Structure Relationship for Binding of Sulfonamides and Penicillins to Bovine Serum Albumin by Fluorescence Probe Technique

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Abstract □ The binding constants and the number of binding sites for the binding of 11 sulfonamide and seven penicillin derivatives to bovine serum albumin were determined using a fluorescence probe method. The results demonstrate that these drugs bind to hydrophobic sites on the serum albumin. The binding affinities of sulfonamide and penicillin are greatly affected by their side-chain substitutions. The binding is enhanced by the hydrophobic substitutions but decreased by the hydrophilic substitutions on the parent molecules.

Keyphrases □ Sulfonamides—relationship between structure and binding to bovine serum albumin, fluorescence probe technique, binding constants □ Penicillins—relationship between structure and binding to bovine serum albumin, fluorescence probe technique, binding constants □ Binding, sulfonamides and penicillins to bovine serum albumin—effect of structure, fluorescence probe technique, binding constants □ Fluorescence probe technique—used to study relationship between sulfonamide and penicillin structure and their binding to bovine serum albumin

The fact that plasma protein-bound sulfonamides and penicillins are devoid of antimicrobial activity is well established (1–3). It has also been found that these drugs bind predominantly to the albumin fraction of plasma (4, 5). Although various binding mechanisms have been proposed (6, 7), the techniques commonly used for binding studies have generally yielded somewhat limited information on the nature involved in the formation of the complex. The use of fluorescence probes has provided insight into the mechanisms and sites of binding of small molecules and has been shown (8) to be a convenient tool for competition studies of small molecules for protein binding sites.

A fluorescence probe (9, 10) is defined as a compound that undergoes changes in one or more of its fluorescence properties when bound to certain proteins. The observation (8) that certain probes bind to a number of highly hydrophobic protein sites led to the proposition that these compounds might be useful for investigation of the hydrophobic nature of protein (9–11). An additional observation (12) suggested that the fluorescence intensity of the probes in protein dispersion is decreased by the introduction of certain drug molecules. This is taken as an indication that competition between drug and probe does occur and that the drug falls into the same type of binding category as characterized by the probe (12).

1-Anilinonaphthalene-8-sulfonic acid has been used as a fluorescence probe to detect binding at the hydrophobic sites of proteins. Competition with a drug for these sites reduces the fluorescence yield of the probe–protein complex. In the present investigation, 11 sulfonamide and seven penicillin analogs were studied by the probe technique; the emphasis was on their structure–activity relationships.

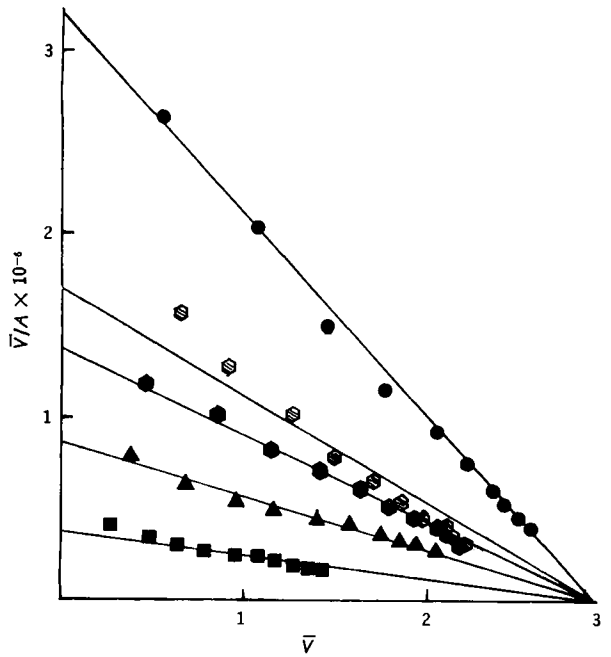


Figure 1—*Scatchard plots of 1-anilinonaphthalene-8-sulfonic acid binding to bovine serum albumin at 27° and pH 7.45. Key: ●, in the absence of drug; ○, in the presence of 2 × 10^{-4} M sulfaphenazole; ○, in the presence of 1 × 10^{-4} M sulfamethoxypyridazine; ▲, in the presence of 5 × 10^{-4} M sulfamethizole; and ■, in the presence of 5 × 10^{-4} M sulfadimethoxine.*

EXPERIMENTAL

Materials—All sulfonamides[1] were recrystallized from ethanol–water mixtures. All penicillins[2] were used without further purification. 1-Anilinonaphthalene-8-sulfonic acid[3] (recrystallized twice from water), spectrograde methanol[4], bovine serum albumin[5] (crystalline), and double-distilled water were also used. All other chemicals[6] were reagent grade and were used without further purification.

