following manner to fit the experimental findings:

$$k_{obs.} = k''_{H_2O} f_{A^{-2}} + k'_{H_2O} f_{HA^-} \quad \text{(Eq. 8)}$$

Or it may be written in the kinetically equivalent:

$$k_{obs.} = k'_{OH^-}[OH^-] f_{HA^-} + k_{OH^-}[OH^-] f_{H_2A} \quad \text{(Eq. 9)}$$

The specific catalytic rate constants for Eqs. 8 and 9 are given in Table III along with other pertinent data.

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ACKNOWLEDGMENTS AND ADDRESSES

Received April 17, 1970, from the *Pharmacy Research and Development Department, Sandoz Pharmaceuticals, Hanover, NJ 07936*

Accepted for publication July 15, 1970.

The authors acknowledge the technical assistance of Virginia Nieman.

* Present address: Hoechst Pharmaceutical Co., Div. American Hoechst Corp., Somerville, N. J.

Competitive Binding of Two Sulfas and Penicillin G to Bovine Serum Albumin Using NMR Techniques

H. ZIA*, R. H. COX, and L. A. LUZZI

Abstract □ NMR spectroscopy was employed to examine the competitive binding of sulfamerazine, sulfacetamide, and penicillin G to bovine serum albumin. Variations in spin-spin relaxation rates were measured, and evidence for the displacement of penicillin by sulfa drugs was obtained.

Keyphrases □ Sulfonamide binding—bovine serum albumin □ Penicillin binding—bovine serum albumin □ Bovine serum albumin—sulfonamide displacement, bound penicillin □ NMR spectroscopy—analysis

The phenomena of drug–protein binding and of the competitive binding of drugs for available protein sites have been the subjects of many investigations (1). In this respect the antibiotics and sulfonamides have received considerable attention. Certain of these medicinal drugs have been shown to bind to bovine serum albumin (BSA). However, the methods of investigation have varied, and the data from competitive binding in certain instances have been inconclusive. Recent studies established the value of nuclear magnetic resonance (NMR) spectroscopy as a powerful tool for conformational determinations of pharmacologically active molecules in solution (2) and for elucidation of possible interactions between small molecules (3, 4). Furthermore, NMR is invaluable in assessing the extent to which various functional groups on small drug molecules participate in drug–protein interactions (5).

Fischer and Jardetzky (6) and Jardetzky and Wade-Jardetzky (7) utilized the extreme sensitivity of spin-spin ($1/T_2$) and spin-lattice ($1/T_1$) relaxation rates to small variations in the molecular environment of a proton species to show clearly how measurement of these parameters reveals formation of intermolecular complexes. In studies on penicillin and sulfa drug binding to BSA, they demonstrated that there were changes in relaxation rates large enough to be measured. This was true in spite of the fact that the concentration of bound molecules did not exceed 1% of the total drug concentration. The ability to detect the differences in relaxation rates upon binding, especially those differences due to diminished molecular motion, particularly rotational motion, made these studies possible. Observation of the relatively large increments in relaxation rate of the phenyl protons of penicillin and the *p*-aminobenzenesulfonamide moiety of sulfonamides lead them to conclude that these portions of the drug molecules are involved in binding.

In another report, Jardetzky and Wade-Jardetzky (8) indicated that there was no displacement of sulfa drugs bound to BSA by penicillin at reasonable concentrations of penicillin. This led them to postulate that the sites of binding on BSA for sulfa drugs and penicillin were different. However, there was no indication of concentration ratios used nor was there any indication that ionic strength and/or pH were controlled. Later, Kunin (9), using dialysis studies, showed that sulfa drugs in general displace certain BSA-bound penicillins.

Jardetzky and coworkers (8) reported that no displacement of sulfa drugs bound to BSA by penicillin occurs; Kunin (9), using different techniques, observed that the reverse displacement does take place. Thus, it appeared desirable to examine both displacement avenues while, at the same time, controlling factors such as ionic strength and pH which are known to be of significance in protein-binding studies. In this paper, the authors report the results of investigations of competitive binding to BSA between sulfamerazine (I) and penicillin G (II) and between sulfacetamide (III) and penicillin G (II) while ionic strength and pH are controlled.

