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Binding of Salicylate and Sulfathiazole by Whole Blood Constituents

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Abstract □ The binding of salicylic acid and sulfathiazole to bovine whole blood, plasma proteins, and purified albumin fraction was investigated using a dynamic dialysis system. The binding profiles for salicylic acid were quite similar in bovine plasma and 4% bovine serum albumin. In contrast, the binding of sulfathiazole was significantly greater in the plasma than in solutions of fraction V bovine serum albumin. Data from dynamic dialysis binding studies of the compounds, conducted in whole blood and suspended erythrocyte systems, did not lend themselves to analysis by classical methods. Hemolysis and alteration in the nature of the protein binding sites during the binding studies were shown to be factors that could explain the unusual binding observed in the whole blood system.

Keyphrases □ Salicylate—binding to bovine whole blood, plasma proteins, and purified albumin fractions compared □ Sulfathiazole—binding to bovine whole blood, plasma proteins, and purified albumin fractions compared □ Binding—salicylate and sulfathiazole to bovine whole blood, plasma proteins, and purified albumin fractions compared □ Plasma proteins—compared to bovine whole blood and purified albumin fraction binding of salicylate and sulfathiazole □ Albumin—purified fractions, compared to bovine whole blood and plasma protein binding of salicylate and sulfathiazole

Numerous drugs and endogenous substances are reported to be bound by serum proteins (1). Plasma albumin is generally thought to be the primary serum protein responsible for the binding of most drugs. Thus, drug binding studies aimed at determining the affinity of a drug for plasma proteins generally have employed albumin as the macromolecule.

In vitro studies with various protein fractions have shown that the summation of the binding to the individual fractions of the plasma may exceed the binding observed in the whole plasma (2). Therefore, binding studies employing purified protein fractions may not accurately reflect the binding that may occur *in vivo*. Several reports have dealt with the binding of drugs to plasma constituents (3–7). However, relatively little consideration has been given to the binding of drugs by erythrocytes (8, 9) or the binding in whole blood systems (10, 11).

The use of classical methods to study the binding of drugs to plasma and whole blood is difficult and subject to error. In equilibrium dialysis, the time necessary to establish equilibrium may be sufficient to permit significant denaturation of the blood and plasma components. The use of ultrafiltration methods for whole blood binding studies may result in separation of the blood components and blockage of the pores of conventional dialysis membranes. In view of these problems, the dynamic dialysis technique of Meyer and Guttman (12) seemed to hold promise for the quantitation of drug binding in whole blood. The time required for the determination of a complete binding profile is relatively short, temperature can be conveniently controlled, and no centrifugation, vacuum, or pressure is required.

Salicylic acid and sulfathiazole were selected as model compounds to test the applicability of the dynamic dialysis technique to the study of drug binding to bovine whole blood, erythrocytes, plasma, and purified serum albumin.

EXPERIMENTAL

Materials—Fraction V bovine serum albumin[1], sodium salicylate[2], and sulfathiazole sodium[3] were obtained from commercial sources. Regenerated cellulose dialysis tubing[4] was conditioned prior to use by rapidly running distilled water through the tubing for several hours, with the tubing immersed in distilled water. The tubing was then stored in distilled water at 4° until used.

Bovine blood was obtained from the jugular vein of freshly slaughtered cattle and was collected into large vessels containing either heparin[5], 50 units/ml of blood, or anticoagulant citrate dextrose solution A USP, 0.15 ml/ml of blood. The collected blood was divided into two portions. One portion was employed in the whole blood studies, and the other portion was centrifuged to separate the plasma and formed elements. The buffy coat was removed from the packed cells, and the erythrocytes were washed several times with pH 7.38, 0.128 *M*, isotonic phosphate buffer solution. The erythrocytes were then resuspended with the phosphate buffer to produce the same volume present before the plasma was removed.

