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Investigation of Drug-Albumin Interactions Using Spin-Labeled Bovine Serum Albumin

J. BLANCHARD,[1] T. N. TOZER, D. L. SORBY,[2] AND L. D. TUCK

School of Pharmacy, University of California, San Francisco, California 94143

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SUMMARY

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The interaction between bovine serum albumin and several anionic and cationic drugs was investigated using the electron spin resonance spin labeling technique. Albumin was covalently labeled with three different spin labels to monitor possible drug-induced conformational changes in its structure. The electron spin resonance spectra of the labeled bovine serum albumin in all cases consisted of two subspectra corresponding to a "partially immobilized" and a "strongly immobilized" label environment. The triazine (2,2,6,6-tetramethyl-4-dichlorotriazine aminopiperidine-1*N*-oxyl)-spin-labeled albumin proved most suitable for quantitation of the observed spectral changes. The anionic drugs tested caused an effect on the electron spin resonance spectra interpretable as a conversion of strongly immobilized sites to partially immobilized sites, probably as a result of unfolding of the bovine serum albumin molecule in the vicinity of the labeled histidine residue(s). The changes induced in the labeled bovine serum albumin spectra by acidic drugs were correlated with the degree of binding up to very high drug to protein ratios. These observations suggest that the spectral changes were due to a drug-induced conformational change in the bovine serum albumin with concomitant exposure of additional binding sites. In contrast to the anionic drugs, three tricyclic basic drugs, imipramine, desmethylimipramine, and fluphenazine, induced changes in the labeled bovine serum albumin spectrum exactly opposite to those observed with the acidic drugs—i.e., conversion of partially immobilized sites to strongly immobilized sites—while three nontricyclic basic drugs caused no detectable spectral changes. On the basis of available binding data, it is proposed that the effects of the basic drugs are also related to their degree of protein binding.

INTRODUCTION

Protein binding has important implications in the action of a drug, since changes in it may alter the normal distribution and elimination of a drug. The protein binding of drugs may be modified by disease states or by the presence of other substances competing for the same binding sites.

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[1] Recipient of United States Public Health Service Traineeship 5T01 GM00728-07. Present address, College of Pharmacy, University of Arizona, Tucson, Arizona 85721.

[2] Present address, School of Pharmacy, University of Missouri, Kansas City, Missouri 64110.

In contrast to investigations of tissue proteins, where little work has been done, the nature of plasma protein binding has been extensively investigated (1–4). Much of this work has been quantitative in nature, with little attempt to elucidate the actual molecular mechanisms involved in the binding process. It is only fairly recently, with the development of sophisticated spectroscopic techniques, that significant insights have been gained into the mechanisms involved in drug-protein interactions. These techniques have been summarized in recent reviews (5, 6).

In the experiments reported here the spin labeling technique (7, 8) was used in an attempt to monitor possible drug-induced conformational changes in bovine serum albumin during the process of drug binding. The binding of a number of acidic drugs tested caused partial conversion of strongly immobilized sites to partially immobilized sites (9), whereas the basic drugs tested produced the opposite conversion. This suggests an inherent difference in the binding characteristics of acidic and basic drugs, with both involving a conformational alteration of the bovine serum albumin structure. A preliminary report of this work was previously communicated (10).

MATERIALS AND METHODS

Materials. The spin labels *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)bromoacetamide and *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide were purchased from Synvar Associates, Palo Alto, Calif. The spin label 2,2,6,6-tetramethyl-4-dichlorotriazine aminopiperidine-1*N*-oxyl was synthesized according to a reported procedure (11). These spin labeles are referred to as bromoacetamide, maleimide, and triazine, respectively.

Crystalline bovine serum albumin, A grade (Calbiochem), was dialyzed against several changes of glass-distilled water at 4°, followed by lyophilization. The concentrations of the solutions of both labeled and unlabeled protein were checked by means of the biuret assay (12) or by measurement of the absorbance at 279 nm, using an $E^{1\%}_{1\ cm}$ value of 6.67 (13). All drugs used in this study were of USP grade or better and were donated by their respective manufacturers. All other chemicals were of reagent grade or better.

