On the Mechanism of the Binding of Sulfonamides to Bovine Serum Albumin[1]

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SUMMARY

The mechanism of the binding of several sulfonamides to bovine serum albumin has been investigated by relaxation time measurements on their high resolution proton magnetic resonance spectra. Upon addition of albumin to the sulfonamide solutions generally a larger increment in the relaxation rate was found for the *p*-aminobenzenesulfonamide moiety than for any of the substituents on N-1, indicating that the parent molecule is the primary binding site. The three-ring compound sulfaphenazole was found to be an exception. Two of the three rings, the *p*-aminobenzene sulfonamide and the phenyl portion of the N-1 substituent, bind independently. The two binding sites can be clearly distinguished since phenylpropanol interferes with the binding of the *p*-aminobenzenesulfonamide moiety and slightly enhances the binding of the phenyl group.

INTRODUCTION

It is generally recognized that the specificity of pharmacologic agents lies in the structure of the complexes which they form with macromolecular receptors. However, the chemistry of such complexes is still poorly understood. Their nature cannot be inferred from structure-activity correlations involving chemical modification of either the drug or the receptor; of necessity chemical modification leads to a simultaneous change of several molecular properties and thereby precludes an unequivocal interpretation of the results. Insight into the structure and function of drug-receptor complexes depends on the development of suitable physical techniques.

We have recently shown (1–4) that differential changes of the relaxation rates in the high-resolution nuclear magnetic resonance (NMR) spectra of complexed organic molecules can provide information on the nature of binding sites. In the present study we have used this technique to determine the part of a sulfonamide molecule which is bound to bovine serum albumin (BSA).

Protein binding of sulfonamides was initially described by Davis (5, 6) and has been extensively studied since (7–16). Klotz and Walker (11), as well as Wöhler (13), have shown that for several sulfonamide derivatives an albumin molecule contains only one binding site. This site appears to be different from the site used for the binding of penicillins (4) but is, as the present findings indicate, common to different sulfonamides. The stability constants for sulfanilamide and several of its derivatives are of the order of $10^3 l \cdot mol^{-1}$ (5).

The dependence of the binding constant (and antibacterial activity) on the nature of the substituent at N-1 is well known. A correlation between chemical constitution and strength of binding has been reported by van Dyke (9). However, this correlation provides no answer to the question whether the effect of a substituent is (a) direct, by its participation in the binding or (b) in-

[1] A preliminary report of a part of this investigation has appeared in *The Pharmacologist* 4, 156 (1962).

direct, e.g., by an alteration of the charge distribution, and hence of the binding properties, of the parent molecule. The present study examines this question. Our findings indicate that the *p*-aminobenzenesulfonamide (PABS) moiety is the primary and probably the sole binding site in the simpler N-1 substituted derivatives of sulfanilamide. In the case of sulfaphenazole binding can occur by two separate, distinguishable mechanisms: via the *p*-aminobenzenesulfonamide ring or via the phenyl portion of the N-1 substituent.

MATERIALS AND METHODS

All measurements were made on a Varian Associates DP 60 high-resolution spectrometer. Spectra were recorded on a Varian G-14 recorder, using field sweep at rates of 10–25 milligauss/min. Chemical shifts were measured by the side-band technique, using a Hewlett Packard model 200 CD wide-range oscillator and an HP 522B electronic counter. All shifts are reported in cps from hexamethyldisiloxane (HMS) as an external standard. No changes in chemical shifts have been observed in any of the solutions upon addition of albumin. The reasons for this have been discussed in detail previously (4).

Line widths were measured on peaks bracketed between two side bands of fixed frequency. Chart speed and field sweep rates were adjusted to give calibrations of approximately 3 cps/cm. Spectra showing deviations greater than ±2.5% from the calibration value because of drift were discarded. Values of the relaxation rate $1/T_2$ were calculated from the spectral line widths using the formula (Eq. 1)

$$\frac{1}{T_2} = \pi\Delta\nu_{1/2} \qquad (1)$$

where $\Delta\nu_{1/2}$ is the line width at ½ of the maximum peak height for single peaks. In the case of symmetrical doublets fused by broadening, it has proved possible to estimate $1/T_2$ within 10% error from the formula

$$\frac{1}{T_2} = \pi(\Delta\nu'_{1/2} - J) \qquad (2)$$

where $\Delta\nu'_{1/2}$ is the width at ½ height of the fused peak and J the peak separation under condition $\Delta\nu_{1/2} \ll J$. Corrections for this case and for the case of overlapping asymmetrical doublets have been evaluated by assuming progressive broadening of Lorentzian lines and were found to be small (V. L. Seery and O. Jardetzky, unpublished calculations). A correction for field inhomogeneity—depending on the resolution during the experiment, usually of the order of 0.3–0.5 cps—has been applied to all lines.

In the case of closely spaced asymmetric peaks which fused upon broadening, notably in the case of sulfaphenazole, measurements of line width were carried out on the difference spectrum. For example, the width of the phenyl peak was estimated after subtracting the *p*-aminobenzenesulfonamide A_2 and pyrazole CH peaks from the observed envelope; the positions of these lines were known from the spectra of the pure sulfaphenazole and could be estimated from the symmetry of the spectrum; their widths were assumed to be identical with those of the B_2 and high field pyrazole CH doublet, respectively. It was found that the widths of the difference spectra were only slightly (5–10%) less than the widths of the phenyl portion of the envelope without the correction. The observed width minus a 10% correction was therefore used as a minimum estimate of the relaxation rate.

Longitudinal relaxation times T_1 were measured by the direct saturation method, using a Varian model 4311 60 Mc RF unit, equipped with a high speed switch bypassing the attenuator and thus allowing changes of the RF power level within 12 μsec. The recovery of the saturated signal was photographed by a Polaroid camera from the screen of a Tektronix model 500 oscilloscope synchronized with the switch and the linear sweep unit. For each peak, T_1 measurements at several rf power levels were made and extrapolation to "zero power" (100 db below 0.5 watt), was carried out. At the levels of attenuation which could be used in this study (50–80 db below 0.5 watt) the variation of T_1 with power level was of the order of 10% over

the entire range. Maximum precautions were taken to minimize distortion of the signal—e.g., by imperfect probe balance—since such factors have large effects on the measured values of T_1. All reported values of the relaxation rates $1/T_1$ or $1/T_2$ are averages of at least six separate measurements. The standard error of the mean is given in parentheses.

Throughout this study, it was assumed that $T_1 = T_2$ and for the sake of consistency all measurements are reported as $(1/T_1)$. The instrumental limits allow measurements of $T_1 > 0.2$ sec, corresponding to $\Delta\nu < 1.6$ cps and of $\Delta\nu > 0.3$ cps, corresponding to $T_1 < 1.1$ sec. The assumption—reasonable on theoretical grounds for the systems in question—was directly verified by simultaneous measurement of T_1 and $\Delta\nu$ in the intermediate range of $0.3 < \Delta\nu < 1.6$.

All solutions were made in D_2O (99.8% isotopic purity). Concentrations are expressed as molar for the sulfonamides and as percentages (W/V) for the protein, i.e., in grams of protein per 100 ml of final solution volume. Adjustments of pH were made with DCl and anhydrous Na_2CO_3. All pH measurements were made on a Beckman model G pH meter. The values given correspond to actual meter readings, uncorrected for the deuterium isotope effect.

