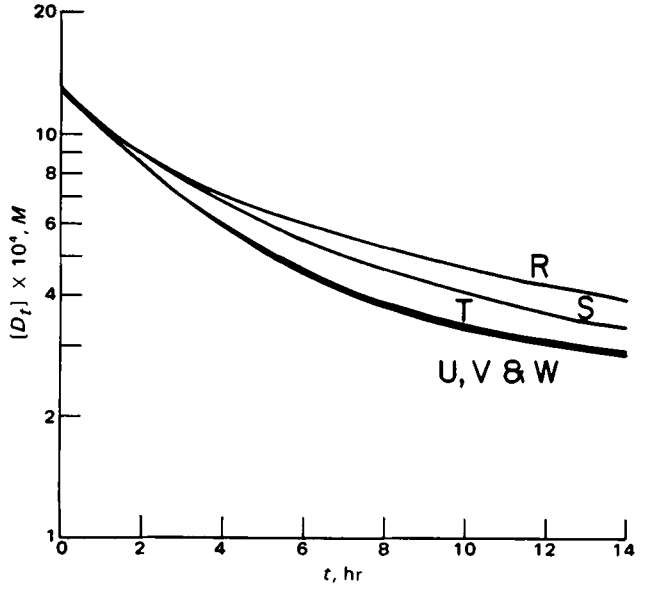

Figure 7—*Loss of drug from inside the dialysis bag in the presence of drug binding by intracellular components of the erythrocyte. See Table I for constants used in generation of individual curves.*

Also, $K = 0.3\ hr^{-1}$ and $[D_t]_0 = 1 \times 10^{-3}\ M$. Therefore, the following values resulted: $K_2 = 1.5$ ml/hr, $(M_1)_0 = 2.093 \times 10^{-6}$ mole, and $(M_2)_0 = 2.907 \times 10^{-6}$ mole.

By using these constants and Eq. 7, the value of $[D_t]$ at any value of t could be found at various values of K_1 in the absence of drug binding by erythrocytes.

By using Eq. 13 and the constants already given, the value of $[D_t]$ at any value of t could be found at various values of k_i and K_1 in the presence of drug binding by intracellular components of erythrocytes. For the construction of Scatchard plots, a 1% concentration of protein of 69,000 molecular weight was assumed, as was done by Cruze and Meyer[1] (2). A different method of constructing Scatchard plots of drug–erythrocyte interactions was proposed recently (13). A single class of sites of $n = 2$ was assumed also.

RESULTS AND DISCUSSION

Figure 2 was obtained using Eq. 7 for the double dialysis in the absence of drug binding by erythrocytes. This figure shows that as drug is "held" in a free state by the erythrocyte, the lower values of K_1 result in slower drug loss from the dialysis bag. This presentation gives the false appearance of drug–erythrocyte binding. This false appearance of binding decreases as K_1 increases and is not visible at $K_1 = 150$ ml/hr where curve D cannot be distinguished from the line obtained for drug loss from the dialysis bag in the absence of protein (curve E). As the ratio of K_1 to K_2 increases, the false appearance of binding decreases.

Scatchard plots of the data obtained using $K_1 = 0.375$ ml/hr and $K_1 = 0.750$ ml/hr indicative of this false binding situation (using Eqs. 1 and 14) are presented as curves F and G, respectively, of Fig. 3. Such unusual Scatchard plots probably result since no binding is occurring. In addition, the value of K of $0.3\ hr^{-1}$ found in the absence of protein in the bag may be adding to the unusual appearance. This value of K, if corrected for the decrease in V_2 that occurs as a result of the presence of erythrocytes, is:

$$\text{corrected } K = \frac{K_2}{V_2} = 0.51600\ hr^{-1} \qquad \text{(Eq. 18)}$$

and, when substituted for the uncorrected K in Eq. 1, results in the Scatchard plots of Fig. 4. Curves H and J are obtained for $K_1 = 0.375$ and 0.750 ml/hr, respectively. These Scatchard plots also have an unusual appearance and could be misinterpreted as resulting from a drug–erythrocyte interaction even though no direct interaction is occurring.

Figures 5–7 show drug loss from inside the dialysis bag in the presence of drug binding by intracellular erythrocyte components. The data were obtained using Eq. 13 for drug binding by erythrocytes. The constants listed in Table I were used to generate the figures.