[1] Sulfisoxazole, Roche, Lot 634069; sulfadimethoxine, Roche, Lot 256050; sulfamethoxazole, Roche, Lot 797031; sulfamethazine, American Pharmaceutical Co., Lot 2915; sulfamerazine, American Pharmaceutical Co., Lot 2915; sulfapyridine, American Pharmaceutical Co., Lot 2915; sulfadiazine, American Pharmaceutical Co., Lot 1784; sulfamethoxypyridazine, Lederle, Lot 1906-JU88; sulfaphenazole, Purdue-Frederick Co., Lot RM9902; sulfamethizole, Ayerst Laboratories, Lot p2935A; and sulfisomidine. Ciba-Geigy, Lot H7531.

[2] Sodium dicloxacillin, Bristol, Lot 71F1026; sodium cloxacillin, Bristol, Lot 71F1009; sodium oxacillin, Bristol, Lot 71F381; sodium methicillin, Bristol, Lot 71F378; potassium penicillin G, Upjohn, Lot 3035K; ampicillin, Wyeth Laboratories, Lot W-703632; and potassium phenoxymethyl penicillin, Abbott, Lot 07-159-CD.

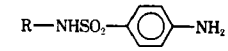
[3] Aldrich Co., Milwaukee, WI 53233

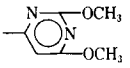
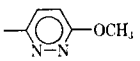
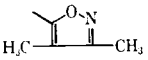
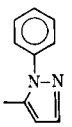
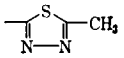
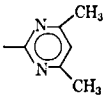
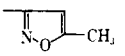
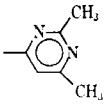
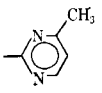
[4] Fisher Scientific Co., Fair Lawn, N.J.

[5] Nutritional Biochemical Co., Cleveland, OH 44128. Concentration was determined by measuring the absorbance of solutions at 280 nm, using $E_{1cm}^{1\%}$ value of 6.6.

[6] J. T. Baker Co.

Table I—Binding Data for Sulfonamides to Blood Fractions



Compound	R	Blood Fraction	Binding Data[a]		Reference
1-Anilinonaphthalene-8-sulfonic acid		Bovine albumin[b]	$n = 2.9$	$k = 1.1 \times 10^6/M$	This study
Sulfadimethoxine		Human serum	$n_1 = 2.12$	$k_B = 7.1$	19
		Bovine albumin	$n = 2$	$\log k = 5.4$	20
		Human plasma	$n = 2.0$	$k = 1 \times 10^4/M$	21
		Bovine albumin	$n = 2.5$	$k = 2.30 \times 10^4/M$	22
		Bovine albumin[b]	$n = 2.9$	$k = 1.5 \times 10^4/M$	This study
Sulfamethoxypyridazine		Human albumin	83%		2
		Human plasma	80%		23
		Human albumin	$n = 70$	$k = 9.78 \times 10^3/M$	24
		Human serum	$n_1 = 1.95$	$k_B = 37.1$	19
		Bovine albumin	$n = 2$	$\log k = 4.8$	20
		Bovine albumin[b]	$n = 2.9$	$k = 1.4 \times 10^4/M$	This study
Sulfisoxazole		Human albumin	84%		2
		Bovine albumin	$n = 2.0$	$\log k = 5.0$	20
		Bovine albumin	$n = 2.5$	$k = 1.47 \times 10^4/M$	22
		Bovine albumin[b]	$n = 2.9$	$k = 9.6 \times 10^3/M$	This study
Sulfaphenazole		Human plasma	90%		23
		Bovine albumin	$n = 2.0$	$\log k = 3.3$	20
		Bovine albumin	$n = 2.5$	$k = 1.07 \times 10^4/M$	22
		Bovine albumin[b]	$n = 2.9$	$k = 5.6 \times 10^3/M$	This study
Sulfamethizole		Bovine albumin	$n = 2.0$	$\log k = 4.3$	20
		Bovine albumin[b]	$n = 2.9$	$k = 5.2 \times 10^3/M$	This study
Sulfamethazine		Human albumin	58%		16
		Human albumin	$m = 0.66$	$\log k = 7.9$	25
		Bovine albumin[b]	$n = 2.9$	$k = 2.9 \times 10^3/M$	This study
Sulfamethoxazole		Human serum	$n_1 = 3.26$	$k_B = 449$	19
		Bovine albumin[b]	$n = 2.9$	$k = 1.6 \times 10^3/M$	This study
Sulfisomidine		Bovine albumin	$n = 2$	$\log k = 3.1$	20
		Human plasma	$n = 1.1$	$k = 5 \times 10^3/M$	21
		Bovine albumin	$n = 2.5$	$k = 3.60 \times 10^3/M$	22
		Bovine albumin[b]	$n = 2.9$	$k = 1.4 \times 10^3/M$	This study
Sulfamerazine		Human albumin	57%		16
		Human albumin	$m = 0.72$	$\log k = 2.8$	25
		Bovine albumin	$n = 2$	$\log k = 3.7$	20
		Bovine albumin[b]	$n = 2.9$	$k = 6.6 \times 10^2/M$	This study
Sulfapyridine		Human plasma	45%		1
		Bovine albumin	$n \times k = 4.2 \times 10^3/M$		26
		Bovine albumin[b]	$n = 2.9$	$k = 4.5 \times 10^2/M$	This study
Sulfadiazine		Human plasma[c]	55%		1
		Human albumin[d]	32%		16
		Human albumin	33%		2
		Human serum	$n_1 = 1.86$	$k_B = 775.6$	19
		Bovine albumin	$n = 2$	$\log k = 2.0$	20
		Human albumin	$n_1 = 1$	$k_1 = 2.4 \times 10^3/M$	27
			$n_2 = 2$	$k_2 = 3.5 \times 10^2/M$	
		Bovine albumin[b]	$n = 2.9$	$k = 4.2 \times 10^2/M$	This study