The sulfonamides used in this study were kindly supplied by Armour Laboratories (sulfacetamide), Hoffman-La Roche Inc. (sulfisoxazole and 5-methyl-3-sulfonamidosoxazole), Eli Lilly & Company (sulfathiazole) Merck Sharp and Dohme (phthalysulfathiazole), Lederle Laboratories (N-1 ethylsulfanilamide), and Ciba Pharmaceutical Company (sulfaphenazole). Crystallized Bovine Plasma Albumin was obtained from Armour Laboratories, Gamma Globulin Bovine Fraction II, from the California Biochemical Corporation, and D_2O from the Bio-Rad Corporation.

RESULTS AND DISCUSSION

The NMR spectra of all sulfonamides are of the A_2B_2 type, characteristic of para-substituted benzenes (17). Occasionally, the pattern approaches A_2X_2. A typical spectrum—that of sulfacetamide—is shown in Fig. 1. The low field (A) peaks were readily assigned to the protons *ortho* to the sulfonamide group and the high field (B) peaks to those *ortho* to the amino group. Following the general procedure developed by McConnell *et al.* (18), and assuming the four strongest lines to represent transi-

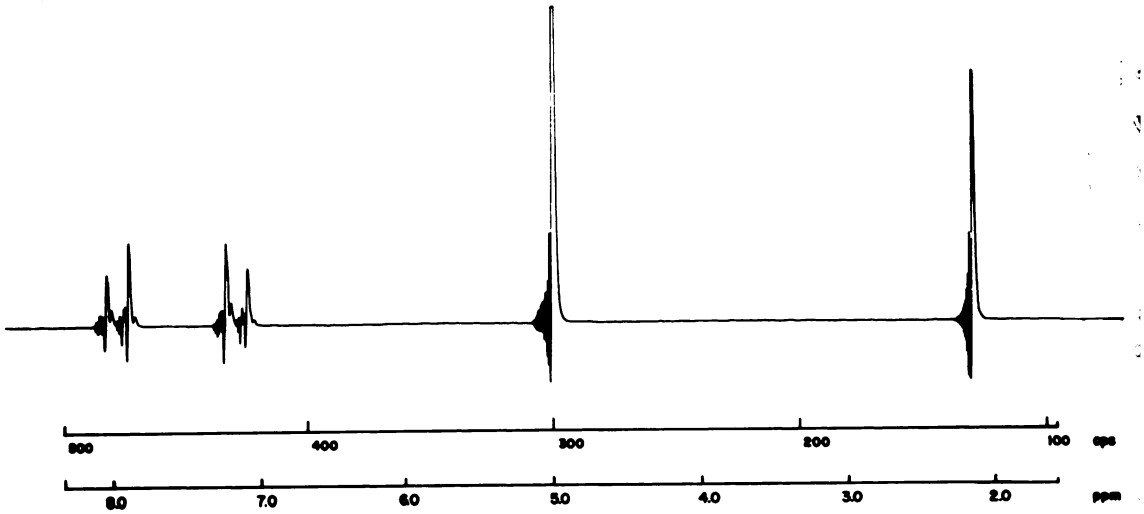
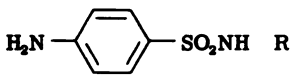


FIG. 1. *NMR spectrum of 0.1 M sulfacetamide in* D_2O, pH 8.6

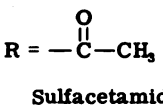
Shifts are from hexamethyldisiloxane as an external standard. Peaks centered at 475.2 and 429.0 cps are from the A_2B_2 system of *p*-aminobenzenesulfonamide; the methyl peak is at 132.5 cps.

tions 1 and 3 for A and B, respectively (forming an AB-like quartet) (19, 20), the spectrum can be calculated from the following parameters: $\Delta_{AB} = 46.2$ cps, $J_{AB} = 8.5$ cps, $J'_{AB} = 0.3$ cps, $J_{AA} = 2.8$ cps, $J_{BB} = 1.4$ cps where Δ_{AB} is the chemical shift between the two pairs of A and B protons, J_{AB} is the *ortho*, J'_{AB} the *para*, and J_{AA} and J_{BB} the two *meta* coupling constants. Spectra of other derivatives can be fitted to a reasonable approximation using similar values of the coupling constants and varying the magnitude of AB in accordance with the positions of lines in the AB quartet.

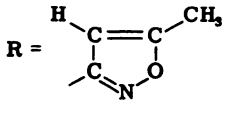
Since an exhaustive study of the binding of a large series of sulfonamides by this method would be prohibitively costly and time consuming, three representative compounds were selected for a detailed investigation. The selection was governed by (a) the nature of the side chain (b) adequate solubility, and (c) relative simplicity of the NMR spectrum. The generality of the conclusions was spot-checked by making relaxation measurements on duplicate samples of other derivatives with and without BSA and calculating the relative increments of the relaxation rates from Eq. (18) of reference (2). The data for the three representative compounds—sulfacetamide (aliphatic side chain), 5-methyl-3-sulfanilamidoisoxazole (single-ring side chain), and 1-phenyl-5-sulfanilamidopyrazole (sulfaphenazole) (two-ring side chain)—are presented under separate headings. The structures of the compounds are:



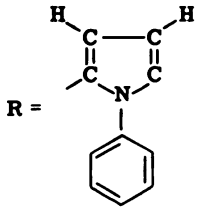
p-Aminobenzenesulfonamide (PABS)



Sulfacetamide



5-Methyl-3-sulfanilamidoisoxazole



Sulfaphenazole

A. *Sulfacetamide*

T_1 measurements for the benzene and methyl peaks of 0.1 M sulfacetamide, with and without 1% BSA at sweep rates of 0.5 cm/sec are shown in Fig. 2 (a)–(d). The

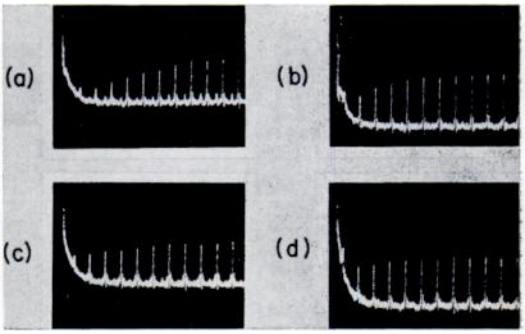


FIG. 2. *T_1 measurements on 0.1 M sulfacetamide in D_2O, pH 8.6*

(a) A_2B_2 system, $T_1 \simeq 3.8$ sec; (b) methyl group, $T_1 \simeq 2.1$ sec, T_1 measurements on 0.1 M sulfacetamide + 1% BSA in D_2O, pH 8.6; (c) A_2B_2 system, $T_1 \simeq 0.6$ sec; (d) methyl group, $T_1 \simeq 1.0$ sec.

fact is immediately apparent that the T_1 of the PABS moiety decreases on addition of BSA by a larger factor than the T_1 of the methyl. Actual measurement shows the following values: (a) 3.8 ± 0.2 sec, (b) 2.1 ± 0.2 sec, (c) 0.6 ± 0.2 sec, (d) 1.0 ± 0.2 sec. As discussed in detail elsewhere (2, 4) such a finding would not result from any of the nonspecific mechanisms for increasing the relaxation rate—e.g., a simple increase in viscosity—but rather indicates a specific interaction with the protein. A nonspecific mechanism will shorten all relaxation times in a molecule by the same factor, whereas in the case of binding the part of the molecule stabilized by the interaction will show a larger effect. The contrast be-

tween the two types of mechanisms is apparent from a comparison of Figs. 3 and 4. As illustrated in Fig. 3, as the concentration of sulfacetamide is increased from 0.1 to 1.0 M the relaxation rate of the PABS peaks increases from 0.25 sec^{-1} to 0.50 sec^{-1} and that of the methyl peak from 0.45 sec^{-1} to 0.9 sec^{-1}, i.e., both change by a factor of 2. In Fig. 4 the relaxation rate of the PABS peaks of an 0.1 M sulfacetamide solution increases from 0.25 sec^{-1} to 10.8 sec^{-1}, i.e., by a factor of 44, whereas that of the methyl increases from 0.45 sec^{-1} to 6.3 sec^{-1}, by a factor of 14, as the protein concentration is increased from 0 to 10%. Thus, the *p*-aminobenzenesulfonamide moiety is preferentially stabilized by the interaction of the sulfacetamide molecule with BSA, whereas the acetyl group retains much greater freedom of motion. The most plausible interpretation of this preferential stabilization is that the PABS ring is the primary binding site.