Dynamic Dialysis Studies—The dynamic dialysis system of Meyer and Guttman (13) was employed with a few modifications. The dialysis behavior of the drug was first characterized with a control solution of the drug in pH 7.38, 0.128 *M* phosphate buffer. A 5-ml sample of the control drug solution was placed inside a 6.5-cm long dialysis sac, secured at one end with a knot and suspended in a jacketed beaker containing 175 ml of the phosphate

[1] Calbiochem, San Diego, Calif.
[2] J. T. Baker, Phillipsburg, N.J.
[3] Sigma Chemical Co., St. Louis, Mo.
[4] Union Carbide dialysis membrane.
[5] The Upjohn Co., Kalamazoo, Mich.

Table I—Binding of Salicylate by 4% Bovine Serum Albumin and Bovine Plasma Proteins

Protein	Buffer	pH	Binding Constants n_1	n_2	K_1, liters/mole	K_2, liters/mole	Temperature
4% bovine serum albumin[a]	Phosphate	7.38	1.04	6.27	5.00×10^4	2.98×10^2	37°
Bovine plasma[a]	Phosphate	7.38	1.01	7.85	6.54×10^4	2.50×10^2	37°
1.61% bovine serum albumin[b]	Phosphate	7.0	1	5	2.0×10^5	1.75×10^3	25°
0.69% bovine serum albumin[c]	Acetate	5.4	0.37	3.5	3.0×10^4	1.00×10^2	4°

[a] This study using pH 7.38, 0.128 *M* phosphate buffer, 37°. [b] Reference 18. [c] Reference 22.

buffer maintained at 37°. The contents of the sac were stirred with a twisted glass rod driven by a constant-speed motor[6], and the external buffer solution was agitated by a magnetic stirring bar.

At various time intervals, 50 ml of the external solution was withdrawn and immediately replaced with 50 ml of fresh buffer solution. The concentration of the small molecule in the external buffer solution was determined spectrophotometrically. The concentration of the small molecule inside the dialysis sac could then be calculated from a knowledge of the initial amount of drug inside the sac and the cumulative amount of drug that had exited the dialysis sac. The binding studies were conducted by replacing the control solution with drug in 5 ml of bovine blood, plasma, suspended erythrocytes, or 4% fraction V purified bovine serum albumin solution.

Binding in Presence of Hemolysis—To study the effects of hemolysis on drug binding, erythrocytes were suspended in an equal volume of 0.128 *M* phosphate buffer and were frozen for 72 hr to produce hemolysis. Upon thawing, 1-, 0.75-, or 0.5-ml aliquots of the resultant solution were added to 4 ml of fraction V bovine serum albumin solutions containing the drug. An appropriate volume of pH 7.38, 0.064 *M* phosphate buffer was added to each solution to maintain the final albumin concentration at 4%, the buffer concentration at 0.013 *M*, and the final volume at 5 ml. Dialysis studies of these solutions were then performed as previously described, using 0.128 *M* phosphate buffer as the external solution.

Analytical Methods—The salicylate and sulfathiazole phosphate buffer solutions were assayed spectrophotometrically for drug at 229 and 255 nm, respectively. Salicylate, in the presence of protein or whole blood, was determined by the method of Trinder (14). The Bratton and Marshall (15) procedure was utilized for the determination of sulfathiazole. Samples obtained from the hemolyzed material, plasma, or whole blood yielded identical blank values when employed in the salicylate or sulfathiazole assays.

The extent of hemolysis was determined spectrophotometrically. A 0.2-ml aliquot of the suspended erythrocytes was diluted to 25 ml with phosphate buffer and incubated for 1 hr in a water bath at 37°. For comparison, 100% hemolysis was achieved by dilution of the suspended erythrocyte samples with distilled water instead of the buffer solution (16).

Total protein and albumin concentrations of the whole blood and plasma were determined by the biuret reaction (17).