Table I—Effect of Variations of Solution Contents on the Relaxation Rate (sec.$^{-1}$) of Selected[a] Protons on the Penicillin G and Sulfamerazine Molecules

Compounds	Merazine[b]	CH_2[c]	CH-COO^-[d]	PABS[e]
0.1 *M* potassium penicillin G alone	—	4.71	1.88	—
0.5 *M* sodium sulfamerazine alone	1.57	—	—	2.51
0.1 *M* potassium penicillin G + 2.5% BSA	—	17.77	4.84	—
0.5 *M* sodium sulfamerazine + 2.5% BSA	3.38	—	—	3.71
0.1 *M* potassium penicillin G + 0.5 *M* sodium sulfamerazine + 2.5% BSA	3.03	10.5	3.45	3.34

[a] Those protons whose $1/T_2$ changes would best elucidate binding characteristics were chosen. [b] Proton 6 on the pyrimidine ring of sulfamerazine. [c] Methylene protons adjacent to the phenyl ring in penicillin G. [d] Methine proton on some carbon as carboxyl group in penicillin G. [e] Protons *ortho* to the sulfonyl group in sulfamerazine.

EXPERIMENTAL

Materials—The following materials were used: crystallized BSA,[1] 99.8% deuterium oxide,[2] potassium penicillin G,[3] sodium sulfacetamide, and sodium sulfamerazine.[4] All chemicals were used without further purification.

Methods—Solutions for NMR studies were made in deuterium oxide (D_2O). Drug concentrations are all expressed as molar (*M*), and protein concentrations are given in percent weight per volume (w/v). Ionic strengths of the solutions were adjusted using reagent grade KCl. The ionic strengths of the final solutions were kept 1 unit in excess of the ionic strength contributed by albumin, phosphate buffer, and potassium penicillin G at pH 7.15 ± 0.05. The pH's of the sample solutions were kept constant using 0.1 *M* phosphate buffer in D_2O to supplement the buffer capacity contributed by albumin. All pH measurements were made on a Radiometer titrator (TTT_{1c}) with microelectrodes. The pH values given are actual meter readings and are uncorrected for deuterium isotope effects.

Spectra—All proton NMR spectra were taken on a Varian Associates HA-100 spectrometer operating in the field-sweep mode with a probe temperature of 29°. Tetramethylsilane (TMS) was used as an external reference. Spectra were calibrated by the usual frequency difference techniques. A scan width of 50 Hz. was employed with a sweep time of 500 sec. Each reported value of $1/T_2$ is the mean of at least four, and usually eight, separate measurements. The values of $1/T_2$ given for the various protons were obtained from the spectral line-widths using Eq. 1:

$$\Delta V = (\pi T_2)^{-1} \qquad \text{(Eq. 1)}$$

where ΔV is the line-width at one-half maximum peak height, and T_2 is the spin-spin relaxation time (10).

RESULTS

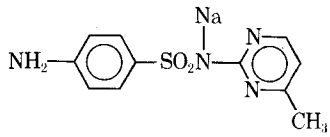
Displacement of Bound Potassium Penicillin G from BSA by Sodium Sulfamerazine—The drug sulfamerazine, which is known to bind rather strongly to serum albumin, was used to displace bound penicillin G from BSA. Spectra of the following five solutions were obtained: (*a* and *b*) potassium penicillin G, 0.1 *M*, alone, and sodium sulfamerazine, 0.5 *M*, alone: these served as controls for the combined solutions; (*c*) penicillin G, 0.1 *M*, and BSA, 2.5%, as a control for the effect of sodium sulfamerazine; (*d*) sodium sulfamerazine, 0.5 *M*, and BSA, 2.5%, as a control for the effect of potassium penicillin G; and (*e*) the combination of all three substances to detect displacement of penicillin G, if any. For penicillin G, $1/T_2$ was calculated for the methylene protons on the side chain and for the methine proton adjacent to the carboxylic group. The phenyl protons were not used since, at 100 MHz., a rather complex pattern is observed for these protons. The two *p*-aminobenzenesulfonamide protons adjacent to the sulfonyl group and a proton (H6) on the pyrimidine ring were used for the calculation of $1/T_2$ values for sodium sulfamerazine. The results obtained from these experiments are given in Table I.[5]

Table II—Effect of Various Concentrations of Sulfacetamide on Relaxation Rates (sec.$^{-1}$) of 0.1 *M* Penicillin G–5% BSA Solution at pH 7.15 ± 0.05 and Constant Ionic Strength