The conclusion that the results given in Fig. 4 represent a specific interaction between sulfacetamide and BSA is further strengthened by the following observations: (a) the increase in viscosity which results from the addition of albumin is insufficient to account for the observed magnitude of the change in T_1; the viscosity of a 5%

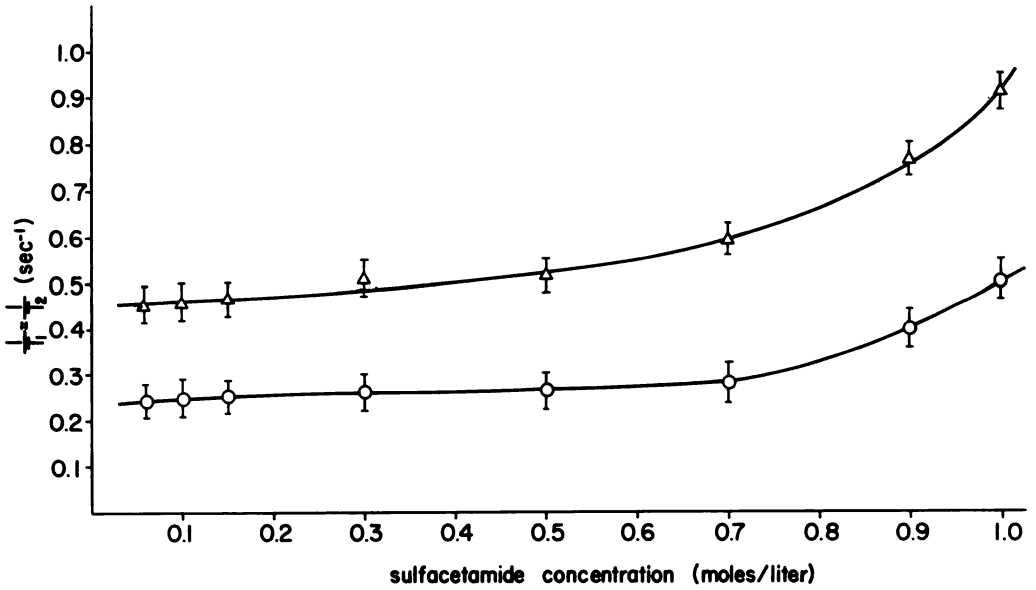


FIG. 3. *Concentration dependence of relaxation rates in sulfacetamide at pH 8.8*
○, PABS protons; △, methyl protons.

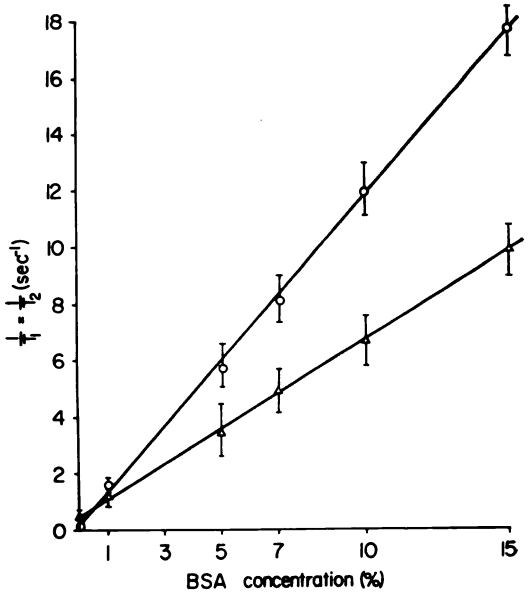


FIG. 4. *Relaxation rates of 0.1 M sulfacetamide peaks, pH 8.8, as a function of BSA concentration*
○, PABS protons; △, methyl protons.

albumin solution is only 1.25 times as large as the viscosity of pure D_2O, but the relaxation rates of the benzene and methyl peaks are respectively 23 and 8 times as large as in D_2O. (b) The effect of γ-globulin, which is known not to bind sulfacetamide, on its relaxation rates is qualitatively different than that of BSA; the increase in the relaxation rates is proportional to the increase in viscosity and is the same for both peaks. This finding exactly parallels the finding in the case of penicillin (4) and is therefore not reproduced here. (c) The relaxation rate depends on the sulfonamide:albumin ratio, rather than the albumin concentration and, for a given albumin concentration, *decreases* with increasing sulfonamide concentration (Fig. 5). As pointed out in the case of penicillin, albumin molecule. By the arguments given previously (4) we can conclude that in the case of sulfacetamide, as in the case of penicillin, the exchange between the free and bound state is rapid, so that the observed relaxation rate is given by

$$\left(\frac{1}{T_1}\right)_{obs} = \alpha \left(\frac{1}{T_1}\right)_{bound} + (1 - \alpha)\left(\frac{1}{T_1}\right)_{free} \quad (3)$$

(case III, ref. 4), where α is the fraction bound. Assuming a single binding site, one can easily find from the law of mass action

$$\alpha \approx \frac{[A]}{[S]} \quad (4)$$

where [A] and [S] are the total concentration of albumin and sulfacetamide, respectively. The approximation is valid for

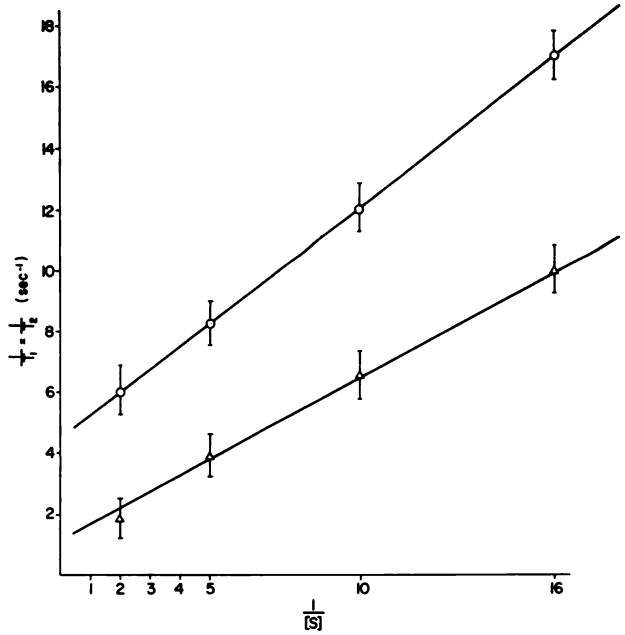


FIG. 5. *Relaxation rates of sulfacetamide peaks as a function of the inverse of sulfacetamide concentration [S] at constant (10%) BSA concentration, pH 7.8*

○, PABS protons; △, methyl protons.

this is exactly the opposite to what one would expect if the broadening were due to one of the nonspecific mechanisms. The findings presented in Fig. 5 also confirm the previous conclusion that there is only one binding site for sulfacetamide on each excess sulfonamide, i.e., $\alpha \ll 1$; $[S] \ll [A] \geqslant K_D$ where K_D is the dissociation constant of the complex. Under these conditions $(1/T_1)_{obs}$ will be directly proportional to [A] at [S] = constant or to 1/[S] at [A] = constant, and it is found to be so.