Curve K of Fig. 5 and the curves of Fig. 7 all have the decreasing slope characteristic of drug–protein interactions. However, curve L of Fig. 5 and the curves of Fig. 6 exhibit first a decreasing and then an increasing slope, similar to those obtained from the dynamic dialysis of both salicylate and sulfathiazole in the presence of either bovine whole blood or suspended erythrocytes (2). These theoretical curves were obtained at a moderate binding strength ($k = 1 \times 10^4\ M^{-1}$) and at all but the lowest K_1 to K_2 ratio. There is only a small change in the curves obtained using a K_1 to K_2 ratio of 4 or 10,000 (Fig. 6). In the case of drug binding by in-

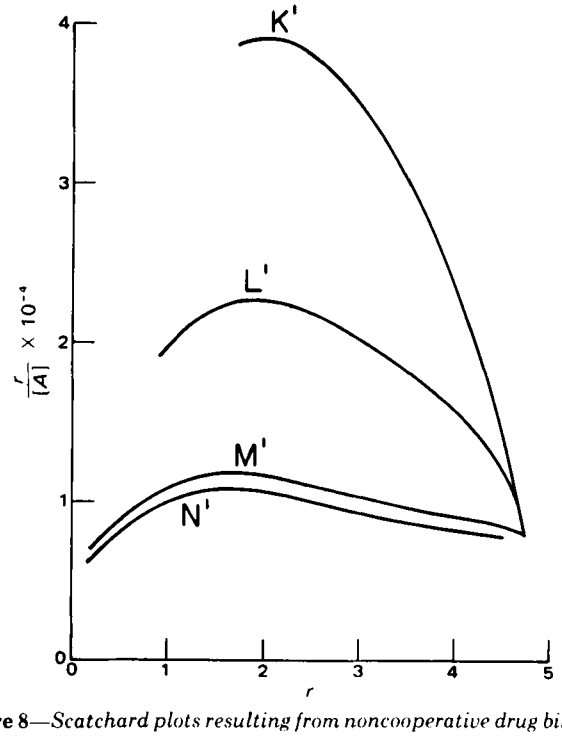

Figure 8—*Scatchard plots resulting from noncooperative drug binding by erythrocyte.*

[1] M. C. Meyer, College of Pharmacy, University of Tennessee Center for the Health Sciences, Memphis, Tenn., personal communication, Oct. 28, 1977.

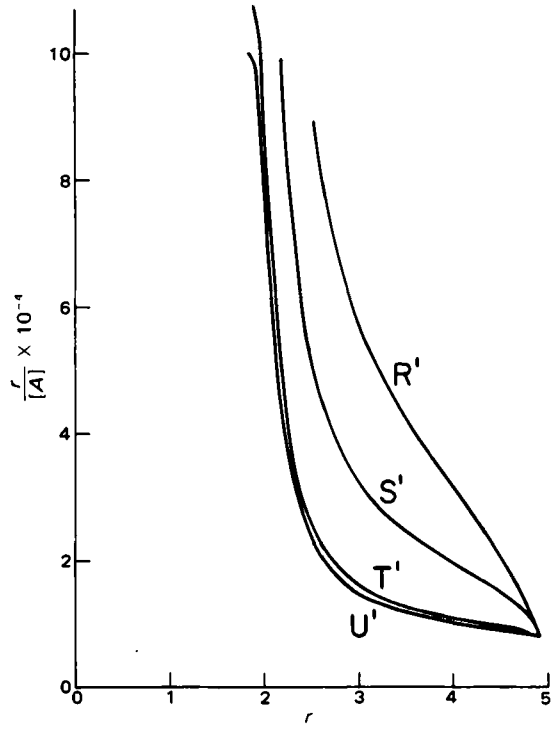

Figure 9—*Scatchard plots resulting from drug binding by a single class of sites on the erythrocyte.*

tracellular erythrocyte components, even at a K_1 to K_2 ratio of 10,000, the double dialysis theory is important, and ignoring it may result in a misinterpretation of dynamic dialysis results. Although the value of K_2 used to obtain these figures may be somewhat low, the results would be similar at higher values of K_2 with the same K_1 to K_2 ratios. In the presence of binding, identical drug escape curves (Figs. 6 and 7) were obtained for all values of the K_1 to K_2 ratio greater than 150.