Methods. Spin-labeled bovine serum albumin derivatives were prepared by reported techniques (11, 14). All solutions were prepared and labeled in phosphate buffer ($\Gamma/2 = 0.0527$) at either pH 6.9 or pH 11. It was necessary to use a Tris buffer to study the effects of some of the basic (cationic) drugs because of their incompatibility with the phosphate buffer. In these studies the bovine serum albumin labeled at pH 6.9 was redialyzed against Tris-HCl buffer, pH 7.0 ($\Gamma/2 = 0.05$), to remove the phosphate ions.

The number of labeled sites on the bovine serum albumin molecule was determined by comparing the spin concentration of the labeled bovine serum albumin solution with a standard aqueous solution of 2,2,6,6-tetramethyl-4-piperidone-1-oxyl oxyl (Tempone). The concentration of this standard solution, which was freshly prepared for each determination, was calculated from its absorbance at 236 nm, $\epsilon_{235\text{-}240} = 2550\ M^{-1}\ cm^{-1}$ (15).

The spin concentration was computed by means of a double integration procedure (16) with the aid of a programmable desk calculator. The method used, reported to be accurate within 5% and to correct for constant baseline drift, has since been refined (17).

Electron spin resonance spectra were recorded on an *x*-band spectrometer which utilized 100-kHz field modulation and Fieldial control of the magnetic field. Titration of triazine-spin-labeled bovine serum albumin with increasing amounts of the various drugs and other agents tested was performed at ambient temperature, using a quartz flat cell and a syringe pump device similar to that described by Holmes and Piette (9). This enabled each spectrum to be recorded under identical instrumental conditions, thereby facilitating direct comparison of successive spectra. The ionic strength and pH were kept constant during the titration, since both are known to affect binding (18–20).

The binding of drugs to bovine serum

albumin was quantitatively determined by centrifugation for 12 hr at 55,000 rpm (400,000 × *g*) (21, 22) using a Beckman preparative ultracentrifuge, model L2-65B, and a type SW 56 swinging bucket rotor. Aliquots of the protein-free supernatant solution were diluted with an appropriate volume of buffer, and the drug concentrations were determined with a Beckman DU spectrophotometer by reference to previously prepared standard curves. The fraction of drug bound to protein was calculated by difference between the concentration in the protein-free supernatant and that in the original solution.

Binding data for phenylbutazone and warfarin were plotted according to the method of Scatchard (23), using the relationship

$$\frac{r}{(D)} = nK_a - rK_a$$

where r = number of moles of drug bound per mole of protein, (D) = molar concentration of free (unbound) drug at equilibrium, K_a = association constant (liters per mole) for the primary binding sites, and n = number of binding sites per molecule of protein.

RESULTS AND DISCUSSION

Selection of labeling conditions and spectral parameter. In the course of the labeling studies the effect of pH changes on the triazine-spin-labeled bovine serum albumin was investigated. Figure 1 shows how the label appears to be more mobile at extremes of pH relative to neutral pH. The increased label mobility at pH 11 is consistent with reported observations (11) with labeled human serum albumin, while the pH 2 spectrum is in agreement with previous observations by McConnell and co-workers (8, 24) concerning bovine serum albumin labeled with either a nitroxide maleimide or a nitroxide isocyanate label. The latter authors observed an increase in the mobility of the attached label as the pH was lowered, with the most dramatic increase in label mobility in the pH 4–2 region. A similar transition with the pH 6.9 triazine-spin-labeled bovine serum albumin was also observed here (Fig. 2).