Considering the fact that the present experiments were done at concentrations of sulfacetamide 10–100 times greater than those used by Klotz and Walker (11), so that one might have expected additional, weaker binding sites to come into play, this result is of some importance. The binding of sulfacetamide to albumin is saturable.

It might also be noted that in the case $(1/T_1)_{bound} \ll (1/T_1)_{free}$ it is possible to write

$$\left(\frac{1}{T_1}\right)_{obs} \approx \frac{[A]}{[S]}\left(\frac{1}{T_1}\right)_{bound} \quad (5)$$

and thus obtain an estimate of $(1/T_1)_{bound}$ directly from plots of $(1/T_1)_{obs}$ vs. [A] at constant [S] or $(1/T_1)_{obs}$ vs. 1/[S] at constant [A]. The calculated values ~900 sec^{-1} for the PABS and ~350 sec^{-1} for the methyl peak are in reasonable agreement with those obtained from a more elaborate calculation (Table 1). As will become apparent later, the small discrepancy may be largely attributed to the difference in pH.

The mass action model for evaluating α and K_D developed in the case of penicillin is directly applicable here. For the case $\alpha \ll 1$ and the number of noninteracting binding sites $n = 1$, the mass action equation can be written in the form

$$[S] = -K_D + \frac{1}{\alpha}[A] \quad (6)$$

Taking an array of the five different drug concentrations each in combination with a similar number of albumin concentrations, one obtains a set of concentration series, of the type shown in Figs. 4 and 5. A plot of all sulfonamide concentrations which give some particular, arbitrarily chosen, line width, against the corresponding albumin concentration, will give a straight line with a slope $1/\alpha$ and intercept $-K_D$, since lines of identical width correspond to identical values of α. Four such lines are shown in Fig. 6. From the intercept, K_D can be estimated within the limits 2×10^{-3} to 9×10^{-3} M. Greater precision is not possible, but the value is in reasonable agreement with the value of 4.9×10^{-3} M, reported by Klotz and Walker. The value of α and the calculations of $(1/T_1)_{bound}$ are summarized in Table 1. The ratio $T_{1\,free}:T_{1\,bound}$, which is the quantity of primary interest (2) shows that upon binding to albumin the relaxation rate of the *p*-aminobenzenesulfonamide moiety increases by a factor of 2800 and that of the methyl by only 600. This substantiates the already stated conclusion that the PABS ring is the binding site (21).

TABLE 1
Calculation of relaxation rates for bound sulfacetamide

Peak	$\left(\frac{1}{T_1}\right)_{obs}$[a]	$\left(\frac{1}{T_1}\right)_{free}$[a]	$\frac{1}{\alpha}$	$\left(\frac{1}{T_1}\right)_{bound}$[a]	Average[a] $\left(\frac{1}{T_1}\right)_{bound}$	$\frac{(T_1)_{free}}{(T_1)_{bound}}$
PABS	3	0.25	266	710	—	—
PABS	6	0.25	80	720	715	2870
CH_3	3	0.45	100	255	—	—
CH_3	6	0.45	60	290	275	610

[a] In sec^{-1}.

The nature of the interaction which leads to the stabilization of the PABS ring is not immediately apparent from the foregoing. The two obvious alternatives are (a) electrostatic attraction of the negatively charged sulfonamide group to a positive charge on the protein and (b) a van der Waals (dipole-dipole) interaction of the ring system with a side chain of the protein, possibly with additional solvent ("hydrophobic") stabilization. Either of these could be reinforced by hydrogen bonds, but it is unlikely that hydrogen bonding per se could account for the observed stability of the complex, since the difference in strength between hydrogen bonds to the protein and to the solvent should be small.

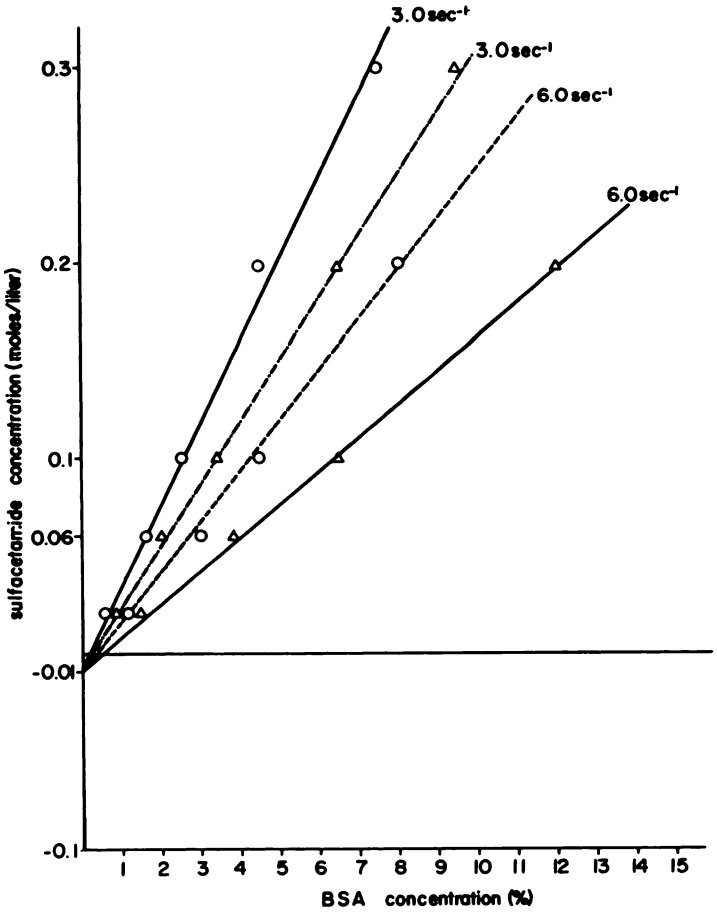


FIG. 6. *Lines of constant width for sulfacetamide, pH 8.8*
○, PABS protons; △, methyl protons.

Although we cannot unequivocally distinguish between the two alternatives, several considerations lead us to prefer the second:

1. There is no obvious reason why a purely electrostatic interaction of the centrally located $SO_2 - N^-$ group, should in the absence of at least some contribution from van der Waals forces lead to a preferential stabilization of one part of the molecule.