Data Treatment—The time course for the loss of drug from each system was analyzed as reported by Meyer and Guttman (18), or a fourth-degree polynomial regression analysis was utilized as described by Crooks and Brown (19). The binding data, computed as previously described from the dialytic rates (18), were employed in the generation of Scatchard plots (20) based on Eq. 1:

$$\bar{V} = \sum_{i=1}^{i=m} \frac{n_i K_i (D_f)}{1 + K_i (D_f)} \qquad \text{(Eq. 1)}$$

where $\bar{V}$ is the moles of small molecule bound divided by the total moles of protein; (D_f) is the free drug concentration; n_i and K_i are the number of binding sites and the corresponding association constant, respectively, for the ith class of binding sites; and m is the number of classes of binding sites.

The binding parameters were evaluated from Scatchard plots with the aid of a nonlinear regression digital computer program based on an algorithm by Marquardt (21). Two classes of binding sites were assumed if the experimentally determined Scatchard plots exhibited curvature.

RESULTS AND DISCUSSION

The binding of salicylate and sulfathiazole was studied to determine the contribution of albumin, plasma, erythrocytes, and whole blood to the binding of each drug *in vitro*.

Binding to Bovine Serum Albumin—Binding studies were ini-

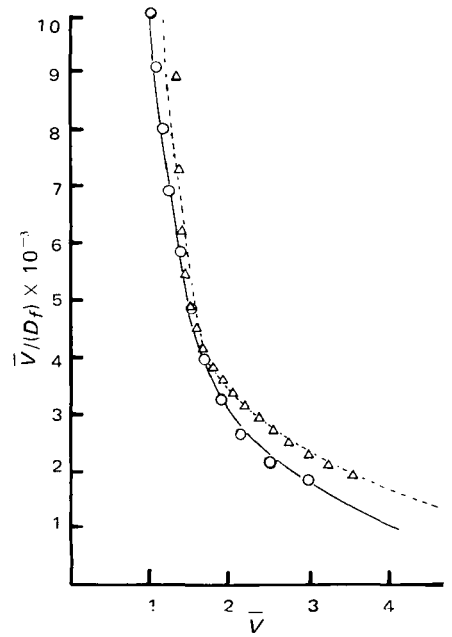

Figure 1—*Scatchard plot of salicylate binding in 4% bovine serum albumin (○) and bovine plasma (△) at 37°.*

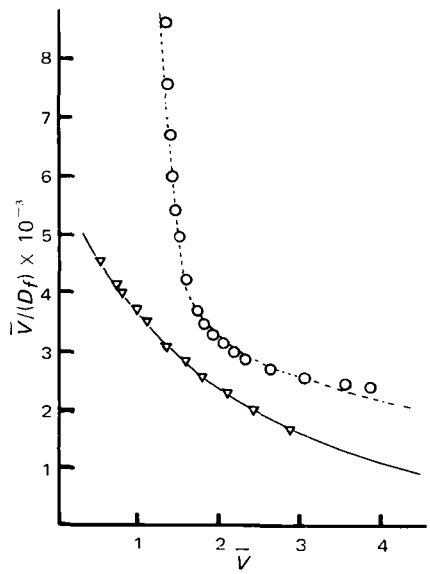

Figure 2—*Scatchard plot of sulfathiazole binding in 4% bovine serum albumin (▽) and bovine plasma (○) at 37°.*

[6] Cole-Parmer Instrument Co., Chicago, Ill.

Table II—Binding of Sulfathiazole to 4% Bovine Serum Albumin and Bovine Plasma Proteins[a]

Protein	Binding Constants			
	n_1	n_2	K_1, liters/mole	K_2, liters/mole
4% bovine serum albumin	1.98	9.77	2.96×10^3	1.21×10^2
Bovine plasma	1.03	8.93	6.51×10^4	2.21×10^2

[a] Using 7.38, 0.128 *M* phosphate buffer, 37°.

tiated with 4% bovine serum albumin solutions, based on the determination that the whole bovine plasma contained approximately 4.1% albumin. Figures 1 and 2 are the Scatchard plots for the binding of salicylic acid and sulfathiazole, respectively, to 4% bovine serum albumin and bovine plasma at 37°. The binding parameters (Tables I and II) were derived from the nonlinear regression analysis of the Scatchard plot.