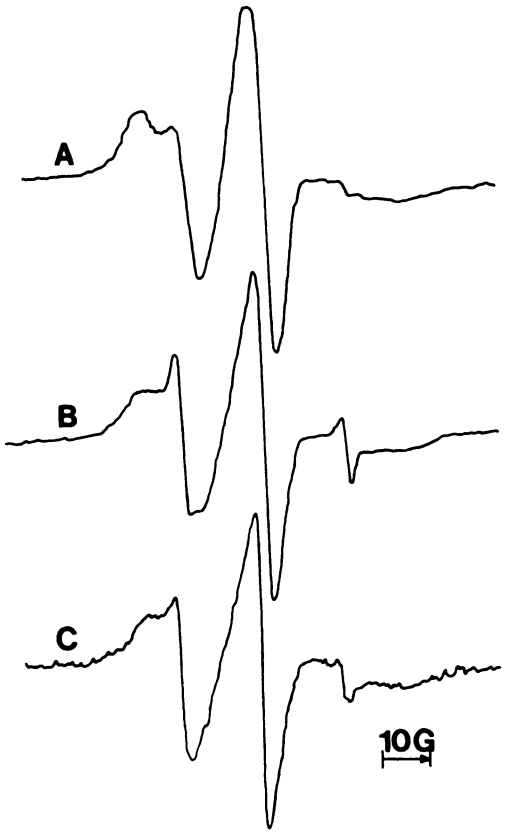

FIG. 1. *Effect of extremes of pH on electron spin resonance spectrum of pH 6.9 triazine-spin-labeled bovine serum albumin*
A. pH 6.9. B. pH 11. C. pH 2.

The pH 4–2 transition seen by McConnell and colleagues and by us using spin labels corresponds quite well with the N–F transition which both human serum albumin and bovine serum albumin have been reported to undergo using other techniques (25). Two distinct conformational transitions occur when albumin is titrated with acid. The first change occurs as the pH is lowered from 4.5 to 3.5. The albumin molecule reportedly undergoes a reversible transformation, in which the N (native or normal) form binds an unknown number of protons and is converted to the F (free) form (25).

A second conformational transition, expansion of the F form, occurs as the pH is lowered from 3.5 to 2.5. Both these changes have been shown by monitoring alterations in the physical properties (26) of the pro-

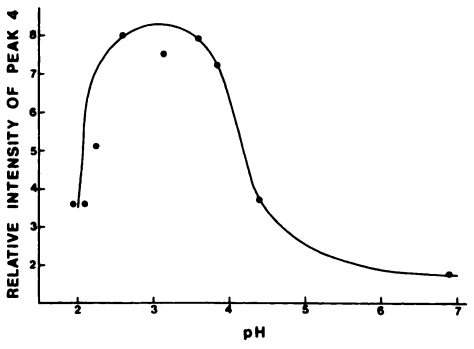

FIG. 2. *Effect of pH change on intensity of high-field peak (peak 4) of pH 6.9 triazine-spin-labeled bovine serum albumin*

teins in solution. The conformational changes probably account for the observed shape of the titration curve of Fig. 2.

Both these acid-induced conformational transitions are reported to have counterparts in the alkaline region. Although no titration on the alkaline side using electron spin resonance spectral techniques has been reported, the spectrum at pH 11 for pH 6.9 triazine-spin-labeled bovine serum albumin (see Fig. 1) is probably due to the reported alkaline transition (25, 27, 28) causing an increase in the mobility of the attached label.

In order to investigate more fully the labeling properties of the triazine spin label, bovine serum albumin was labeled at pH 11 as well as pH 6.9, since Likhtenshtein and Bobodzhanov (11) reported that the triazine spin label bonds solely to histidine groups at pH 6.9, whereas at pH 11 it bonds to both histidine and lysine ϵ-amino groups. Comparison of the effects of added drugs on the electron spin resonance spectra of pH 6.9 and pH 11 triazine-spin-labeled bovine serum albumin revealed that (a) the pH 11 labeled spectrum was more intense, indicating that more groups were labeled per molecule of bovine serum albumin; and also (b) the drug-induced spectral changes were more pronounced in the pH 6.9 labeled sample. The latter observation is made readily apparent when the ratio of the intensity of peak 4 (the high-field peak of the partially immobilized spectrum)[3] in the presence and absence of drug for both pH 6.9 and pH 11 labeled bovine serum albumin samples is plotted against the drug to protein ratio on the same graph (Fig. 3). The increase in the height of this peak was taken as a measure of the conversion of strongly immobilized sites to partially immobilized sites due to the interaction of the drug with the bovine serum albumin.

This was possible because the use of the syringe pump device enabled successive spectra to be recorded under identical instrumental conditions. Among the affected peaks, peak 4 was least altered by any overlap of the strongly immobilized spectrum, and provided data points with a minimum of scatter. The desirability of using the high-field peak of the isotropic spectrum has previously been noted (29).