2. The pH dependence of the relaxation rate observed in the presence of BSA (Fig. 7) shows that although the binding is greatly diminished in the region below the pK of sulfacetamide (pK = 5.35), it is not abolished altogether, as it should have been, were the interaction purely ionic. This fact is also apparent from the data of Davis (6). The finding of a pH optimum resulting from an increase in binding with increasing ionization on one hand and an increase in binding with the increase in positive charge on the protein on the other is equally compatible with either of two possibilities: (a) a predominantly ionic interaction or (b) a predominantly dipolar interaction, since the magnitude of the permanent dipole across the ring would be increased by ionization. In either case the repulsive forces would diminish with increasing protonation of the protein, accounting for the pH dependence above pH 6. It is of interest to note that the direction of the pH dependence in this region is the same here as in the case of penicillin, where

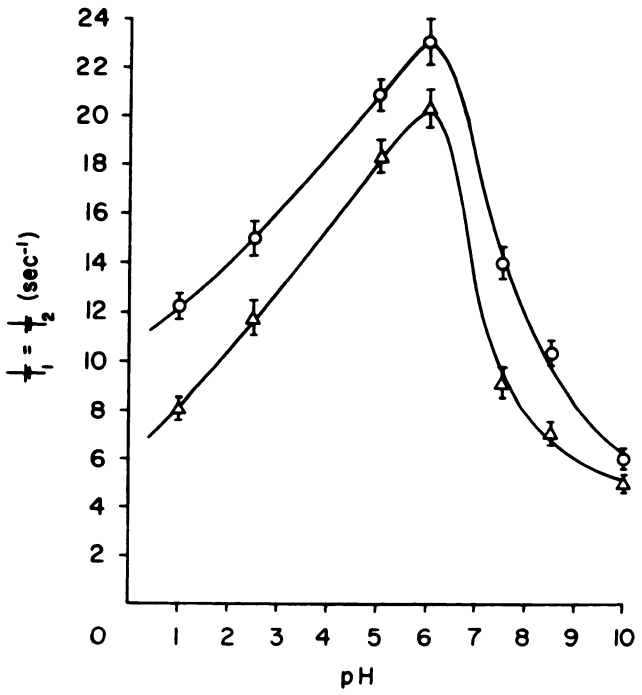


FIG. 7. *pH dependence of relaxation rates in 0.1 M sulfacetamide–10% BSA*
In the absence of BSA, relaxation rates of 0.1 M sulfonamide peaks show no pH dependence. ○, PABS protons; △, methyl protons.

the binding is beyond doubt predominantly hydrophobic.

3. The effect of increasing the ionic strength of the solution (Table 2) is to enhance, rather than diminish the binding. This one would not expect were the interaction predominantly coulombic in nature.

TABLE 2
Effect of increasing salt concentration on the relaxation rates in 0.1 M sulfacetamide–10% BSA solutions, pH 7.8[a]

NaCl molarity	$\left(\frac{1}{T_1}\right)_{obs\ A_2B_2}$	$\left(\frac{1}{T_1}\right)_{obs\ CH_3}$
0	11.2 (±0.3)	6.5 (±0.3)
1	12.1 (±0.5)	7.0 (±0.5)
2	13.2 (±0.5)	8.6 (±0.5)
3	14.6 (±0.6)	11.5 (±0.5)
4	21 −23	17.2 (±0.6)

[a] Values in sec^{-1}.

The temperature dependence of the relaxation of 0.1 M sulfacetamide with 10% BSA has also been examined, and the results are shown in Fig. 8. It is likely that the narrowing of lines observed with an increase in temperature largely reflects the temperature dependence of the equilibrium constant. Since at present it is not possible accurately to assess the temperature dependence of the correlation time, calculations of thermodynamic parameters are deferred until reliable measurements of the relaxation times of protein side chains become available.

B. *5-Methyl-3-sulfanilamidoisoxazole*

A priori the possibility exists that the binding behavior of sulfacetamide is not typical of the majority of sulfonamide derivatives, which predominantly have aromatic substituents at N-1. It was therefore decided to investigate in detail two additional compounds, 5-methyl-3-sulfanilamidoisoxazole and sulfaphenazole, bearing on N-1 one and two unsaturated rings, respectively. The experimental design is identical to that previously described, and to avoid needless repetition only the data essential to the argument are presented. The spec-

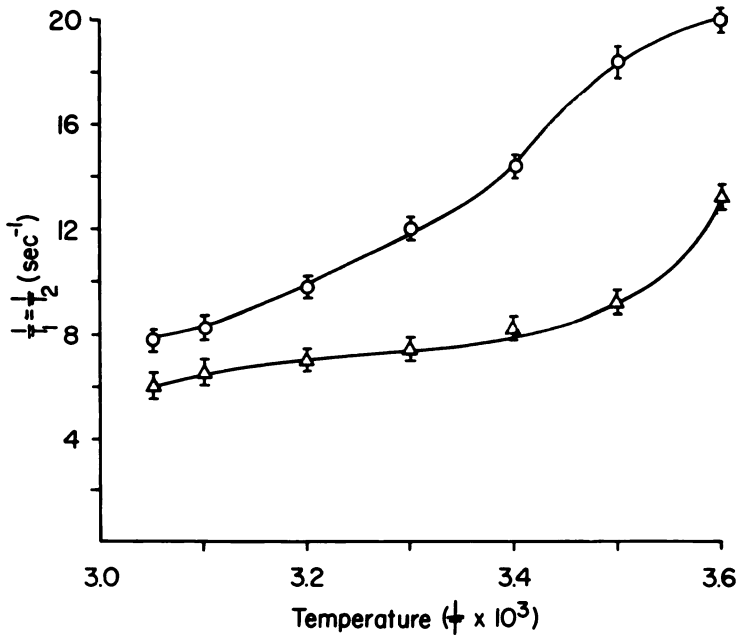


FIG. 8. *Temperature dependence of relaxation rates in 0.1 M sulfacetamide at pH 8.3*
○, PABS protons; △, methyl protons.

trum of 5-methyl-3-sulfanilamidoisoxazole consists of three groups of lines, centered at 445.1 cps (PABS), 356.2 cps (oxazole proton), and 134.5 cps (methyl). The coupling constant for the oxazole-methyl proton interaction is 0.9 cps.

The variation of the relaxation rates of the three methylsulfanilamidoisoxazole peaks with increasing albumin concentration is shown in Fig. 9. The lines of constant width calculated from an array of 0.05, 0.1, 0.15, and 0.3 M sulfonamide each with 2.5%, 5%, 10%, and 15% albumin, are presented in Fig. 10. The calculation of $(1/T_1)_{bound}$ and of $(T_1)_{free}:(T_1)_{bound}$ is given in Table 3. The result is nearly identical to that obtained in the case of sulfacetamide, confirming the conclusion that the *p*-aminobenzenesulfonamide moiety is the primary binding site.

C. *Other sulfonamides*

Relaxation rate measurements for six other simple sulfonamides with and without albumin are summarized in Table 4. It is obvious from Eq. (3) that the ratio $(T_1)_{free}:(T_1)_{observed}$ is a linear function of the ratio $(T_1)_{free}:(T_1)_{bound}$. In the case of a single binding site it is therefore possible to infer preferential stabilization from a comparison of the ratios $(T_1)_{free}:(T_1)_{observed}$ for two different peaks, without the detailed quantitative analysis presented in the case of sulfacetamide and methylsulfanilamidoisoxazole. The data in Table 4 are consistent with the conclusion that the binding of these compounds occurs at the same site and by the same mechanism as the binding of sulfacetamide. It should be emphasized that this conclusion is tentative insofar as it is drawn from these data, because in the case of multiple nonequivalent binding sites the observed result could occur by coincidence. In the case of sulfisoxazole, sulfadiazine, and sulfathiazole several concentration series were carried out with BSA and γ-globulin and the findings were identical to those reported for 5-methyl-3-sulfanilamidoisoxazole, so that the assumption of a single site appears justified. In other cases it is made by analogy.