[a] n, n_1, and n_2 = number of binding sites; k, k_1, k_2, and k_B = binding constants; % = percentage of drug bound to blood fractions; and m = slope for the Freundlich-type equation (17), obtained by plotting logarithmic concentrations of bound drug *versus* free drug. [b] The pH and temperature of the solutions were 7.45 and 27°, respectively. [c] Drug and plasma concentrations were 10 and 7%, respectively. [d] Drug and plasma albumin concentrations were 10 and 3%, respectively.

Methods—The binding of the probe, 1-anilinonaphthalene-8-sulfonic acid, to bovine serum albumin was determined by measuring the increase in fluorescence following the titration of the protein solution with the probe as described by Brand *et al.* (8) and modified as described below. The fluorescence measurements were made at 475 nm with a spectrophotofluorometer[7], using an excitation wavelength of 375 nm. The temperature of all measurements was maintained at 27 ± 1°.

Solutions of bovine serum albumin were prepared in 0.05 *M* phosphate buffer at pH 7.4. The probe, 1-anilinonaphthalene-8-sulfonic acid, was dissolved in methanol at a concentration of 1×10^{-3} *M*. Successive aliquots of 2 μl of probe solution were added directly to the cell, which contained 2 ml of bovine serum albumin; addition was made by means of a microsyringe[8]. Titrations with probe were carried out at both low (1.38×10^{-6} *M*) and high (1.38×10^{-5} *M*) bovine serum albumin concentrations.

Drug binding was determined by titrating 2 ml of the low protein solution with successive additions of 2 μl of 1×10^{-3} *M* 1-anilinonaphthalene-8-sulfonic acid in the presence of drug compound.

Because of their low solubilities in water and alcohol, all of the sulfonamides examined, except sulfisoxazole and sulfaphenazole, were dissolved in 0.1 *N* NaOH to make a concentration of 0.1 *M* with ensuing conversion to their sodium salts. Ten microliters of each sulfonamide solution was injected separately into 2 ml of 1.38×10^{-6} *M* bovine serum albumin prior to titration as pre-

[7] Aminco-Bowman, American Instrument Co., Silver Spring, Md.

[8] Hamilton, Hamilton Co., Whittier, Calif.