Determination of strongly immobilized to partially immobilized ratio. In order to quantitate the differences between the pH 6.9 and pH 11 triazine-spin-labeled bovine serum albumin samples, the fractions of strongly immobilized and partially immobilized sites in each sample were calculated. The procedure employed for this was as follows.

First, a spectrum of a 2-ml volume of each triazine-spin-labeled bovine serum albumin sample was recorded using the syringe pump device previously described. Next, 1.4 ml of 0.2000 N HCl were added to produce a pH of approximately 2, which is reported to be the optimal pH for pepsin (30). The sample was thoroughly mixed, and the electron spin resonance spectrum was again recorded. Approximately 10 mg of pepsin were added to the system, and after thorough mixing the spectrum was recorded. The electron spin resonance spectrum of the labeled bovine serum albumin sample treated with pepsin gradually became more isotropic with time. A spectrum sufficiently isotropic to analyze was obtained in 2-3 hr.

In order to measure the fraction of label in strongly immobilized and partially immobilized sites, the heights of peaks 1 and 4 were utilized. The height of peak 1 is proportional to the number of strongly immobilized sites present; its value is measured before (SI_1) and after (SI_2) denaturation of the bovine serum albumin with

[3] See Fig. 1 of ref. 10 for the peak-numbering convention.

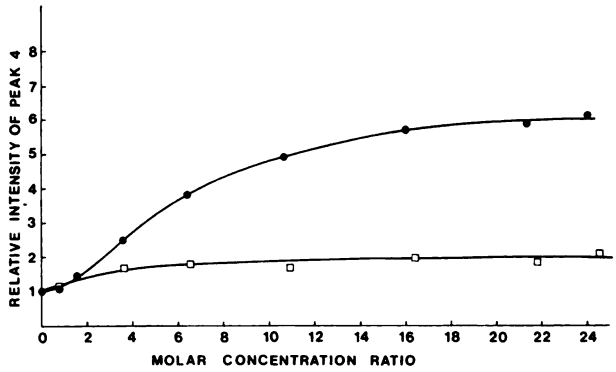

FIG. 3. *Plot of relative intensity of peak 4 against molar concentration ratio of phenylbutazone to bovine serum albumin*

●——●, pH 6.9 triazine-spin-labeled bovine serum albumin; □——□, pH 11 triazine-spin-labeled bovine serum albumin (5%, w/v).

pepsin. The values of PI_1 and PI_2 (the heights of peak 4) represent the relative number of partially immobilized sites present on the labeled albumin before and after pepsin denaturation, respectively.

Since the formation of partially immobilized sites occurs at the expense of strongly immobilized sites, and vice versa, the total concentration of sites labeled (T) does not change. Consequently the concentration of strongly immobilized sites before (a_1) and after (a_2) denaturation and the concentration of partially immobilized sites before (b_1) and after (b_2) denaturation may be determined by solving the following equations:

$$a_1 + b_1 = T$$
$$a_2 + b_2 = T$$
$$\frac{a_1}{a_2} = \frac{SI_1}{SI_2}$$
$$\frac{b_1}{b_2} = \frac{PI_1}{PI_2}$$

By substituting the measured values of SI_1, SI_2, PI_1, and PI_2 into the above equations, the values of a_1, a_2, b_1, and b_2 can be solved in terms of T. In a typical experiment it was determined that the pH 6.9 triazine-spin-labeled bovine serum albumin sample had about 97.5% of its total labeled sites in a strongly immobilized environment and 2.5% in a partially immobilized environment, whereas the pH 11 triazine-spin-labeled bovine serum albumin sample had 99.5% of its total labeled sites in a strongly immobilized environment and 0.5% in a partially immobilized environment.

It was found, using spin concentration measurements, that approximately one spin label is bonded to bovine serum albumin when labeled at pH 6.9, and between two and three when labeled at pH 11. These results lend support to the observations (11) that at pH 11 the triazine spin label bonds to lysine residues in addition to the histidines labeled at pH 6.9.

