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* Present address: Laboratory of Pharmaceutical Chemistry, University of Athens, Athens, Greece.

† To whom all requests for reprints and information should be directed.

‡ Present address: Division of Dermatology, School of Medicine, University of California, San Francisco Medical Center, San Francisco, Calif.

In Vitro Binding Study of Epinephrine and Bovine Serum Albumin

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

Abstract □ The binding of epinephrine to bovine serum albumin and to acetylated bovine serum albumin was studied with respect to evaluation of the active site on the small molecule and to the exchange rate. The effects of varying concentrations of bovine serum albumin on the relaxation rate and chemical shift of 0.1 M (molal) epinephrine were examined. Temperature and pH effects on the relaxation rate of various epinephrine lines were examined. A comparison of relaxation rates between epinephrine–bovine serum albumin and epinephrine–acetylated bovine serum albumin was made. NMR instrumentation was used to follow the reactions. It was found that binding between epinephrine and bovine serum albumin was detectable with this instrumentation and that the most probable active site on the small molecule was the alkyl side chain. It also appeared that there was a fast exchange rate between bound and unbound epinephrine.

Keyphrases □ Epinephrine binding—bovine serum albumin □ Bovine serum albumin, acetylated bovine serum albumin—epinephrine binding □ Temperature, pH effects—epinephrine–bovine serum albumin relaxation rates □ NMR spectroscopy—analysis

The interaction of hormones with plasma proteins is attracting increasing interest because of the involvement of binding in events that are of considerable physiological and pharmacological importance. The numerous studies in this area have been the subjects of several extensive review articles (1–4). During the last decade, there have been a number of conflicting reports concerning whether or not the endogenous hormones, epinephrine and norepinephrine, are bound to plasma proteins. In 1958, Antoniades *et al.* (5), employing dialysis, cationic exchange-resin techniques, and chemical and biological assays, concluded that although epinephrine was completely bound to plasma proteins an appreciable amount of norepinephrine seemed to be unbound, and that the plasma protein responsible for the binding and transport of these hormones was albumin.

In 1962, Bickel and Bovet (6) utilized the method of "crossing paper electrophoresis" and postulated that there was no interaction between these hormones and dog serum albumin. However, during the same year, Litt (7) published the results of his equilibrium dialysis studies and indicated that there was binding between epinephrine and bovine serum albumin (BSA).

In 1968, Cohen *et al.* (8), using paper electrophoresis, showed that isotopically labeled epinephrine and norepinephrine were bound by rabbit serum albumin and also by α- and β-globulin. Neither this nor any of the previous studies has defined the active site of binding on the adrenergic molecules.

Recent studies have established the value of NMR spectroscopy as a tool for conformational determinations of pharmacologically active molecules in solution (9–11), for the elucidation of interactions between small molecules (10, 11), for studying protein small molecule interactions, and in assessing the extent to which various functional groups on the small molecule participate in the interaction (12–16).

In the study reported here, NMR spectroscopy was used to investigate the binding of epinephrine to BSA and to acetylated BSA. The results of this study suggest that epinephrine is bound to BSA and that the active site for binding is located on the aliphatic side chain of epinephrine.