D. *1-Phenyl-5-sulfanilamidopyrazole (sulfaphenazole)*

Sulfaphenazole was selected for detailed study because the likelihood of finding mul-

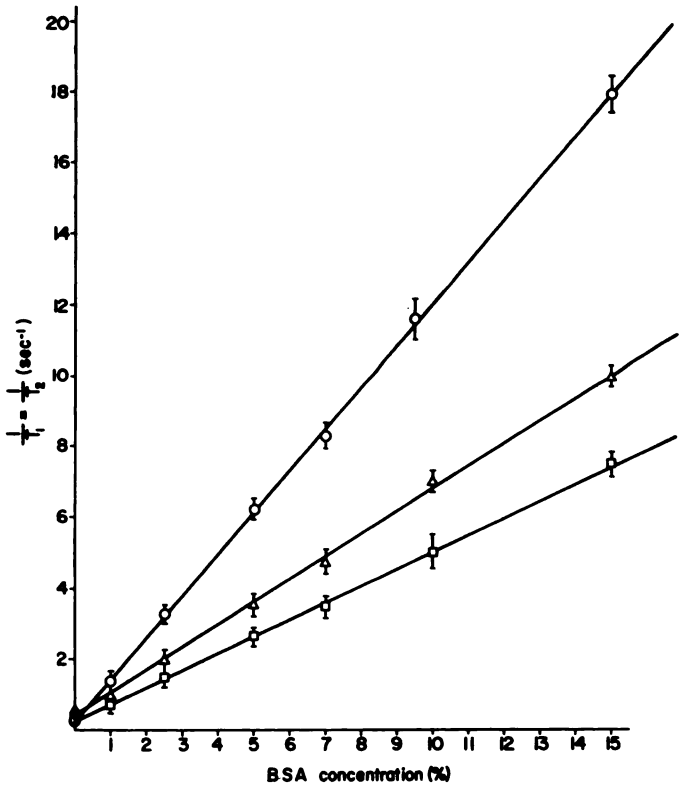


FIG. 9. *Relaxation rates of 0.1 M methylsulfanilamidoisoxazole peaks, pH 9.1, as a function of BSA concentration*

○, PABS protons; □, oxazole proton; △, methyl protons.

tiple binding sites was thought to increase with the number of substituent rings. The spectrum of the compound is shown in Fig. 11(a). In addition to the usual A_2B_2 lines at 413.6, 422.1, 464.0, and 471.5 cps, there are two single hydrogen doublets from the pyrazole at 362.1 and 461.0 cps, the latter partially obscured by the singlet phenyl of the N-1 substituent side chain at 462.1 cps. The pyrazole doublet at lower field is probably due to the $C_{(3)}H$ and the doublet at higher field to the $C_{(4)}H$. The coupling constant for the two protons is 2.3 cps.

In solutions of free sulfaphenazole the relaxation rates for the protons of the three rings are almost equal, i.e., 0.32 sec^{-1}

TABLE 3

Calculation of relaxation rates for bound methylsulfanilamidisoxazole

Peak	$\left(\frac{1}{T_1}\right)_{obs}$[a]	$\left(\frac{1}{T_1}\right)_{free}$[a]	$\frac{1}{\alpha}$	$\left(\frac{1}{T_1}\right)_{bound}$[a]	Average[a] $\left(\frac{1}{T_1}\right)_{bound}$	$\frac{(T_1)_{free}}{(T_1)_{bound}}$
PABS	4	0.34	220	810	785	2320
PABS	6	0.34	133	760		
Isoxazole	4	0.30	93	340	325	1080
	6	0.30	54	310		
CH_3	4	0.50	120	420	430	860
	6	0.50	80	440		

[a] In sec^{-1}.

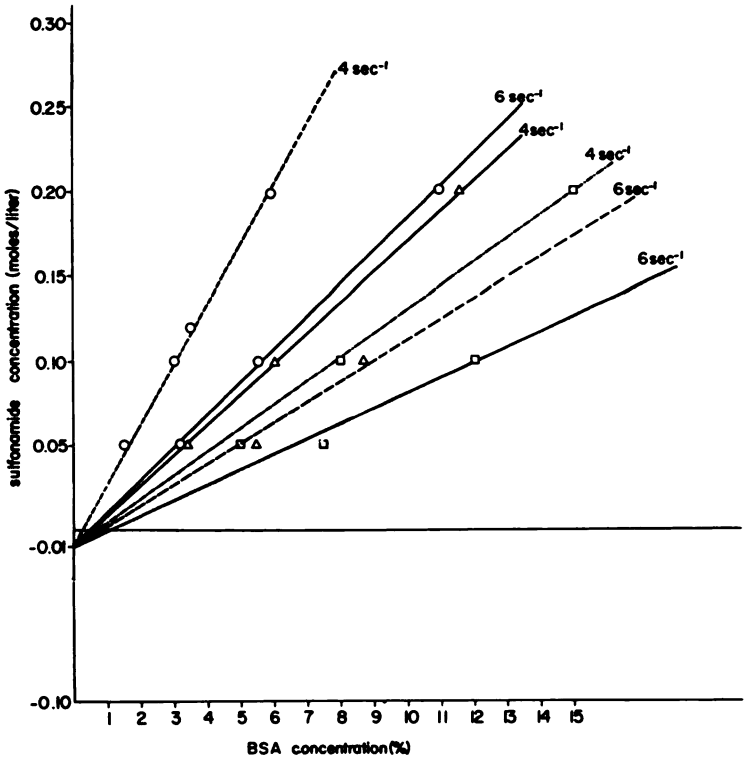


FIG. 10. *Lines of constant width for methylsulfanilamidoisoxazole, pH 9.1*
○, PABS protons; □, oxazole proton; △, methyl protons.

TABLE 4
Observed relaxation rates of sulfonamide derivatives (0.1 M) with and without bovine serum albumin (7%)

Compound peak	$\left(\frac{1}{T_1}\right)_{\text{obs free}}$[a]	$\left(\frac{1}{T_1}\right)_{\text{obs BSA}}$[a]	$\frac{(T_1)_{\text{free}}}{(T_1)_{\text{BSA}}}$	pH
Sulfadiazine				
PABS	0.25 ± 0.02	9.1 ± 0.3	36.4	9.3
Pyrimidine	0.83 ± 0.02	6.6 ± 0.3	7.9	
Sulfamethazine				
PABS	0.23 ± 0.02	11.5 ± 0.3	50.0	9.5
CH_3	1.0 ± 0.02	8.5 ± 0.3	8.5	
Sulfadimethoxine				
PABS	0.43 ± 0.02	10.4 ± 0.3	24.2	9.6
CH_3	1.0 ± 0.02	8.1 ± 0.3	8.1	
Sulfathiazole				
PABS	0.26 ± 0.03	13.6 ± 0.3	52.3	9.6
Thiazole	0.66 ± 0.03	11.5 ± 0.3	17.4	
Sulfaisoxazole				
PABS	0.31 ± 0.02	15.0 ± 0.5	48.4	8.2
Oxazole	0.59 ± 0.02	12.4 ± 0.5	21.0	
N-1 Ethylsulfanilamide				
PABS	0.29 ± 0.08	11.3 ± 0.6	38.9	8.0
CH_3	1.1 ± 0.05	10.2 ± 0.6	9.3	

[a] In sec^{-1}.