Previously reported salicylate binding data are also given in Table I for comparison. The binding of the salicylate was somewhat weaker than that reported by Meyer and Guttman (18) but was stonger than found by Davison and Smith (22). The difference may be the result of buffer composition, protein source, degree of ionization, or temperature effects.

Binding to Bovine Plasma—It can be seen from Fig. 1 and Table I that the binding of salicylic acid was quite similar in both bovine plasma and 4% bovine serum albumin. The albumin concentration of the plasma was 4.1%, and the total protein concentration was 8.25%. In view of the comparable salicylate binding in the 4% bovine serum albumin and plasma, it appears that salicylic acid primarily interacts with the albumin.

In contrast, Fig. 2 and Table II illustrate a significant difference in the binding of sulfathiazole in bovine plasma and 4% bovine serum albumin, with a stronger interaction occurring in the plasma. It is apparent from these data that the use of purified fraction V bovine serum albumin may not adequately reflect the extent of binding that may occur for some drugs.

Binding to Whole Blood—As shown in Figs. 3 and 4, the decline of salicylate or sulfathiazole concentration within the dialysis sac with time, in the presence of 4% bovine serum albumin, followed the nonlinear behavior observed by Meyer and Guttman (13) for the dialysis of drugs bound by protein. The whole blood

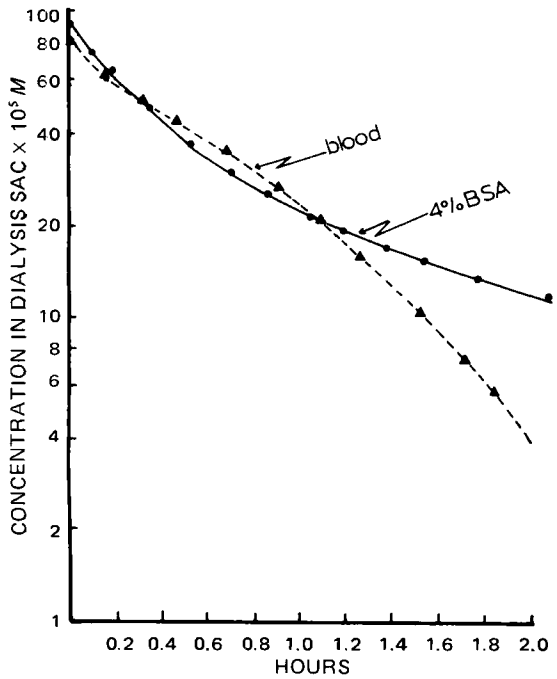

Figure 3—*Loss of salicylate from inside a dialysis sac in the presence of bovine whole blood (▲) and 4% bovine serum albumin (●) at 37°.*

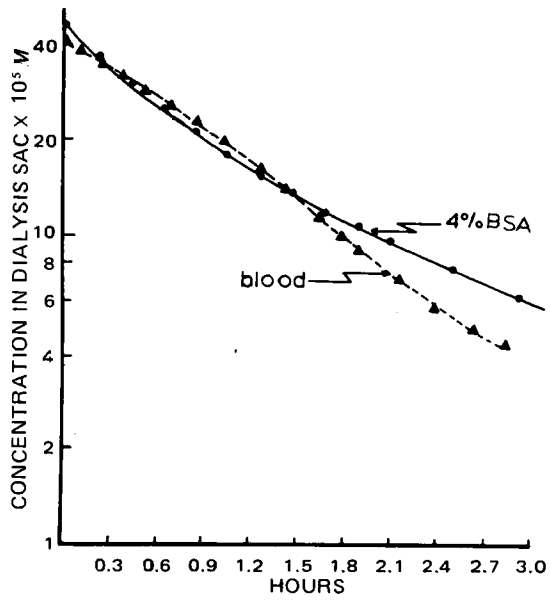

Figure 4—*Loss of sulfathiazole from inside the dialysis sac in the presence of bovine whole blood (▲) and 4% bovine serum albumin (●) at 37°.*

data, however, are most unusual. Instead of a constantly decreasing slope, as was observed for the 4% bovine serum albumin or plasma, the whole blood dialysis exhibited first a decreasing and then an increasing slope. This same phenomenon was observed when the drugs were dialyzed in the presence of suspended erythrocytes.