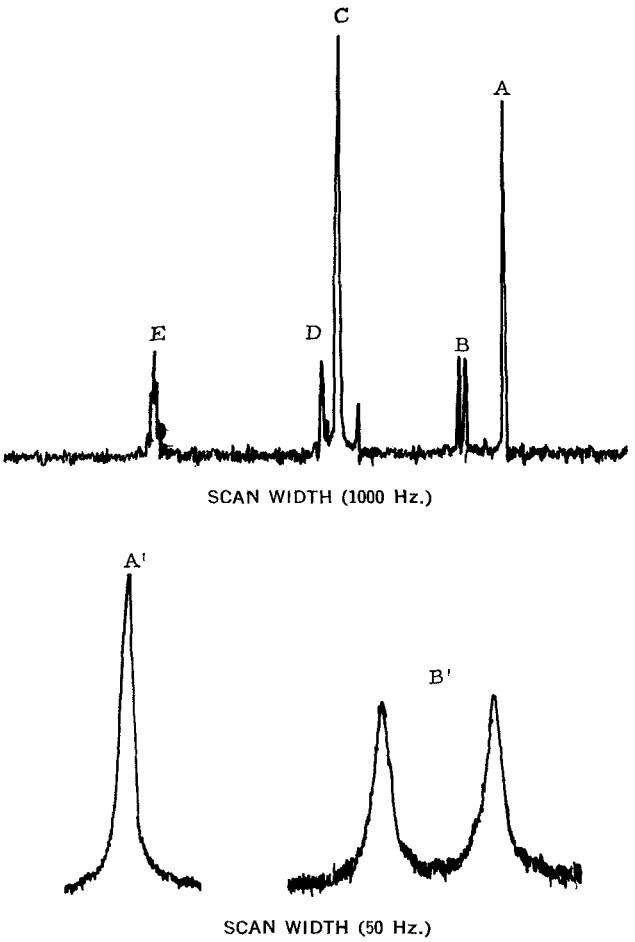

Figure 1—*Proton magnetic resonance spectrum of 0.1 M epinephrine at pH 5.8. At 1000* Hz., *A* = *N—CH₃, B* = *CH₂, C* = *HOD, D* = *CH, and E* = *phenyl protons. At 50 Hz., A'* = *N—CH₃, and B'* = *CH₂ (obtained at different instrument settings).*

EXPERIMENTAL

Materials and Methods—The following materials were used: crystallized BSA,[1] *l*-epinephrine,[2] and 99.8% deuterium oxide.[3] All chemicals were used without further purification.

Acetylated BSA was prepared according to the procedure of Fraenkel-Conrat *et al.* (17). Preliminary purification of acetylated BSA from ions and excess reagents was immediately followed by dialysis of the reaction products at 4° against 8 l. of deionized distilled water for 36 hr. with four changes of distilled water. The purified preparation was then lyophilized.

The extent of acetylation was estimated by treating the sample when *p*-toluene sulfonic acid and distillation of the acetic acid released, followed by titration of the acetic acid with barium hydroxide. The percentage of acetylation was estimated to be 2.5%.

Adjustments of pH were made with DCl and NaOD. All measurements were made on a Radiometer titrator (TTT_1c), with microelectrodes calibrated with standard buffer solutions. All pH values given are actual meter readings and are uncorrected for deuterium isotope effects. All samples were run within 2–3 hr. of preparation.

SPECTRA

Solutions for NMR studies were made in deuterium oxide. Drug concentrations are expressed as molal (M), and protein concentrations are given in percent weight per volume (w/v). All spectra were obtained on a Varian Associates HA-100 spectrometer operating in the field-sweep mode. A probe temperature of 29° and a tetramethylsilane (TMS) external reference were used. 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 six, separate measurements reliable within ± 0.3 sec.$^{-1}$. The values of $1/T_2$ for the various protons were obtained from the spectral line widths using the formula

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

where ΔV is the line width at one-half maximum peak height, and T_2 is the spin-spin relaxation time (18). Chemical shifts are reported in cycles per second downfield from TMS and are uncorrected for bulk susceptibility differences.

RESULTS

Effect of Various Concentrations of BSA on Spin-Spin Relaxation Rates of 0.1 M Epinephrine—The proton magnetic resonance spectrum of 0.1 M epinephrine in D_2O at pH 5.8 is shown in Fig. 1. The aromatic protons are easily identified by their characteristic low-field position (736.5 Hz.) and the single proton on the β-carbon by a triplet (563.3 Hz.) close to the DOH peak (516.0 Hz.). The methylene protons are identified by a doublet (370.5 Hz.), and the N—CH_3 protons by a sharp single peak (320.0 Hz.). A typical example of the effect of albumin on the line width of 0.1 M epinephrine and 5% BSA is shown in Fig. 2. It may be seen from this figure that all lines are broadened to some extent. However, the effect is maximum for the methylene protons. The results of several concentrations of BSA on the relaxation rates of 0.1 M epinephrine at pH 5.8 are summarized in a plot of $1/T_2$ of the side-chain protons *versus* percent (w/v) of BSA (Fig. 3).