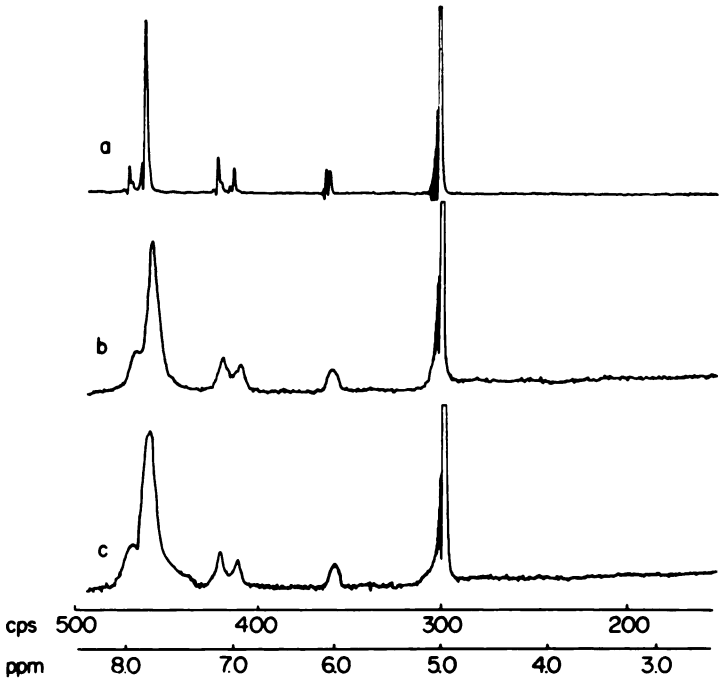


FIG. 11. *NMR spectra of 0.15 M sulfaphenazole, pH 8.5*

(a) Sulfaphenazole in D_2O. Shifts are from hexamethyldisiloxane as an external standard. Peaks centered at 467.8 and 417.9 cps are from the PABS protons; doublets at 461.0 and 362.1 cps are from pyrazole protons; large peak at 462.1 cps is from phenyl protons. (b) Sulfaphenazole + 10% BSA. (c) Sulfaphenazole + 10% BSA + 0.1 M phenylpropanol.

for the *p*-aminobenzene, 0.33 sec^{-1} for the pyrazole, and 0.38 sec^{-1} for the phenyl. The change of relaxation rates upon addition of albumin is shown in Fig. 12.

Several models for the binding of sulfaphenazole are incompatible with the observed result: Model 1: Binding primarily by the PABS ring as in the case of sulfacetamide; if this were the case the increase in the relaxation rate should be largest for the PABS lines and much less for both the pyrazole and the phenyl peaks. Assuming this model, the slope of $(1/T_1)_{obs}$ vs [A] for the pyrazole might be equal to or larger than that for the phenyl, but considering the geometry of the molecule the reverse is unreasonable. Model 2: Binding primarily by the phenyl ring. The expected order of slopes in this case would be $(1/T_1)_{phenyl} > (1/T_1)_{pyrazole} \geqslant (1/T_1)_{PABS}$. Model 3: Simultaneous binding of the PABS and phenyl rings. Model 4: Simultaneous binding of all three rings. In either of the last

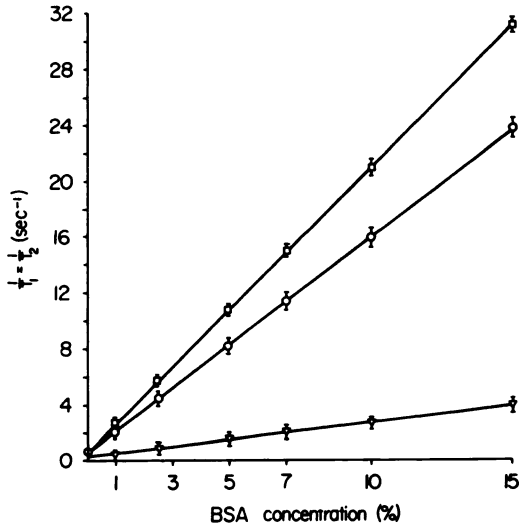


FIG. 12. *Relaxation rates of 0.15 M sulfaphenazole peaks, pH 9.9, as a function of BSA concentration*

○, PABS protons; ▽, pyrazole protons; □, phenyl protons.

two cases the slopes of all three lines should nearly coincide since the values of $(1/T_1)_{free}$ are almost identical and since the molecule would be bound as a rigid unit. Examination of a space-filling molecular model (Pauling-Corey) readily establishes the last point.

The simplest model compatible with the result is that there are two nonequivalent binding sites, one for the PABS ring and another for the phenyl. Sulfaphenazole molecules bound at either site exchange rapidly with the molecules free in solution, so that for each peak the observed relaxation rate is the weighted average of three different values. Thus, if we denote the two sites I and II, we have for any given peak

$$\left(\frac{1}{T_1}\right)_{obs} = \alpha\left(\frac{1}{T_1}\right)_{bI} + \beta\left(\frac{1}{T_1}\right)_{bII} + (1 - \alpha - \beta)\left(\frac{1}{T_1}\right)_{free} \quad (7)$$

where α is the fraction bound at site I and β the fraction bound at site II.

If this model is correct, it should be possible to find an agent that would selectively inhibit the binding of one of the rings without preventing the binding of the other. Then, a selective narrowing of either the PABS or the phenyl peak should be observed in the presence of the agent. Such an effect is shown in Fig. 11,b and c. When 0.1 M phenylpropanol is added to a solution of 0.15 M sulfaphenazole + 10% BSA (Fig. 11c), a narrowing of the PABS B_2 lines and a broadening of the phenyl line is seen by comparison with the control (Fig. 11b). The existence of two distinguishable binding sites is thus apparent.

The specificity of phenylpropanol for the PABS site is rather surprising. The compound was tested on the assumption that it would interfere with the binding of the phenyl group. Nevertheless the narrowing of the PABS peaks upon addition of phenylpropanol has been found, without exception, in the case of all sulfonamide derivatives examined. This strongly suggests that the site on the protein to which the PABS ring binds is the same for all sulfonamide derivatives. The observation of competition between different sulfonamides is also in accord with this conclusion, but the competition experiments were limited in scope and number by solubility and overlap of spectra. It is worth noting that in the cases of sulfacetamide and methylsulfanilamidoisoxazole the narrowing produced by the addition of phenylpropanol is nonselective, affecting the PABS and the N-1 substituent to a comparable extent. This provides additional support for the view that in these cases the PABS ring is the sole binding site.

Several other compounds, among them benzene, phenol, phenylacetic acid, picric acid, and penicillin were tested and found not to interfere with the binding of either the PABS or the phenyl ring at reasonable concentrations. This is of some interest, since it has been shown previously (4) that the phenyl groups of penicillin, phenylacetic and phenoxyacetic acid are bound to BSA. The results thus suggest that phenyl groups with different side chain substituents can bind at different, noninteracting, saturable sites. The binding of all phenyl groups, in-

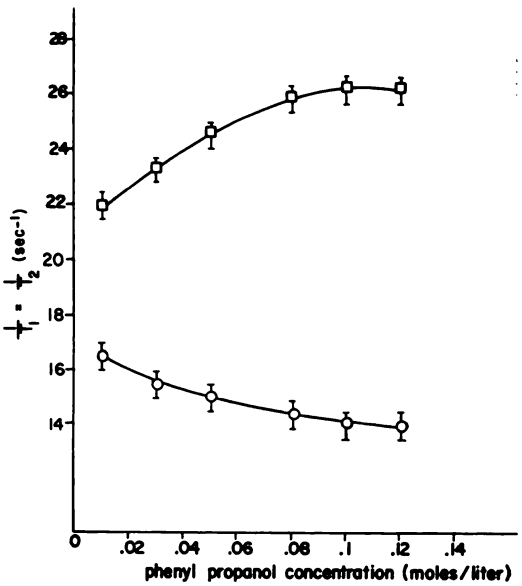


FIG. 13. *Dependence of relaxation rates of 0.15 M sulfaphenazole peaks on concentration of phenylpropanol at constant (10%) BSA concentration, pH 9.9*

○, PABS protons; ▽, pyrazole protons; □, phenyl protons.

cluding that of sulfaphenazole is enhanced by the addition of salt.