Perturbation of binding caused by label. The introduction of the label may be considered to be a "tolerable" perturbation if the property of interest, in this case drug binding, is comparable in the labeled and unlabeled systems (31). In order to resolve this question the binding affinities of pH 6.9 and pH 11 triazine-spin-labeled bovine serum albumin were compared with unlabeled bovine serum albumin. Two drugs, warfarin and phenylbutazone, were chosen for this study, as they are both known to possess a high affinity for human serum albumin (32). Binding studies by means of ultracentrifugation were performed. The solutions used were the same as those used in the electron spin resonance titrations. Table 1 summarizes these results and shows that the binding was only slightly reduced as a result of labeling. The extent of drug binding to the bovine serum albumin samples followed the order unlabeled > pH 6.9 labeled > pH 11 labeled—a reasonable order since there are more sites labeled at pH 11 than at pH 6.9. This

indicates that the labeling does cause a small but "tolerable" perturbation of the binding process, perhaps by masking a small fraction of the groups to which these two acidic drugs would normally bind. This is consistent with the suggestion by Skidmore and Whitehouse (33) that the ϵ-amino group of lysine is the binding site for anionic drugs.

It does not appear that the labeling produces a conformational change in the bovine serum albumin, since it is highly unlikely that the drug binding would be so slightly altered by a major conformational change. Moreover, the conformational changes with pH and with the addition of urea (14) are the same as with unlabeled bovine serum albumin. While ultracentrifugal binding studies were not performed on all drugs used, it seems likely that these results are typical at least for acidic drugs similar to those tested.

Electron spin resonance drug titration studies. The acidic drugs used in this study were selected because each was reported to have a high affinity for serum albumin. The results of the titrations of labeled bovine serum albumin with acidic drugs are summarized in Fig. 4. All these drugs seemed to induce comparable changes in the electron spin resonance spectrum, which manifested themselves as a conversion of strongly immobilized sites to partially immobilized sites, as illustrated by Fig. 1 of ref. 10, which is typical of all the acidic drugs tested.

The effect of weakly bound agents on the spectrum of labeled bovine serum albumin was obtained by titrating pH 6.9 and pH 11 triazine-spin-labeled bovine serum albumin with antipyrine. Antipyrine was chosen because it is reported to bind very weakly to albumin (34). Ultracentrifugal analysis demonstrated that about 14% of antipyrine was bound to bovine serum albumin at a 24:1 molar ratio, a binding somewhat higher than that reported. Antipyrine differs from the previously studied acidic drugs in that it is a weak base (pK_a 1.4) and at pH 6.9 it is essentially totally un-ionized whereas the acidic drugs were all predominantly in the ionic form. For both pH 6.9 and pH 11 triazine-labeled bovine serum albumin samples the changes induced by antipyrine in the labeled bovine serum albumin spectra, up to drug to bovine serum albumin molar ratios of 24:1, were very small compared to equivalent molar ratios of phenylbutazone. Since the weakly bound antipyrine caused only small spectral changes relative to strongly bound agents, it may be inferred that the spectral changes observed with the strong binders may be related to the binding process.

In order to correlate the degree of binding with the observed spectral changes, binding profiles were obtained for the two structurally dissimilar acids warfarin and phenylbutazone. The results of these studies are shown in Fig. 5, where the relative intensity of peak 4 is plotted against r (the

TABLE 1
Protein binding data

Protein	Drug bound					
	Warfarin			Phenylbutazone		
	2:1[a]	4:1	9.1:1	2:1	4:1	9.1:1
	%	%	%	%	%	%
Unlabeled bovine serum albumin	97.5	91.2	68.7	97.1	90.3	—[b]
pH 6.9 triazine-spin-labeled bovine serum albumin	96.3	87.0	—	95.2	—	69.9
pH triazine-spin-labeled bovine serum albumin	92.9	81.2	58.1	89.5	78.5	60.3

[a] Drug to protein ratios, all with 5% (w/v) bovine serum albumin.
[b] The dashed lines indicate that no binding data were obtained.