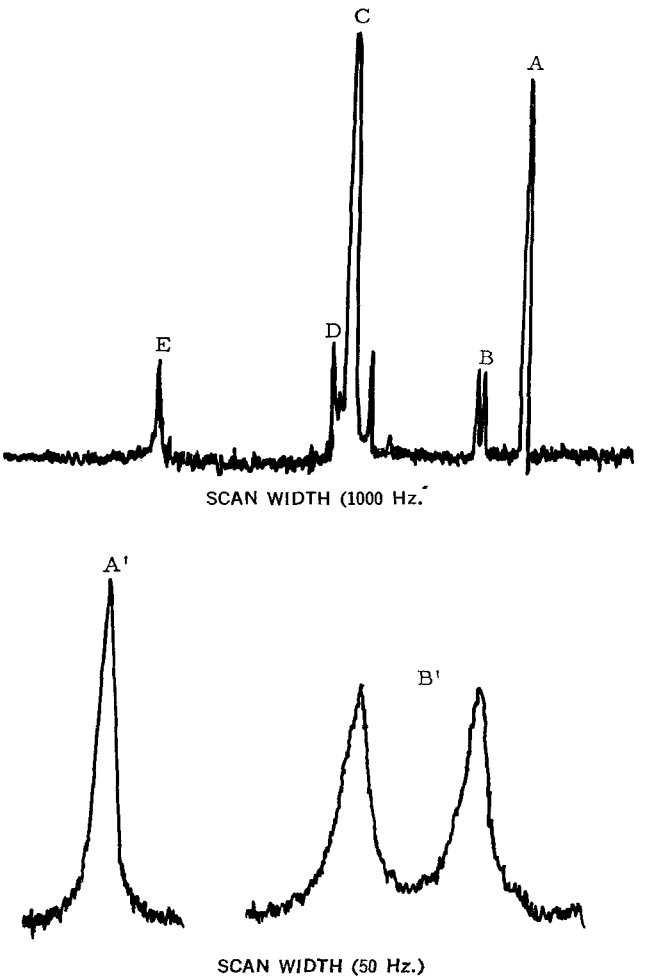

Figure 2—*Proton magnetic resonance of 0.1 M epinephrine–5% BSA in D_2O at pH 5.8. A, A', B, B', C, D, and E bear the same designations as in Fig. 1.*

[1] Nutritional Biochemical Corp.
[2] Wilson Laboratories.
[3] Mallinckrodt.

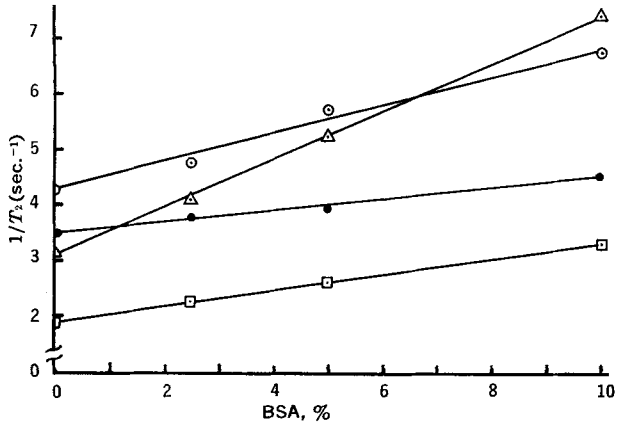

Figure 3—*Effect of BSA on the relaxation rates of 0.1 M epinephrine spectral lines at pH 5.8. Key:* ⊙ = *CH*, △ = CH_2, ⊡ = CH_3, *and* ● = *phenyl protons.*

It can be seen from Fig. 3 that the addition of BSA to a solution of epinephrine increased the relaxation rates of all peaks. However, the effect was more pronounced in the case of methylene protons.

For solutions in which oxidized epinephrine was present, an increase in $1/T_2$ values for the phenyl protons was found which was similar to the increase in the methylene protons. Therefore, it was important to maintain the pH of the epinephrine solutions below 5.8 to prevent oxidation. At pH 5.8, no oxidation of the drug occurred during the experiment.

Effect of BSA on Chemical Shifts of Epinephrine Lines—Chemical shifts of 0.1 M epinephrine for several concentrations of BSA showed no marked differences. In the range of concentrations used here, only a small fraction of epinephrine molecules can be in the bound form and, consequently, changes in chemical shift may not be detectable. Findings such as these have been discussed by Fisher and Jardetzky (14).

Effect of Temperature on Relaxation Rates of Epinephrine–BSA Solution—To confirm the existence of a specific interaction between epinephrine and BSA, several corollary experiments, including temperature studies, were conducted. The effect of variations in temperature on the relaxation rates of 0.1 M epinephrine–2.5% BSA over a temperature range of 10–39° and pH 5.8 is given in Table I. A plot of these data is shown in Fig. 4. Although there were no significant changes in the chemical shifts of epinephrine–BSA solution, it may be seen from Table I and Fig. 4 that the relaxation rates were increased two to threefold as the temperature was lowered. Since the effect of temperature on the relaxation of free and bound molecules has been reported to be small (14), it seems reasonable to assume that the major difference in the relaxation rate observed here as temperature was decreased was due to an increase in the fraction of epinephrine bound.