The dialysis data were fitted to a fourth-degree polynomial regression equation to permit the generation of Scatchard data from the dialytic rates (19). The Scatchard plots for the binding of salicylate in 4% bovine serum albumin, whole blood, and suspended erythrocytes are shown in Fig. 5. Similar profiles were obtained for the binding of sulfathiazole in these systems. In an attempt to rationalize the unusual appearance of the Scatchard plots, several possible explanations were explored. The Scatchard data treatment assumes that the various binding sites are mutually independent. If, in fact, the association of a small molecule with binding sites on the protein affects the binding of subsequent small mole-

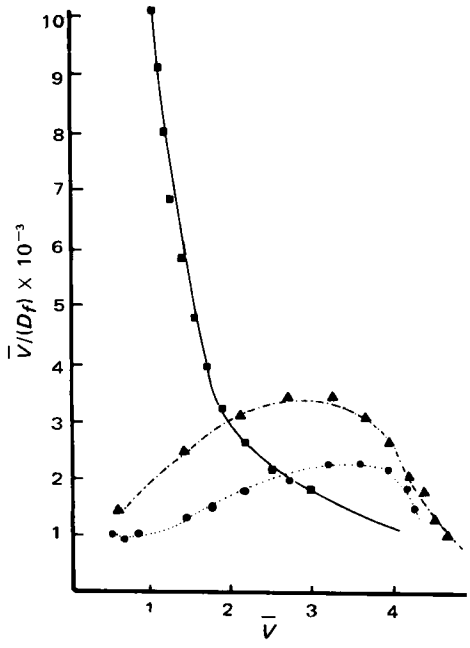

Figure 5—*Scatchard plot of salicylate binding in bovine whole blood (▲), suspended erythrocytes (●), and 4% bovine serum albumin (■) at 37°.*

Table III—Theoretical Scatchard Data for a Typical Drug[a]

$\bar{V}$	(D_f), moles/liter	$\bar{V}/(D_f)$, (liters/mole) × 10^{-5}
5.5	1.1×10^{-4}	0.5
5.0	5.0×10^{-5}	1.0
4.0	2.0×10^{-5}	2.0
3.0	1.0×10^{-5}	3.0
2.0	5.0×10^{-6}	4.0
1.0	2.0×10^{-6}	5.0
0.5	9.1×10^{-7}	5.5

[a] $n = 6$, and $K = 1.0 \times 10^5$ liters/mole.

Table IV—Effect on Binding of a Decreasing Number of Binding Sites[a]

$\bar{V}$	(D_f), (moles/liter) × 10^5	$\bar{V}/(D_f)$, (liters/mole) × 10^{-5}	n
5.0	5.0	1.0	6
4.5	3.0	1.5	6
4.0	2.0	2.0	6
3.5	1.4	2.5	6
3.0	1.5	2.0	5
2.5	1.7	1.5	4
2.0	2.0	1.0	3
1.5	3.0	0.5	2
0.5	1.0	0.5	1

[a] $K = 1.0 \times 10^5$ liters/mole.

cules, the classical mass balance data treatment is not applicable. Similar considerations apply if configurational alterations in the protein structure occur during the drug–protein interactions. For example, a rather complex computer analysis of Scatchard plots exhibiting both positive and negative slopes was described (23–25).