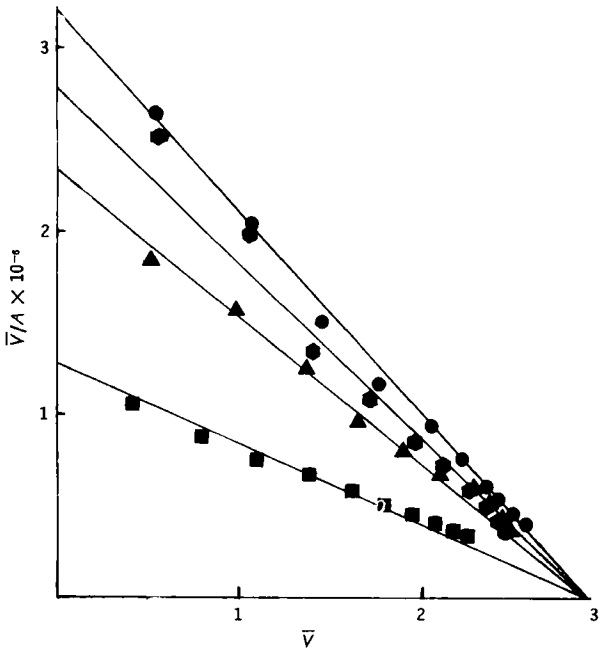


Figure 2—*Scatchard plots of 1-anilinonaphthalene-8-sulfonic acid binding to bovine serum albumin at 27° and pH 7.45. Key: ●, in the absence of drug; ○, in the presence of 5 × 10⁻⁴ M sulfadiazine; ▲, in the presence of 5 × 10⁻⁴ M sulfamerazine; and ■, in the presence of 5 × 10⁻⁴ M sulfamethazine.*

viously described (13). The final concentration of each sulfonamide solution was 5×10^{-4} *M*, except for sulfaphenazole and sulfamethoxypyridazine which were 2×10^{-4} and 1×10^{-4} *M*, respectively. The final pH ranged from 7.40 to 7.45. Sulfisoxazole and sulfaphenazole were dissolved in methanol; the final pH and the ionic strength of the protein solutions, to which sulfisoxazole and sulfaphenazole were added, were controlled by the addition of 0.1 *N* NaOH to the buffer solution to eliminate those variables.

In the case of ampicillin, dimethyl sulfoxide was used to effect dissolution of the drug and the remaining penicillins were dissolved in double-distilled water. All solutions were 0.1 *M* with respect to the individual penicillin.

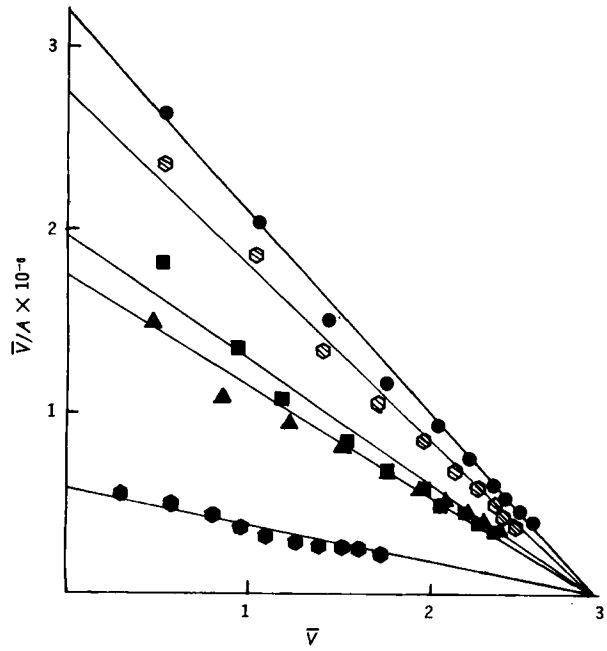


Figure 3—*Scatchard plots of 1-anilinonaphthalene-8-sulfonic acid binding to bovine serum albumin at 27° and pH 7.45. Key: ●, in the absence of drug; ○, in the presence of 5 × 10⁻⁴ M sulfapyridine; ■, in the presence of 5 × 10⁻⁴ M sulfisomidine; ▲, in the presence of 5 × 10⁻⁴ M sulfamethoxazole; and ○, in the presence of 5 × 10⁻⁴ M sulfisoxazole.*