Compounds	PABS[a]	CH_2[b]	CH-COO^-[b]	C_6H_5[c]
0.1 *M* potassium penicillin G + 5% BSA only	—	25.75	6.63	16.64
0.1 *M* potassium penicillin G + 0.1 *M* sulfacetamide + 5% BSA	3.17	23.14	6.65	15.13
0.1 *M* potassium penicillin G + 0.3 *M* sulfacetamide + 5% BSA	3.60	21.38	5.37	15.07
0.1 *M* potassium penicillin G + 0.5 *M* sulfacetamide + 5% BSA	4.34	20.72	5.97	15.57
0.1 *M* potassium penicillin G + 1.0 *M* sulfacetamide + 5% BSA	4.24	20.47	6.37	15.39

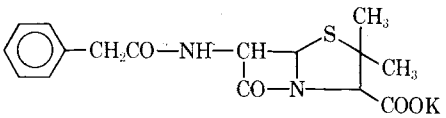
[a] Protons *ortho* to the sulfanyl group in sulfacetamide. [b] The same as in Table I. [c] Phenyl protons of penicillin G.

Displacement of Potassium Penicillin G from BSA by Sodium Sulfacetamide—Since it has been reported that the relaxation rates of penicillin and sulfa drugs are both pH and ionic strength dependent (6, 8), further experiments were designed to show that the decrease in $1/T_2$ obtained in potassium penicillin G with the addition of sodium sulfamerazine was not due to these factors. To measure relaxation rates in the pH region where pH effects on the $1/T_2$ of penicillin G are minimum (6), it was necessary to choose another sulfa drug that did not have the limitations of solubility inherent with sulfamerazine. In this series of experiments, the intent was to displace potassium penicillin G (0.1*M*) from BSA solutions (2.5 and 5%) by adding various concentrations of sodium sulfacetamide. The pH and ionic strength of these sample solutions were adjusted



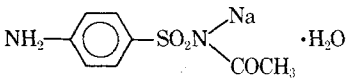
sodium sulfamerazine

I



potassium penicillin G

II



sodium sulfacetamide

III

[1] Nutritional Biochemical Corp.
[2] Mallinckrodt.
[3] Bristol Laboratories.
[4] Abbott Laboratories.

[5] To measure accurately the relaxation times for a proton, one must be able to observe an isolated single signal due to a transition from that proton. At 100 MHz., the phenyl protons of penicillin G give rise to a complex AA'BB'C NMR spectrum. Since there is considerable overlap of the signals from the phenyl protons, a single transition, due to the phenyl protons, could not be measured with accuracy. Therefore, the methylene protons, adjacent to the phenyl ring, of penicillin G were used for calculating relaxation times.

Table III—Effect of Various Concentrations of Sulfacetamide on Relaxation Rates (sec.$^{-1}$) of 0.1 *M* Potassium Penicillin G–2.5% BSA Solution at pH 7.15 ± 0.05 and Constant Ionic Strength

Compounds	PABS[a]	CH_2[a]	CH-COO^-[a]	C_6H_5[a]
0.1 *M* potassium penicillin G + 2.5% BSA only	—	15.70	4.36	14.62
0.1 *M* potassium penicillin G + 0.1 *M* sulfacetamide + 2.5% BSA	2.54	14.79	3.83	13.85
0.1 *M* potassium penicillin G + 0.3 *M* sulfacetamide + 2.5% BSA	2.91	13.84	3.96	13.63
0.1 *M* potassium penicillin G + 0.5 *M* sulfacetamide + 2.5% BSA	3.47	13.41	4.21	13.19
0.1 *M* potassium penicillin G + 1.0 *M* sulfacetamide + 2.5% BSA	3.58	13.22	4.18	13.47

[a] The same as in Table II.

according to the method reported in the *Experimental* section.

The data obtained with these experiments are given in Tables II and III. The relaxation times for the phenyl protons of penicillin G are given in these tables. However, since it cannot be ascertained with certainty that the values were calculated from a single transition, no conclusions could be drawn from the $1/T_2$ value for the phenyl protons. The values for the phenyl protons are given here to demonstrate the inconclusiveness of these data.[5] A typical plot of $1/T_2$ against concentration of sodium sulfacetamide is in Fig. 1.