An alternative explanation for the unusual binding observed in the systems containing whole blood or suspended erythrocytes could be the presence of some agent competing with the small molecule for protein binding sites. If the competitor was dialyzed out of the dialysis sac at a different rate than the drug molecule, and/or if the competitor was being released in the dialysis sac during dialysis, the continually changing concentrations of drug and competitor could conceivably produce the observed phenomenon. One source of a potential competitor could be material released from the erythrocytes upon hemolysis. It has been shown, for example, that hemolysis alters the radioimmunoassay for insulin by competitive displacement mechanisms (26).

To test these possible explanations, theoretical data were computed in an attempt to simulate the experimental data. In addition, experiments were designed to study the influence of hemolysis on the binding of salicylate. Theoretical data were generated to simulate binding values that may be obtained if the number of binding sites or the affinity constant characterizing the sites varies over the time course of the dialysis. In addition, data were generated based on the hypothesis that a competitor could be present in increasing concentrations while the drug was continually decreasing in concentration within the dialysis sac.

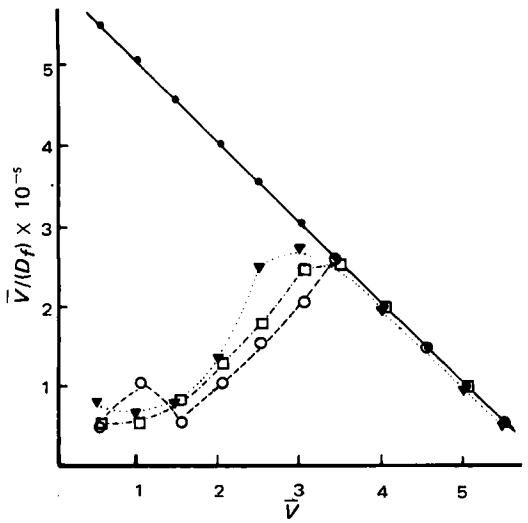

Figure 6—*Scatchard plot of theoretical drug binding data. Key: ●, normal (Table III); ○, decreasing number of sites (Table IV); □, decreasing association constant (Table V); and ▼, increasing competitor concentration (Table VI).*

Table V—Effect on Binding of a Decreasing Association Constant[a]

$\bar{V}$	(D_f), moles/liter	$\bar{V}/(D_f)$, (liters/mole) × 10^{-5}	K, (liters/mole) × 10^{-4}
5.0	5.00×10^{-5}	1.00	10.0
4.5	3.00×10^{-5}	1.50	10.0
4.0	2.00×10^{-5}	2.00	10.0
3.5	1.40×10^{-5}	2.50	10.0
3.0	1.25×10^{-5}	2.40	8.0
2.5	1.43×10^{-5}	1.75	5.0
2.0	1.67×10^{-5}	1.20	3.0
1.5	1.67×10^{-5}	0.90	2.0
1.0	2.00×10^{-4}	0.50	1.0
0.5	1.00×10^{-4}	0.49	0.9

[a] $n = 6$.

For simplicity, the theoretical data were derived assuming that a single class of binding sites was involved. Thus, free drug concentrations (D_f) could be computed, based on a rearrangement of Eq. 1:

$$(D_f) = \frac{\bar{V}}{nK - \bar{V}K} \qquad \text{(Eq. 2)}$$

The binding parameters were arbitrarily fixed at $n = 6$ and $K = 1 \times 10^5$ liters/mole. Table III gives the Scatchard data generated over a free drug concentration range of 10^{-4}–10^{-7} M, assuming constant values for n and K.

Table IV is based on the assumption that the number of available binding sites declines with a decrease in drug concentration. The data in Table V were obtained assuming that the affinity constant increases with decreasing drug concentration.

For the case where a competitor is present, Eq. 3 may be readily derived from the Scatchard equation, as presented by Meyer and Guttman (18):

$$(D_f) = \frac{\bar{V}_D + \bar{V}_D K_C (C_f)}{n_D K_D - \bar{V}_D K_D} \qquad \text{(Eq. 3)}$$

where K_D and K_C are the affinity constants for the drug and competitor, respectively; and (C_f) is the free competitor concentration. Table VI summarizes data generated with the use of Eq. 3, based on the assumption of an increasing competitor concentration occurring as drug concentration declines.