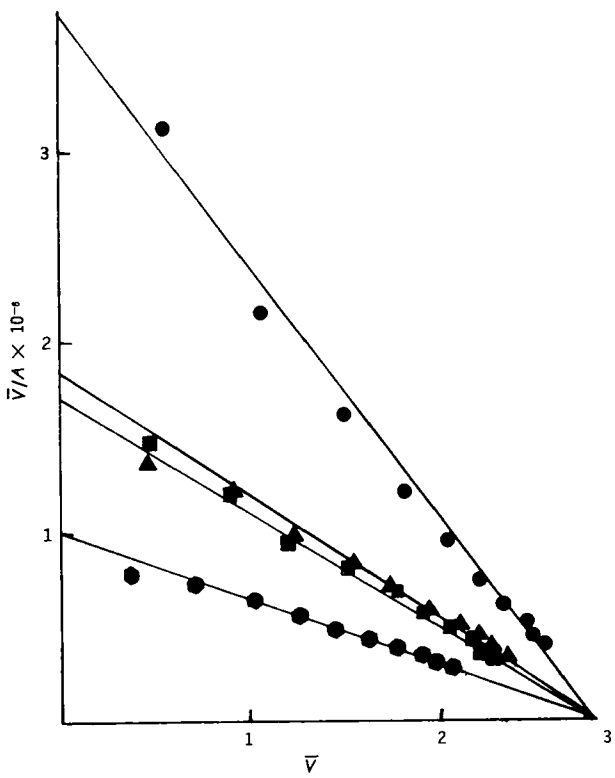


Figure 4—*Scatchard plots of 1-anilinonaphthalene-8-sulfonic acid binding to bovine serum albumin at 27° and pH 7.40. Key: ●, in the absence of drug; ▲, in the presence of 5 × 10⁻⁴ M potassium phenoxymethyl penicillin; ■, in the presence of 5 × 10⁻⁴ M potassium penicillin G; and ○, in the presence of 5 × 10⁻⁴ M ampicillin.*

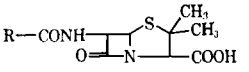
RESULTS AND DISCUSSION

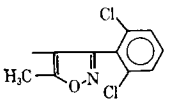
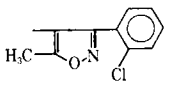
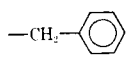
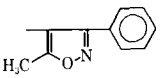
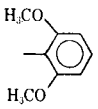
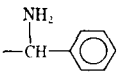
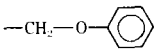
Binding of Sulfonamides to Bovine Serum Albumin—Scatchard plots of the results with sulfonamides (Figs. 1–3) were constructed by using the Scatchard equation (14) as previously described (13). The common intercept indicates that binding occurs at the same sites on the albumin where the probe is bound. The binding affinities are characterized by the binding constants calculated from the equation of Klotz *et al.* (15). These binding constants are compared with the results from published data (Table I). Although the results varied, the rank order of all data seems to be in agreement.

Sulfadimethoxine, sulfamethoxypyridazine, sulfisoxazole, sulfaphenazole, and sulfamethizole can be classified as highly bound, with affinities higher than $5 \times 10^3/M$. Sulfamethazine, sulfamethoxazole, and sulfisomidine can be classified as moderately bound, with affinities between 5×10^3 and $1 \times 10^3/M$; sulfamerazine, sulfapyridine, and sulfadiazine are weakly bound, with affinities below $1 \times 10^3/M$. These differences in affinity may be due to differences in structure (Table I). With sulfamethazine, sulfamerazine, and sulfadiazine, it is seen that the binding affinities increase from 4.2×10^2 to 6.6×10^2 to $2.9 \times 10^3/M$ with the increasing number of methyl groups on the pyrimidine ring. This indicates that methyl groups on the pyrimidine ring increase the phenomenon of protein binding. A similar conclusion was also given by Van-Dyke *et al.* (16).