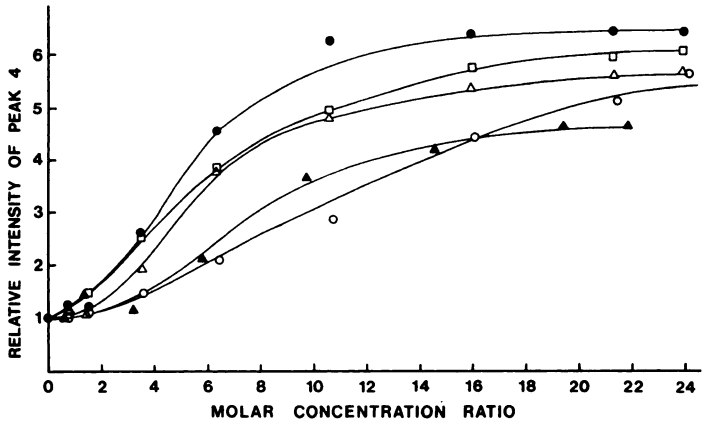

FIG. 4. *Plot of relative intensity of peak 4 against drug to protein molar concentration ratio for several acidic drugs*

●——●, flufenamic acid; □——□, phenylbutazone; △——△, sulfaethidole; ▲——▲, oxyphenbutazone; ○——○, warfarin.

moles of drug bound per mole of bovine serum albumin). The linear relationship obtained with the phenylbutazone binding data, together with the high correlation coefficient (0.99), is strong evidence that the observed spectral changes are related to the degree of binding. The linearity of response up to high molar ratios of drug to bovine serum albumin indicates that the spectral change, which is probably a result of a conformational alteration of the protein, occurs via a mass-action process such as protein binding. There seems little doubt that the observed effects are due to a conformational change in the bovine serum albumin molecule by a mechanism similar to that suggested by Stone *et al.* (8) to account for changes in the spectra of labeled bovine serum albumin due to pH.

The curve for warfarin, while not a linear relationship, nevertheless indicates a definite correlation between the degree of binding and the observed spectral change. The difference between the shape of this curve and the straight line observed for phenylbutazone may result from differences in the nature of the binding of warfarin and phenylbutazone; i.e., they may bind at different sites or at the same site, but with a different spatial orientation relative to the protein, as has previously been suggested by Chignell, using circular dichroism (35). Chignell also noted a linear

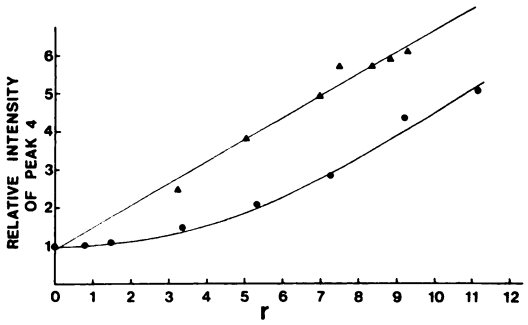

FIG. 5. *Relationship between extent of binding of warfarin and phenylbutazone and their effect on electron spin resonance spectrum of pH 6.9 triazine-spin-labeled bovine serum albumin (5%, w/v)*

●——●, warfarin; ▲——▲, phenylbutazone. The abscissa, *r*, is moles of drug bound per mole of protein.

relationship between the molar ellipticity and the moles of drug bound in a phenylbutazone–human serum albumin system. The observed ellipticity reached a maximum at a molar ratio of about 2.6 moles of phenylbutazone per mole of human serum albumin, for which Chignell (36) offered no explanation. It was noted that human serum albumin possessed one high-affinity binding site ($K_a = 10^5$) and two others of lower affinity ($K_a = 4 \times 10^4$) for phenylbutazone.

Scatchard (23) plots of our binding data for phenylbutazone and warfarin are shown in Fig. 6. These results indicate that there

are more than two binding sites for phenylbutazone on the bovine serum albumin molecule. This is shown in Fig. 6 by the lack of a well-defined intercept on the abscissa up to an r value of 11. If this is also true for human serum albumin, a possible explanation for Chignell's data, in which the molar ellipticity leveled off after addition of 2.6 moles of phenylbutazone per mole of human serum albumin, may be that a large number of low-affinity sites exist whose interaction with phenylbutazone is not sufficiently strong to hold the drug rigidly in a preferred conformation upon binding. Another possibility is that the low-affinity sites may lack certain symmetry elements necessary for the generation of optical activity.