Although qualitatively the effect of phenylpropanol is clear-cut, its quantitative interpretation is not simple. The dependence of the effect on the concentration of phenylpropanol is seen in Fig. 13. The narrowing of the PABS peaks is accompanied by a *broadening* of the phenyl peak as the concentration of the alcohol is increased. This could reflect either an increase in the number of phenyl binding sites, or a slight increase in the relaxation rate of the bound form, or partially compensating changes in both. From Eq. (7) it is apparent that even under the conditions $\alpha \approx \beta \ll 1$ the slope of a plot of $(1/T_1)_{obs}$ vs. [A] or 1/[S] no longer reflects $(1/T_1)_{bound}$ but the weighted sum of $(1/T_1)_{bound}$ for two different sites. It is reasonable to assume $n_1 = 1$ for the PABS site (I), but the possibility of several equivalent noninteracting phenyl sites (II) should not be excluded, i.e., $n_2 \geqslant 1$. We then have

$$\left(\frac{1}{T_1}\right)_{obs} \approx \left[\left(\frac{1}{T_1}\right)_{bI} + n_2\left(\frac{1}{T_1}\right)_{bII}\right]\frac{[A]}{[S]} \qquad (8)$$

Where the subscripts bI and bII denote the relaxation rate for the bound form of any given group when the binding occurs at the PABS and the phenyl site, respectively. The evaluation of the individual terms for each peak is possible only under simplifying assumptions. Such calculations are precarious, but we have been interested in examining the result for the simplest possible case. The calculation is summarized inTable 5.

Comparison of the slopes in Figs. 12 and 14 indicates that Eq. (8) is valid, and Fig. 15 suggests that a similar linear relationship

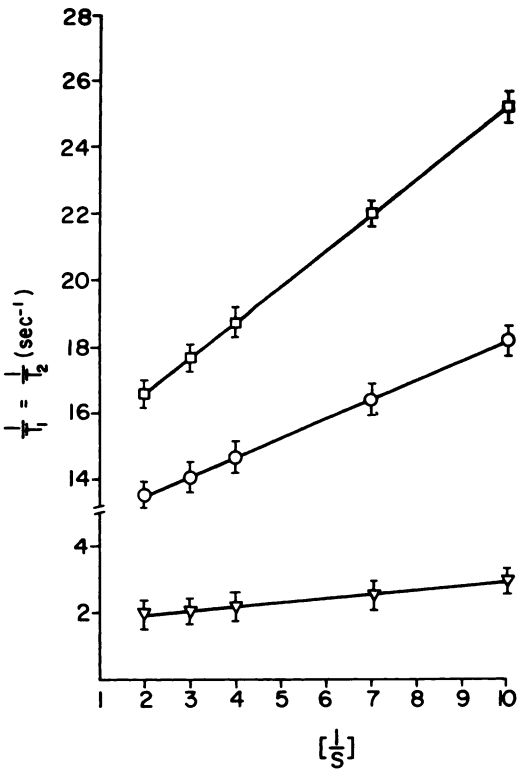


FIG. 14. *Relaxation rates of 0.15 M sulfaphenazole peaks as a function of the inverse of sulfaphenazole concentration [S] at constant (10%) BSA concentration, pH 9.9*

○, PABS protons; ▽, pyrazole protons; □, phenyl protons.

holds in the presence of high concentrations of phenylpropanol. Column (a) gives the value of the bracket of Eq. (8) estimated from Figs. 12 and 14; in column (b) it is assumed that $(1/T_1)_{bI}$ has values similar to those obtained for sulfacetamide and methylsulfanilamidoisoxazole and given in Tables 1 and 3. (The actual numbers were

TABLE 5

Calculation of relaxation rates for bound sulfaphenazole[a]

Peak	(a) $\left[\left(\frac{1}{T_1}\right)_{bI} + n_2\left(\frac{1}{T_1}\right)_{bII}\right]$	(b) Assumed $\left(\frac{1}{T_1}\right)_{bI}$	(c) $n_2\left(\frac{1}{T_1}\right)_{bII}$	(d) $n_2^*\left(\frac{1}{T_1}\right)_{bII}$	(e) $n_2^*\frac{n_2^*}{n_2}$
PABS	1200	700	500	600	1.3
Pyrazole	500	350	150	180	1.2
Phenyl	1700	300	1400	2200	1.5

[a] Values for columns a-d are stated in sec^{-1}.

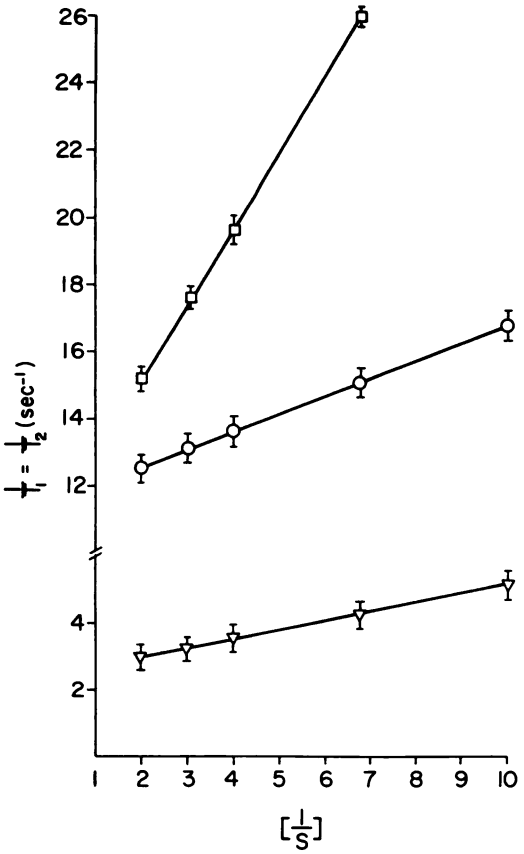


FIG. 15. *Relaxation rates of 0.15 M sulfaphenazole peaks as a function of the inverse of sulfaphenazole concentration [S] at constant (10%) BSA concentration in the presence of 0.1 M phenylpropanol, pH 9.9*

○, PABS protons; ▽, pyrazole protons; □, phenyl protons.

obtained by multiplying the relaxation rates of free sulfaphenazole by the $(T_1)_{free}$: $(T_1)_{bound}$ for the PABS ring and side chain, respectively.) The assumption is arbitrary, but not unreasonable; the second term of the bracket, obtained by difference is given in column (c). Assuming that at a high concentration of phenylpropanol all PABS sites are obliterated, $(1/T_1)_{bI} = 0$ and the slope of Fig. 15 yields the term in column (d). It is expressly *not* assumed that $n_{2*} = n_2$ or that $(1/T_1)_{bII*} = (1/T_1)_{bII}$; the starred values refer to the solutions containing phenylpropanol. The ratio (e) is then a measure of the change in the relaxation term for the phenyl binding site, accompanying the saturation of PABS sites by phenylpropanol. If one assumes in addition that the relaxation rate of the bound form is not affected by phenylpropanol, (e) is the ratio of the numbers of phenyl binding sites with and without the alcohol.

The close agreement between the values of (e) for the different peaks may well be fortuitous, but the fact that the ratio (e) is not very different from unity suggests that the addition of phenylpropanol causes only a small change in either the number of phenyl binding sites or the relaxation rate of the bound form. Its effects can be understood in terms of the simple model of only two binding sites on each albumin molecule, one specific for the PABS and the other for the phenyl ring.

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