Sulfisomidine, having two methyl groups on its pyrimidine ring, has a higher binding constant than sulfapyridine, which has none. Sulfisoxazole has two additional methyl groups on its oxazole ring when compared with sulfamethoxazole and, therefore, exhibits a higher binding constant even though the *p*-aminobenzenesulfonamide moiety is substituted at a different position on the oxazole ring. Sulfadimethoxine, having two methoxyl groups

Table II—Binding Data for Penicillins to Blood Fractions



Compound	R	Blood Fraction	Binding Data[a]	Reference
Dicloxacillin		Human serum	97.9%	28
		Bovine albumin[b]	$n = 2.8$ $k = 7.5 \times 10^3/M$	This study
Cloxacillin		Human serum	94%	29
		Human serum	95.2%	28
		Bovine albumin[b]	$n = 2.8$ $k = 7.2 \times 10^3M$	This study
Penicillin G		Human albumin	58%	7
		Human albumin, fraction V	60%	7
		Bovine albumin	52%	7
		Human serum	60.7%	30
		Human serum	66.7%	31
		Human serum	59%	29
		Bovine serum	37.7%	32
		Human serum	48%	33
		Human serum	48.7%	34
		Bovine albumin[b]	$n = 2.8$ $k = 5.2 \times 10^3/M$	This study
Oxacillin		Human serum	94.2%	28
		Human serum	93.1%	29
		Human serum	87%	35
		Bovine serum	58.4%	32
		Bovine albumin[b]	$n = 2.8$ $k = 4.5 \times 10^3/M$	This study
Methicillin		Human serum	49%	30
		Human serum	37.3%	28
		Human serum	49.3%	29
		Human serum	22%	35
		Bovine albumin[b]	$n = 2.8$ $k = 2.9 \times 10^3/M$	This study
Ampicillin		Human serum	18%	30
		Human serum	22.5%	28
		Human serum	18%	29
		Bovine serum	24.2%	32
		Bovine albumin[b]	$n = 2.8$ $k = 2.1 \times 10^3/M$	This study
Phenoxymethyl penicillin		Human serum	79.5%	30
		Bovine albumin, fraction V	$n_1 = 1$ $n_2 = 3$ $n_3 = 82$ $k_1 = 1887/M$ $k_2 = 14/M$ $k_3 = 15.2/M$	36
		Human serum	83.4%	31
		Human serum	78.5%	28
		Human serum	79.7%	29
		Bovine serum	53.8%	32
		Human serum	75.5%	33
		Human serum	74.4%	34
		Bovine albumin[b]	$n = 2.8$ $k = 1.7 \times 10^3/M$	This study

[a] n, n_1, n_2, and n_3 = number of binding sites; k, k_1, k_2, and k_3 = binding constants; and % = percentage of drug bound to blood fractions. [b] The pH and temperature of the solutions were 7.40 and 27°, respectively.

on its pyrimidinyl ring, has a higher binding constant than sulfamethoxypyridazine, which has one on its pyridazinyl ring.

Scholtan (17), studying the structure effects of sulfonamides on their binding to serum, also found that binding is strengthened by the presence of alkyl, alkoxyl, and halogen groups in the sulfonamide structure. Furthermore, he reported that binding was strengthened by the substitution of an oxygen atom for a sulfur atom but was reduced by amino substitution. These effects suggest that the binding of sulfonamide to serum proteins is of a hydrophobic nature.

Jardetzky and Wade-Jardetzky (18) used an NMR relaxation technique to investigate the binding of sulfonamides and found that sulfaphenazole may bind to albumin either by its *p*-aminobenzenesulfonamide moiety or by its phenyl ring. The fact that sulfaphenazole is highly bound to albumin is probably due to the phenyl substitution on the pyrazole ring as a result of strengthening hydrophobic bonding.

Binding of Penicillins to Bovine Serum Albumin—The Scatchard plots of the data for each penicillin are shown in Figs. 4 and 5. The plots, both in the absence and presence of penicillin, intercept at the same point on the X axis, indicating the competition between the probe and penicillins at the same or adjacent hydrophobic sites. The binding affinities, calculated from the Klotz equation (12, 15), are shown in Table II.

According to Kunin (31, 34), penicillins with binding constants less than $4.0 \times 10^3/M$ do not bind to a therapeutically significant degree. In the present studies, sodium dicloxacillin, sodium cloxacillin, potassium penicillin G, and sodium oxacillin are strongly bound to albumin, with binding constants greater than $4.0 \times 10^3/M$. Sodium methicillin, ampicillin, and potassium phenoxymethyl penicillin are less strongly bound, with binding constants of less than $4.0 \times 10^3/M$.

A comparison of published data with the data reported here shows that the relative binding affinities of these drugs (Table II) are generally in good agreement.

The binding affinities of penicillins vary with different side-chain substituents. These side-chain groups all possess a phenyl ring, which has been shown by NMR studies (37) to be the primary binding site.