In further comparing our electron spin resonance results on acidic drugs with those obtained by Chignell using optical rotatory dispersion-circular dichroism techniques, it was noted from Chignell's data that warfarin (37) and sulfonamides (35) did not generate any extrinsic Cotton effects upon binding to human serum albumin; apparently they form a rather loose complex with human serum albumin. Our results with bovine serum albumin (Fig. 4) indicate that both warfarin and sulfaethidole, a sulfonamide, cause a dramatic change in the electron spin resonance spectrum of the albumin. The correlation of the binding data of warfarin with the observed spectral change up to high molar ratios of warfarin to bovine serum albumin (Fig. 5)

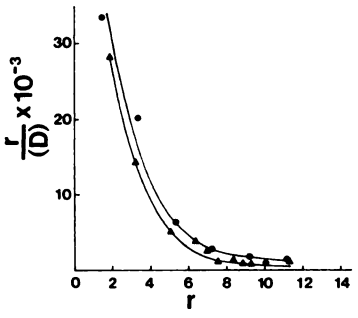

FIG. 6. *Scatchard plot of binding of phenylbutazone (▲——▲) and warfarin (●——●) to pH 6.9 triazine-spin-labeled bovine serum albumin (5%, w/v)*

r and (D) are defined in the text.

is strongly suggestive of a drug-induced conformational change in the bovine serum albumin molecule, with concomitant exposure of additional binding sites. Such a possibility has previously been suggested by O'Reilly (38) to account for the high capacity of human serum albumin for warfarin.

Oxyphenbutazone was less effective than phenylbutazone in converting strongly immobilized to partially immobilized sites (see Fig. 4). This observation concurs with that of Chignell (36), who noted a markedly lower extrinsic optical activity for oxyphenbutazone relative to phenylbutazone.

The similarities and apparent differences seen with these two procedures illustrate the complementarity of these and other spectroscopic methods and reinforce the notion that a combination of probe techniques is desirable in ascertaining the nature of ligand-macromolecule interactions.

The first basic drug titrated against the pH 6.9 triazine-spin-labeled bovine serum albumin was the tricyclic antidepressant desmethylimipramine, which has been postulated to induce a conformational change in human serum albumin (39, 40). The effect of desmethylimipramine on the electron spin resonance spectrum of pH 6.9 triazine-spin-labeled bovine serum albumin was directly opposite to the effect observed with the acidic drugs, in that it caused a conversion of partially immobilized sites to strongly immobilized sites (Fig. 7). Since peak 4 exhibited little change here, the height of peak 2 (the low-field peak of the partially immobilized spectrum) was the spectral parameter measured. A plot of the relative intensity of peak 2 against the molar concentration ratio of drug to bovine serum albumin (Fig. 8) clearly illustrates the decrease in the relative number of partially immobilized sites present as drug is added.

Several other basic drugs were tested in a qualitative manner by observing the spectrum of the pH 6.9 labeled bovine serum albumin in Tris buffer before and after addition of 17 moles of drug per mole of

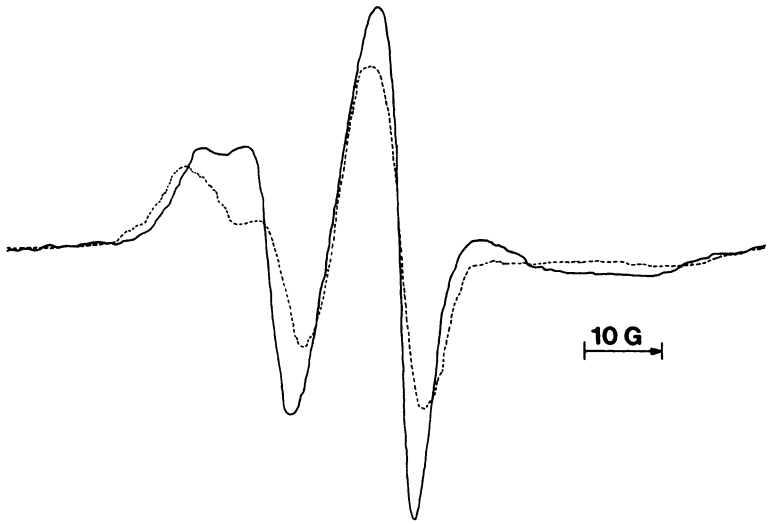

FIG. 7. *Electron spin resonance spectra of pH 6.9 triazine-spin-labeled bovine serum albumin in Tris buffer, pH 7, before (——) and after (-----) addition of 24 moles of desmethylimipramine per mole of bovine serum albumin (5%, w/v)*