The Scatchard plots shown in Figs. 8 and 9 were obtained for the data shown in Figs. 5–7. Each Scatchard plot is denoted by the prime of the letter used to identify the curve for drug loss from the dialysis bag. The

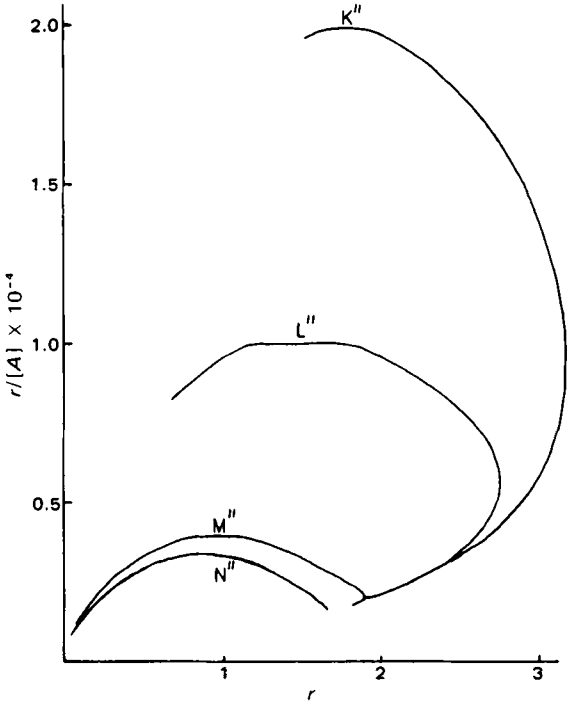

Figure 10—*Scatchard plots resulting from Figs. 5 and 6 using* K = *0.3* hr^{-1}.

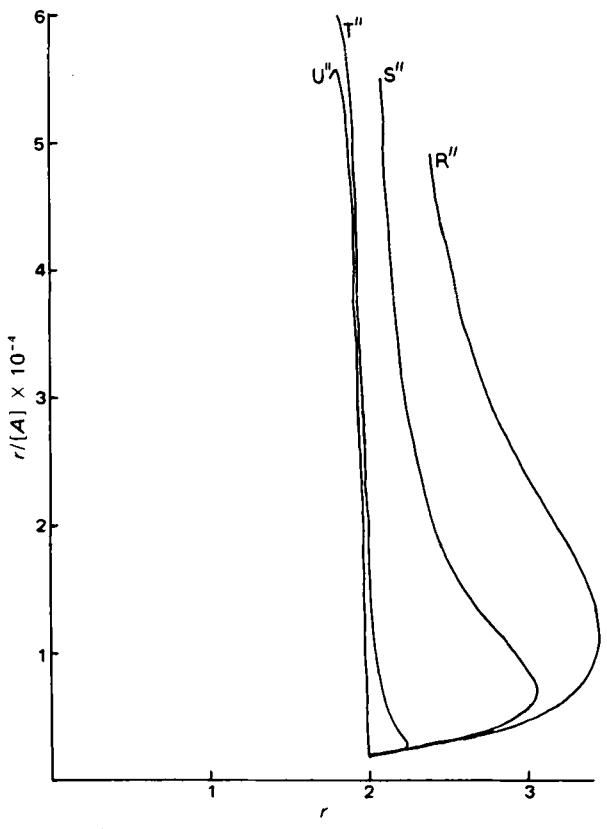

Figure 11—*Scatchard plot resulting from Fig. 7 using* K = *0.3* hr^{-1}.

Scatchard plots were obtained using Eqs. 13 and 1 with the corrected value of K.

The Scatchard plots of Fig. 8 show how the noncooperative drug binding by intracellular erythrocyte components could be misinterpreted as a cooperative drug–erythrocyte interaction. Again, this occurs even at a K_1 to K_2 ratio of 100.

The Scatchard plots of Fig. 9 could also be misinterpreted as a result of ignoring the double dialysis. These curves, especially T′ and U′ obtained at the higher ratios of K_1 to K_2, could be misinterpreted as the interaction of drug with two classes of erythrocyte binding sites. This misinterpretation would be even more likely since the corresponding curves for drug loss from the dialysis bag (Fig. 7) have the constantly decreasing slope that normally results from a drug–protein interaction.

Scatchard plots obtained for the data shown in Figs. 5–7 using the uncorrected K value of 0.3 hr^{-1} in Eq. 1 are shown in Figs. 10 and 11. Each Scatchard plot is denoted by the double prime of the letter used to identify the curve for drug loss from the dialysis bag. The curves may be misinterpreted as being cooperative at $k = 1 \times 10^4$ or as linear but with a slope unequal to $-k$ at $k = 1 \times 10^6$.