Effect of pH on the Relaxation Rates of Epinephrine–BSA Solution—To study the effect of changes in pH on the relaxation rates of epinephrine–BSA, a solution of 0.05 M epinephrine and 5% BSA was chosen. The pH range over which such a study could be carried out was restricted due to oxidation of epinephrine at pH values above 5.8 and a reversible configurational change below pH 4.2 (19).

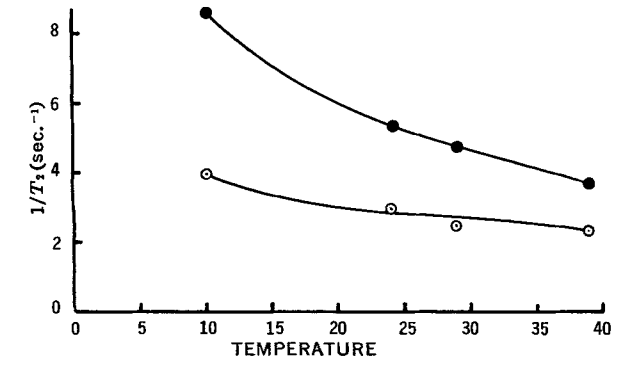

Figure 4—*Effect of variations in temperature on relaxation rates of 0.1 M epinephrine–2.5% BSA at pH 5.8. Key:* ● = CH_3, *and* ⊙ = CH_2.

Table I—Effect of Temperature on Relaxation ($1/T_2$ sec.$^{-1}$) Rates of 0.1 M Epinephrine–2.5% BSA at pH 5.8

Temperature	Phenyl[a] Ring	CH_3	CH[b]	CH_2
10°	4.05	3.99	Overlap	8.67
24°	3.77	3.11	5.81	5.34
29°	3.77	2.51	5.34	4.71
39°	3.61	2.30	4.87	3.77

[a] Indicates measurement taken on the center of the multiplet. [b] Indicates measurement taken on the first line of triplet due to overlap of other peaks in the triplet with the HOD peak.

A plot of pH *versus* $1/T_2$ for methylene protons is shown in Fig. 5. A general increase of relaxation rates was observed, with the methylene peak again exhibiting the greatest change. With the present data, it is difficult to determine the exact mechanism leading to the increase in relaxation rates. However, it seems likely that an increase in pH alters the conformational characteristics of the protein so as to favor formation of the complex and, thus, increase the $1/T_2$ values.

Effect of Temperature on Relaxation Rates of Epinephrine–Acetylated BSA Solution—To elucidate further the nature of the epinephrine–BSA interaction, BSA was acetylated in order to partially block ε-ammonium side chains of the BSA and, thereby, increase the net negative charge on the protein.

As the results in Table II indicate, there was a larger fraction of epinephrine bound to the protein in the 0.1 M epinephrine–2.5% acetylated BSA system than in the 0.1 M epinephrine–2.5% BSA system. This finding is consistent with the proposed mechanism that epinephrine binds to BSA through its side chain by an electrostatic force that may, or may not, be reinforced by the hydrogen bonding of hydroxyl groups.

DISCUSSION

The spin-spin relaxation rate measurements obtained using concentrations of 0.1 M and lower of epinephrine, both with and without varying the BSA concentration, demonstrated that addition of BSA increased the $1/T_2$ of the methylene protons to a greater degree than it did for other epinephrine protons (Fig. 3). Fischer and Jardetzky (14), upon examination of a system consisting of penicillin and BSA, postulated that such findings indicate a specific type of interaction as opposed to one in which nonspecific mechanisms, such as viscosity and intermolecular interaction, may operate. Nonspecific mechanisms, such as those described, should increase the $1/T_2$ of all lines in a given molecule to the same extent. However, if binding is the cause for the increase in relaxation rates, then that part of the molecule that is bound will exhibit the greatest change. Therefore, the findings reported here indicate that not only does a specific interaction take place between epinephrine and BSA, but they also suggest that the side chain of epinephrine is directly involved in the binding process.

The speculation that the results shown in Fig. 3 represent a specific interaction between the epinephrine side chain and BSA is further strengthened by data gathered in the temperature-variation experiments. It can be seen from Table II and Fig. 4 that temperature variations affected the $1/T_2$ of all lines to some degree but that the

Table II—Effect of Temperature on Relaxation Rates ($1/T_2$ sec.$^{-1}$) of 0.1 M Epinephrine–2.5% Acetylated BSA at pH 5.8

Temperature	Phenyl[a] Ring	CH_3	CH	CH_2
3°	—	11.24	Overlap	Not measurable
29°	4.71	5.84	Overlap	15.20
38°	4.55	6.03	Overlap	13.25
46°	4.71	5.93	Overlap	11.68

[a] Indicates measurement taken on the center of the multiplet.