From the binding affinities determined by the probe technique for sodium dicloxacillin, sodium cloxacillin, and sodium oxacillin, it is seen that the binding is enhanced by halogen substitution on the phenyl ring. Since the oxacillins are shown generally to have greater binding affinities than the group of penicillins at large, it is clear that the methoxazole ring has an enhancing effect on the binding of these drugs to proteins. The methoxazole ring is also present in both sulfisoxazole and sulfamethoxazole, and they also bind more strongly than the class as a whole.

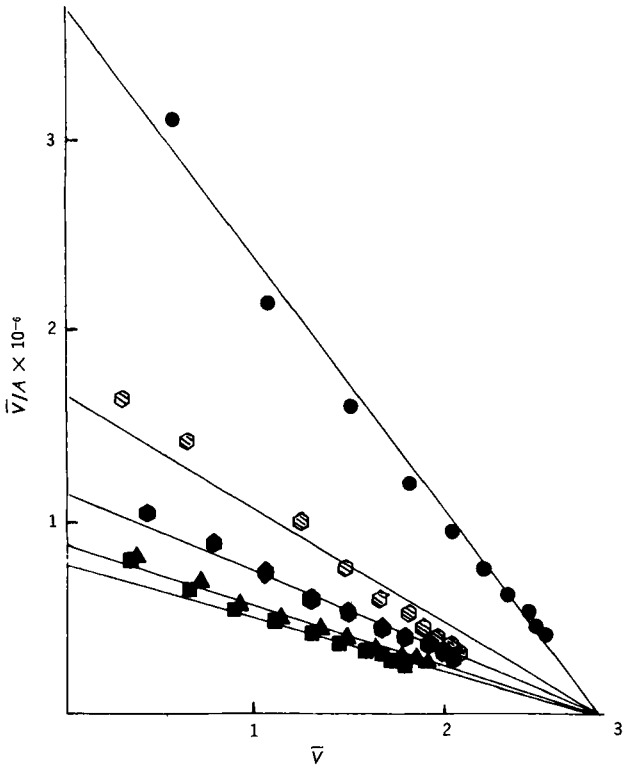


Figure 5—*Scatchard plots of 1-anilinonaphthalene-8-sulfonic acid binding to bovine serum albumin at 27° and pH 7.40. Key: ●, in the absence of drug; ○, in the presence of 5 × 10⁻⁴ M sodium oxacillin; ▲, in the presence of 5 × 10⁻⁴ M sodium cloxacillin; ■, in the presence of 5 × 10⁻⁴ M sodium dicloxacillin; and ○, in the presence of 5 × 10⁻⁴ M sodium methicillin.*

In contrast to the effect of halogen substitution, an amino substitution may reduce the binding affinity. As shown in Table II, the binding constant of potassium penicillin G was found to be $5.2 \times 10^3/M$ at pH 7.40 while that for ampicillin was found to be $2.0 \times 10^3/M$.

Phillips *et al.* (38) used the "hydrated electron" to detect the ionic binding of the carboxylic group of penicillin. They found that ionic binding was greatly reduced with similar compounds without the phenyl ring. This led them to suggest that hydrophobic binding may facilitate ionic binding. It is suggested at this time that the reverse may also be true and, as suggested by Brand and Gohlke (28), 1-anilinonaphthalene-8-sulfonate may act as an anion to augment the binding constants to the value found in the present study (Table I).

SUMMARY AND CONCLUSIONS

In summary, it seems that the R groups may play an important role in the binding of sulfonamides to hydrophobic protein sites. The methyl groups on the pyrimidine rings of sulfamerazine and sulfamethazine, the methyl groups on the pyrimidine ring of sulfisomidine, and the methyl group on the isoxazole ring of sulfisoxazole significantly increase the binding to albumin. The benzene substitution of sulfaphenazole and methoxy substitutions of sulfadimethoxine and sulfamethoxypyridazine may also enhance the binding to albumin.

The binding of penicillins is enhanced by halogen substitution on the side chain while being greatly reduced by amino substitution. Since the binding constants of penicillins with identical parent molecules differ with different side chains, it may also be concluded that the side chains of penicillin are involved in the binding to albumin.

These relationships between the structure and binding affinities and the agreement between the present study and previously published data show that the fluorescence probe technique can rapidly yield valuable binding information.

REFERENCES

(1) B. D. Davis, *Science*, **95**, 78(1942).