DISCUSSION

The results presented in Table I show that the relaxation rates of both the methylene and methine protons of potassium penicillin G are increased by approximately two to threefold in the presence of 2.5% BSA as compared to the same protons of potassium penicillin G without BSA. Furthermore, addition of 0.5 *M* sodium sulfamerazine decreases significantly the $1/T_2$ value of the potassium penicillin protons. This decrease in relaxation rate indicates displacement of potassium penicillin G from BSA. Although the relaxation times reported in this investigation are smaller than those reported previously for potassium penicillin G (6), the increase in relaxation rates upon addition of BSA is approximately the same. This difference in relaxation rates in the two investigations is thought to arise from the differences in resolution of the two instruments used.

Although sodium sulfacetamide has less affinity for BSA than sodium sulfamerazine (11), addition of increasing amounts of sodium sulfacetamide to potassium penicillin G–BSA solutions results in a displacement of potassium penicillin G from BSA (Tables II and III). The decrease in relaxation rates for the penicillin protons is smaller than the decrease observed in the experiments with sulfamerazine, and this reflects the relative affinities of the two sulfa drugs for BSA. The results in Tables II and III show, however, that higher concentrations of sulfacetamide do not yield proportional increases in penicillin G displacement.

The results obtained from this investigation (Tables I–III) indicate that both sulfamerazine and sulfacetamide are able to displace penicillin G from BSA. It is also apparent from these results that both the concentration and affinity of sulfa for BSA seem to be important factors in the degree of displacement of penicillin G from BSA, except at higher sulfa concentrations where displacement is not proportional to concentration. This may be interpreted, in part, on the basis that addition of a high concentration of the sulfa drug (0.5 *M* or more) increases the viscosity of the solution to the extent that decreases in relaxation rates due to displacement are offset with increases in the relaxation rate produced by the viscosity of the solution.

Although the addition of sulfa drugs to the system may change the conformation of the BSA molecules and this change may in part be

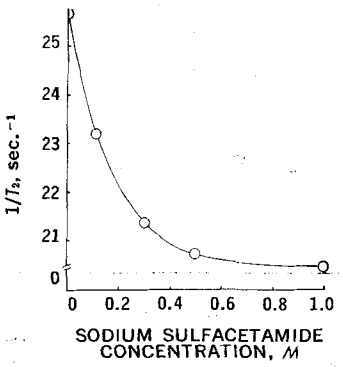


Figure 1—*Effect of concentration of sulfacetamide on relaxation rate of methylene protons for 0.1* M *potassium penicillin G–5% BSA solution at pH 7.15 ± 0.05 and constant ionic strength.*

responsible for the decrease in penicillin–BSA binding, it does not preclude that penicillin is displaced from its protein-bound position.

The possibility that penicillin G may displace sulfacetamide at constant pH and ionic strength was also examined. A 10-fold excess of penicillin G resulted in only a small decrease in the relaxation rates for the sulfacetamide protons compared to the relaxation rate for sulfacetamide–BSA solutions. However, due to the narrow linewidths obtained for the sulfa protons compared to that of the methylene protons from penicillin G and due to the experimental error incurred with relaxation rates, a fair statement concerning whether or not penicillin G displaces sulfacetamide or sulfamerazine cannot be made. It was an aim of this experimentation to establish a definite relationship in this case; however, it was not possible within the limitation of the instrumentation. The results should not be taken to indicate that penicillin is binding to the same site on BSA as the sulfa drugs. The sulfa drugs could be binding to a site adjacent to the penicillin-binding site on BSA and inhibit the binding of penicillin. It is also true that the binding or presence of sulfa drugs could cause a change in the conformation of BSA which could inhibit the binding of penicillin. The data do not allow one to distinguish between these possibilities.

The results of the studies reported here are in agreement with the dialysis data reported by Kunin (9) and, in this respect, further substantiate the usefulness of NMR in studying drug–protein binding. The major advantage of this method compared with dialysis methods is the rapidity with which reliable results may be obtained. No conclusive evidence indicating that penicillin inhibits the binding of sulfa drugs to BSA was found, and this is in agreement with previous studies.

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ACKNOWLEDGMENTS AND ADDRESSES

Received August 4, 1969, from the *Department of Pharmacy, School of Pharmacy, and Department of Chemistry, University of Georgia, Athens, GA 30601*

Accepted for publication July 28, 1970.

* Present address: University of Isfahan, Isfahan, Iran.