Figure 6 graphically summarizes the data presented in Tables III–VI. These data clearly illustrate that Scatchard plots exhibiting both positive and negative slopes can arise from the assumptions employed in the generation of these data. The similarity be-

Table VI—Effect on Binding of an Increasing Competitor Concentration[a]

$\bar{V}$	(D_f), moles/liter	(C_f), moles/liter	$\bar{V}/(D_f)$, liters/mole
5.5	1.10×10^{-4}	0.00	5.00×10^4
5.0	5.03×10^{-5}	5.00×10^{-9}	9.95×10^4
4.0	2.02×10^{-5}	1.00×10^{-8}	1.96×10^5
3.0	1.10×10^{-5}	1.00×10^{-7}	2.73×10^5
2.5	1.43×10^{-5}	1.00×10^{-6}	1.75×10^5
2.0	1.50×10^{-5}	2.00×10^{-6}	1.33×10^5
1.5	1.67×10^{-5}	4.00×10^{-6}	9.00×10^4
1.0	1.40×10^{-5}	6.00×10^{-6}	7.15×10^4
0.5	6.36×10^{-6}	6.00×10^{-6}	7.90×10^4

[a] $n_D = 6$, $K_D = 1.0 \times 10^5$ liters/mole, $n_C = 6$, and $K_C = 1.0 \times 10^6$ liters/mole.

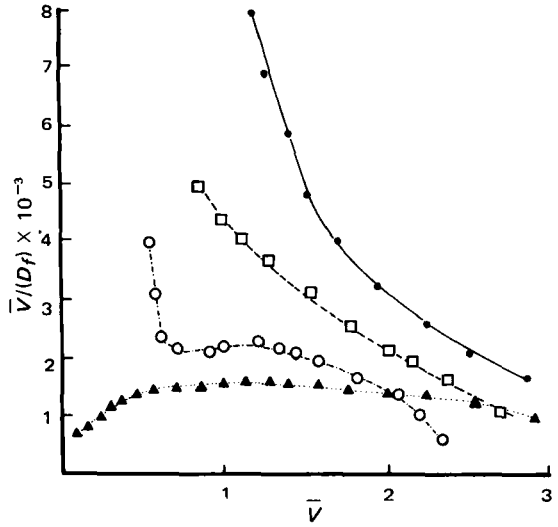

Figure 7—*Effect of hemolysis products on the binding of salicylate by 4% bovine serum albumin. Key: ●, no hemolysate; □, 0.5 ml of hemolysate; ○, 0.75 ml of hemolysate; and ▲, 1.0 ml of hemolysate.*

tween the profiles for these theoretical curves and the experimental data obtained for the binding of salicylate to whole blood and suspended erythrocyte systems is readily apparent. Thus, it appears that the unusual salicylate binding data could be attributed to an alteration in the binding parameters (n's and K's) during dialysis and/or the presence of a competitor species that interferes with the binding of salicylate.

In vitro binding studies were also initiated to investigate further the possibility that hemolysis was contributing to the observed effects. It was determined that between 2 and 10% erythrocyte hemolysis occurred during a dynamic dialysis study. Most hemolysis appeared to be due to the mechanical agitation of the stirring rod rotating within the dialysis sac. Various aliquots of hemolyzed cell constituents were added to solutions of salicylate in 4% bovine serum albumin. The pH of these samples was 7.38, and the phosphate concentration inside the dialysis sac was 0.013 M. The resulting decrease in salicylate binding with increasing amounts of hemolysis product is apparent from Fig. 7.

These results indicate that the characterization of drug binding in whole blood systems is difficult using common methodology and data treatment. The assumption of mutually independent binding sites apparently may not hold for the more complex whole blood systems, and/or hemolysis may produce products that may compete for drug binding sites.

The results of these studies suggest that plasma samples employed in drug binding studies should be obtained only from freshly drawn, nonhemolyzed blood specimens.

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