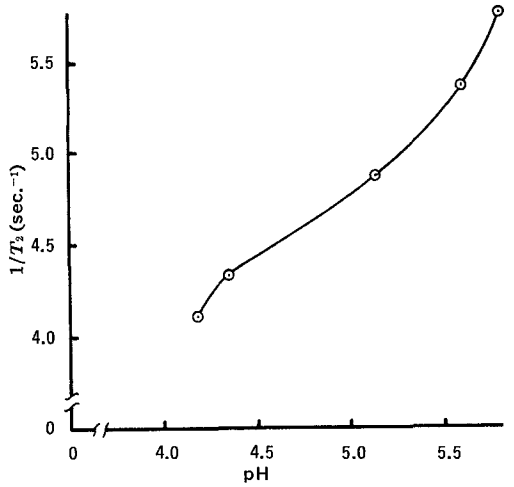

Figure 5—*Effect of pH on relaxation rate of methylene protons for 0.05 M epinephrine–5% BSA solution.*

effect was more pronounced in the case of the methylene lines. These results seem to lend further credence to the previous position that the binding of epinephrine to BSA occurs through the side chain portion of the epinephrine molecule. These results also tend to rule out nonspecific mechanisms as a major factor. Thus, the major increase observed in the relaxation rate, when temperature was the variable, may be attributed to an increase in the fraction of bound epinephrine molecules.

The postulated mechanism that a predominately electrostatic force (which may or may not be reinforced by H-bonding of the hydroxyl group) was operating tends to be supported by the data obtained from the acetylated BSA studies. One would expect that the interaction between BSA and epinephrine would be enhanced by the increased negative charge on the protein if the proposed mechanism is correct. This indeed seems to be the case; the results shown in Table II indicate that BSA showed greater affinity for the drug when acetylated.

A question which remains unanswered is why should the relaxation rate involving epinephrine and BSA increase when pH is increased over the limited range of 4.2–5.8. One possibility would be a change in the conformation of the BSA molecule so as to favor formation of the complex. This reasoning is based on the supposition that in the range of concentration used here, the limiting factor is the number of protein binding sites and not the quantity of ionized drug. At the pH values indicated, ionized epinephrine molecules should be in sufficient supply since the pKa of the amine function has been reported to be at a value in excess of 8.0 (20).

Under the experimental conditions used here, there seemed to be no measurable contribution from the aromatic portion of the epinephrine molecule to the binding process. This may or may not be true if one is considering the binding of epinephrine to serum albumin in a biological system. It is well known that this class of compounds has outstanding chelate-forming properties (21). Trace metal ions in serum and their biological importances have been known for many years. Thus, one may not be able to rule out the possible formation of a three component (epinephrine–metal–albumin) complex. However, as Klotz and Loh-Ming (22) pointed out, such mediation of metal may not be true in all systems.

In 1957, Zimmerman and Brittin (23), while studying absorption of water on silica gel, developed a theory to explore the characteristic relaxation rates of small molecules existing in equilibrium in two or more phases (*i.e.*, bound and free). The observed relaxation rates for such molecules were found to be dependent upon the rate of exchange of molecules between the bound and free forms, as well as on the equilibrium binding constant. Therefore, considering a system where small molecules can exist in only two forms, bound and free, and assuming that the relaxation rate for the bound state is much faster than for the free, three possible cases may be described: slow exchange, intermediate exchange, and fast exchange. Fischer and Jardetzky (14) discussed each case in relation to NMR studies.

The results obtained in this study seem to demonstrate a fast exchange mechanism for the epinephrine–BSA system. The slow exchange mechanism is not acceptable, since no double lines were observed. Neither does intermediate exchange seem to be indicated, since all protons do not have the same relaxation rate. Thus, the only remaining mechanism which explains the nature of epinephrine–BSA interaction is fast exchange. Since the broadening of the lines is not as large as is found in the case of penicillin–BSA (14), one may speculate that the fraction of bound molecules in the penicillin–BSA system is greater than in the epinephrine–BSA system.

In conclusion, it has been shown in the work reported here that binding does occur between epinephrine and BSA. Furthermore, the results obtained suggest that the alkyl side chain is the active site for binding to BSA. Finally, it appears that bound epinephrine is exchanging rapidly with epinephrine in the unbound state.

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