(2) A. H. Anton, *J. Pharmacol. Exp. Ther.*, **129**, 282(1960).

(3) R. Tompsett, S. Shultz, and W. McDermott, *J. Bacteriol.*, **53**, 581(1947).

(4) F. J. DiCarlo, S. G. Malament, L. J. Haynes, and G. E. Phillips, *Toxicol. Appl. Pharmacol.*, **5**, 61(1963).

(5) T. Murakawa, T. Y. Wakai, Y. Toi, and M. Nishida, *Jap. J. Antibiot.*, **22**, 387(1969).

(6) A. Goldstein, *Pharmacol. Rev.*, **1**, 102(1949).

(7) M. C. Meyer and D. E. Guttman, *J. Pharm. Sci.*, **57**, 895(1968).

(8) L. Brand, J. R. Gohlke, and S. Rao, *J. Biochem.*, **6**, 3510(1967).

(9) L. Stryer, *J. Mol. Biol.*, **13**, 482(1965).

(10) W. O. McLure and G. M. Edelman, *Biochemistry*, **5**, 1908(1966).

(11) G. Weber and L. B. Young, *J. Biol. Chem.*, **239**, 1415(1965).

(12) H. W. Jun, R. T. Meyer, C. M. Himel, and L. A. Luzzi, *J. Pharm. Sci.*, **60**, 1821(1971).

(13) H. W. Jun, L. A. Luzzi, and P.-L. Hsu, *ibid.*, **61**, 1835(1972).

(14) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660(1949).

(15) I. M. Klotz, H. Triwuish, and F. M. Walker, *J. Amer. Chem. Soc.*, **70**, 2935(1948).

(16) H. B. Van-Dyke, N. A. Tupikova, B. F. Chow, and H. A. Walker, *J. Pharmacol. Exp. Ther.*, **83**, 203(1945).

(17) W. Scholtan, *Arzneim.-Forsch.*, **14**, 469(1964).

(18) O. Jardetzky and N. G. Wade-Jardetzky, *Mol. Pharmacol.*, **1**, 214(1965).

(19) P. Spring, *Arzneim.-Forsch.*, **16**, 346(1966).

(20) I. Moriguchi, S. Wada, and J. Nishizawa, *Chem. Pharm. Bull.*, **16**, 601(1968).

(21) S. R. Walker, *J. Pharm. Pharmacol.*, **22**, 574(1970).

(22) M. Nakagaki, N. Koga, and H. Terada, *Yakugaku Zasshi*, **84**, 516(1964).

(23) B. B. Newbould and R. Kilpatrik, *Lancet*, **11**, 887(1960).

(24) J. Clausen, *J. Pharmacol. Exp. Ther.*, **153**, 167(1966).

(25) W. Scholtan, *Arzneim.-Forsch.*, **14**, 1234(1964).

(26) I. M. Klotz and F. M. Walker, *J. Amer. Chem. Soc.*, **70**, 943(1948).

(27) W. Scholtan, *Antibiot. Chemother.*, **12**, 103(1964).

(28) L. Brand and J. R. Gohlke, *Ann. Rev. Biochem.*, **41**, 843(1972).

(29) G. N. Rolinson and R. Sutherland, *Brit. J. Pharmacol.*, **25**, 638(1965).

(30) A. E. Bird and A. C. Marshall, *Biochem. Pharmacol.*, **16**, 2275(1967).

(31) C. M. Kunin, *J. Lab. Clin. Med.*, **65**, 416(1965).

(32) G. Y. Kivman and V. P. Yokovlev, *Antibiotiki*, **9**, 151(1964).

(33) C. I. Smith, J. D. Levin, and D. R. Embody, *Antibiot. Ann.*, **1957**, 306.

(34) C. M. Kunin, *Proc. Soc. Exp. Biol. Med.*, **107**, 337(1961).

(35) W. M. M. Kirby, L. S. Rosenfeld, and J. Brodie, *J. Amer. Med. Ass.*, **181**, 739(1962).

(36) P. M. Keen, *Biochem. Pharmacol.*, **15**, 447(1966).

(37) J. Fisher and O. J. Jardetzky, *J. Amer. Chem. Soc.*, **87**, 3237(1965).

(38) G. O. Phillips, D. M. Power, C. Robinson, and J. V. Davies, *Biochim. Biophys. Acta*, **215**, 491(1970).

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