Dynamic dialysis studies of drug–erythrocyte interactions could be misinterpreted if double dialysis is ignored. Therefore, such quantitative analysis of drug–erythrocyte interactions should be conducted carefully and compared to results from another experimental method. Equation 15 could be used to interpret correctly the interaction of drug and erythrocytes by dynamic dialysis. However, with the availability of less complex experimental methods such as equilibrium dialysis, the use of dynamic dialysis may prove less than satisfactory. One problem that immediately surfaces with dynamic dialysis is deciding whether a plot of drug loss from the dialysis bag that has a constantly decreasing slope is due to the double dialysis effect alone (Fig. 2) or to drug binding in addition to the double dialysis effect (Fig. 7). Again, equilibrium dialysis should be able to distinguish between these two effects, although this method is not totally free of technical difficulties.

Erythrocytes may be important reservoirs for some drugs without the drug being bound. The drug may exist within the erythrocyte in a free, diffusible form. Thus, no true drug–protein interaction occurs. At equilibrium, the drug concentration within the erythrocyte would be equal to the free drug concentration in the extracellular fluid. Such an entrapment of drug may have the same effect as drug binding by plasma

proteins. These results show that entrapment of drug could resemble a drug–protein interaction at a sufficiently low ratio of K_1 to K_2. This results from the decrease in drug available for diffusion from V_2 to the external sink. At low values of the K_1 to K_2 ratio, as drug is removed from V_2, it is not immediately replaced by drug from within the erythrocytes; the end result is the same as if the drug had actually been bound by the erythrocyte.

Dynamic dialysis may be a useful method for the study of drug entrapment since equilibrium methods cannot be used to determine transport rate constants of drug out of erythrocytes. However, it is important not to confuse drug entrapment by erythrocytes with true drug–erythrocyte complexation.

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High-Performance Liquid Chromatographic Separation and Identification of Epimeric 17-Ketone Impurities in Commercial Sample of Dexamethasone Sodium Phosphate

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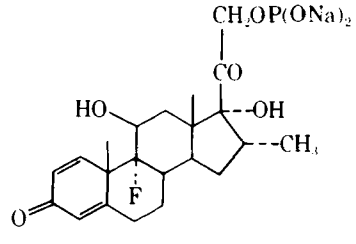
Abstract □ A commercial sample of dexamethasone sodium phosphate solution for injection was found to contain 56% of the label concentration and to be extensively contaminated (~50%) with a white insoluble solid, which was identified as a mixture of the 16α- and 16β-methyl epimers of 9-fluoro-11β-hydroxy-16-methylandrosta-1,4-diene-3,17-dione. High-performance liquid chromatography (HPLC) was used to separate, identify, and quantitate these epimers and to determine their presence in commercial samples. One epimer was identified by HPLC comparison with a synthesized specimen of 9-fluoro-11β-hydroxy-16α-methylandrosta-1,4-diene-3,17-dione. The second peak was identified as the 16β-epimer by epimerization of the synthesized α-component with alkali to obtain a product whose chromatogram matched that of the impurity. These conclusions are supported by data obtained by IR and UV spectrophotometry, TLC, and the blue tetrazolium test.

Keyphrases □ Dexamethasone sodium phosphate—analysis, high-performance liquid chromatography, identification of impurities in a commercial sample, epimerization □ High-performance liquid chromatography—analysis of dexamethasone sodium phosphate and impurities in a commercial sample □ Epimerization—dexamethasone sodium phosphate in a commercial solution

Drug samples contain various impurities: drug intermediates, by-products, and degradation products introduced during manufacture, packaging, or storage, as well as products of the interaction of such substances with excipients in the sample or with the drug itself. These impurities constitute physiological and mechanical hazards. For example, during degradation, the structure of the compound may retain active or modified active centers that, through structural relationship to the intact drug, may bestow undesirable physiological activity or toxic properties on the decomposition product. An example of a physical or mechanical hazard is the presence or formation of insoluble substances during intravenous injection.



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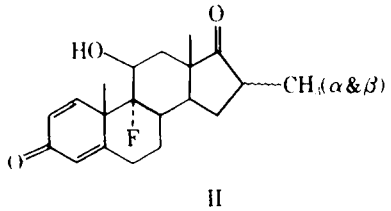

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