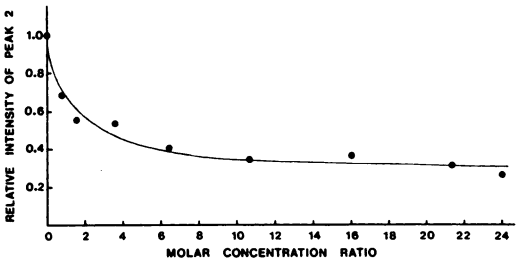

FIG. 8. *Relative intensity of peak 2 plotted against molar concentration ratio of desmethylimipramine to bovine serum albumin (5%, w/v)*

bovine serum albumin. Two other tricyclic basic drugs tested, imipramine hydrochloride and fluphenazine dihydrochloride, caused changes qualitatively similar to those seen with desmethylimipramine. Conversely, three nontricyclic basic drugs, codeine phosphate, pilocarpine hydrochloride, and homatropine hydrobromide, caused no detectable changes in the labeled bovine serum albumin spectrum. This observation can be explained by the relatively weak binding affinity of the drugs for the protein or by binding of these three drugs at a site too far removed from the triazine spin label to affect its mobility. This latter speculation is partially substantiated by previous experiments wherein atropine, a closely related analogue of homatropine, was shown not to competitively inhibit the binding of warfarin (41). The three tricyclic basic drugs caused qualitatively similar effects, because they probably share a common binding site as a result of their close structural similarity. Although quantitative binding studies for these basic drugs were not performed, available binding data indicate relatively strong binding for the tricyclic antidepressants (39, 40, 42) and relatively weak binding for codeine,[4] pilocarpine (43–45), and homatropine.[5]

In addition to the effects of acidic and basic drugs and weakly bound, un-ionized antipyrine, the effect of strongly bound non-ionic drugs on the system was also deemed to be highly desirable. Unfortunately, a drug un-ionized at pH 6.9 and possessing the desired properties of high water solubility and strong affinity for bovine serum albumin could not be found.

Holmes and Piette (9), using a spin-labeled bovine erythrocyte ghost system, observed the conversion of partially immo-

[4] Many of the binding data for these agents are old and semiquantitative.

[5] The association constant for the structurally related analogue, atropine, is about 10^3 (46).

bilized sites to strongly immobilized sites with several phenothiazine analogues, including imipramine and desmethylimipramine, whereas they noted exactly the opposite effect (conversion of strongly immobilized to partially immobilized sites) with sodium lauryl sulfate. Since this agent is anionic, as are the acidic drugs used here, we decided to investigate its effect on this system. Sodium lauryl sulfate, like the other anions, caused the conversion of strongly immobilized to partially immobilized sites. The similarity of the spectral changes of these two structurally diverse labeled protein systems indicates that both albumin and erythrocyte ghosts undergo conformational changes (at least in part) via electrostatic effects. How universal these effects of anions and cations are on the conformation of proteins has yet to be determined.

One possible explanation for the poor sensitivity of the pH 11 relative to the pH 6.9 labeled bovine serum albumin can now be postulated. In titrating the labeled bovine serum albumin with phenylbutazone, conversion of strongly immobilized sites to partially immobilized sites was observed as the drug concentration increased. If the additional (lysine) sites labeled at pH 11 are immobilized much more strongly than those labeled at pH 6.9, as the data seem to indicate, it is quite possible that the pH 11 sites are buried and thus inaccessible to the action of added drug. In other words, the greater sensitivity of the pH 6.9 labeled bovine serum albumin samples may be due to their containing a greater proportion of sites accessible to the action of the drug. It is worth noting here that the terms strongly immobilized and partially immobilized are merely qualitative, and that not all strongly immobilized sites necessarily possess the same degree of immobilization. These observations are consistent with those of Holmes and Piette (9), who noted that when bovine erythrocyte ghost membranes were labeled with the iodoacetamide spin label the electron spin resonance spectrum indicated a single population of fairly mobile spin labels that were sensitive to the addition of phenothiazines. In contrast, the maleimide-spin-labeled membranes contained a large population of strongly immobilized spin labels which were too insensitive for adequate monitoring of any drug-induced spectral changes.

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