sodium carbonate mixture could be predicted to be the most stable blend after 1 day of testing. The prediction was clearly confirmed by results of a classical stability study requiring up to 6 months of storage at 50°.

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Drug-Albumin Interactions Using Spin Labeling

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Abstract ☐ Three spin labels were used to monitor possible drug-induced conformational changes in bovine serum albumin. The electron spin resonance spectra of labeled bovine serum albumin in all cases consisted of two subspectra corresponding to labels in a partially immobilized environment and a strongly immobilized environment. Only bovine serum albumin with the triazine spin label was suitable for quantitation of data. The strongly protein-bound acidic drug, phenylbutazone, caused a conversion of strongly immobilized sites to partially immobilized sites, an effect that was proportional to the amount of drug added. This probably was due to the initial drug binding inducing a conformational change in the bovine serum albumin, thereby exposing additional binding sites.

Keyphrases ☐ Drug–albumin interactions—use of three spin labels to monitor drug-induced protein conformational changes, electron spin resonance spectra ☐ Albumin–drug interactions—use of three spin labels to monitor drug-induced protein conformational changes, electron spin resonance spectra ☐ Spin labeling—monitoring drug-induced conformational changes in bovine serum albumin, three spin labels studied

The technique of spin labeling was recently applied to a number of macromolecular systems where conformational changes were suspected (1, 2). This report describes, for the first time, an attempt to monitor drug-induced changes in bovine serum albumin utilizing this method. Although various analogous systems have been studied similarly (3–5), the pharmacodynamically important drug–albumin system (6, 7) has not been investigated; preliminary data are reported here.

EXPERIMENTAL

Materials and Methods—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[1]. The spin label 2,2,6,6-tetramethyl-4-dichlorotriazineaminopiperidine-1-*N*-oxyl was synthesized according to a reported procedure (8). These spin labels are referred to here as bromoacetamide, maleimide, and triazine, respectively.

Prior to use, the crystalline bovine serum albumin[2] was dialyzed against several changes of glass-distilled water at 4° followed by lyophilization. Protein concentration was checked by the biuret assay (9) or by measurement of absorbance at 279 nm., using a = 0.667 (10). Spin-labeled derivatives of bovine serum albumin were prepared using techniques similar to those previously reported (8, 11). All solutions were prepared and labeled in phosphate buffer of pH 6.9 (μ = 0.0527). Electron spin resonance spectra were recorded on an *X*-band spectrometer, utilizing 100-kHz. field modulation and Fieldial control of the magnetic field. The titration of triazine spin-labeled bovine serum albumin with increasing amounts of phenylbutazone was performed at ambient temperature using a quartz flat cell and a syringe pump set up similarly to one described elsewhere (3). Each spectrum could be recorded under identical instrumental conditions, thereby facilitating direct comparison of successive spectra.

RESULTS AND DISCUSSION

The electron spin resonance spectra obtained with both maleimide and bromoacetamide spin-labeled bovine serum albumin did not change appreciably when interacted with phenylbutazone. This could be due to the fact that these two sulfhydryl group-directed labels were located at a site far removed from the binding locus for phenylbutazone. Another possibility is that these labels may have been present in too low a concentration to have their spectra detectably altered by the binding process. It is also possible that the sulfhydryl grouping may be in a region of the protein that results in the attached labels being too strongly immobilized for their spectra to be adequately perturbed by the binding process.

In contrast, the amino group-directed triazine spin label, which at pH 6.9 is linked solely to histidine residues (8), appears to contain a ratio of strongly immobilized sites amenable to quantitation. Figure 1A shows that the spectrum of triazine spin-labeled bovine serum albumin actually consists of two types of signals, a sharp three-line spectrum corresponding to a label at a partially immobilized site and a superimposed broadened spectrum characteristic of the label in a strongly immobilized environment. Figure 1B shows the effect of adding 24 moles of phenylbutazone/mole of labeled bovine serum albumin. This spectrum represents the endpoint in the titration, because this was the maximum amount of phenylbutazone added. Spectra of several intermediate ratios of phenylbutazone to labeled bovine serum albumin were also recorded. Figure 2 shows a plot of the relative intensities of the high field peak of the partially immobilized spectrum (peak 4) *versus*

[1] Synvar Associates, Palo Alto, Calif.

[2] A grade, Calbiochem.

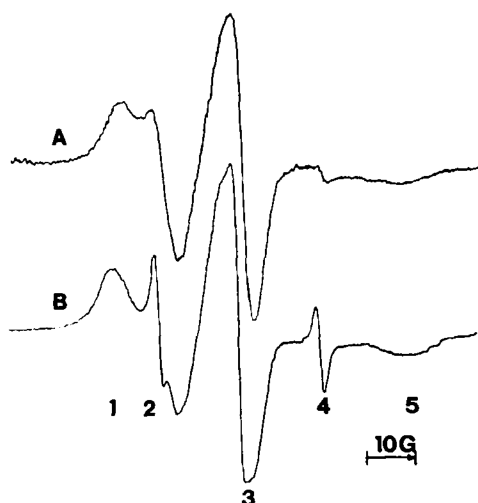

Figure 1 *Electron spin resonance spectrum of triazine spin-labeled bovine serum albumin before (A) and after (B) addition of 24 moles of phenylbutazone. Peaks 1, 3, and 5 correspond to strongly immobilized sites and peaks 2, 3, and 4 correspond to partially immobilized sites.*

increasing drug concentration. The increase in this peak was taken as a measure of the conversion of strongly immobilized sites to partially immobilized sites as a result of the interaction of the phenylbutazone with bovine serum albumin. This parameter was chosen since it appeared to be little affected by overlap of the strongly immobilized spectrum and, therefore, was a relatively accurate measure of the number of partially immobilized sites actually present.

The fact that this effect seems to increase monotonically up to molar ratios of 20:1 or more before leveling off seems to indicate that there is indeed more than one binding site for phenylbutazone on bovine serum albumin. This could possibly be due to the initial binding of the drug inducing a conformational change in the albumin (reflected by the triazine spin label as a conversion of strongly immobilized sites to partially immobilized sites), thereby enabling the macromolecule to accommodate successive drug molecules. The possibility of these secondary binding sites becoming available only after occupation of the primary binding site without a conformational alteration of the bovine serum albumin seems unlikely.

Chignell (12) and Chignell and Starkweather (13), using circular dichroism spectrometry, correlated the molar ellipticity of the complex between phenylbutazone and human serum albumin with the binding of phenylbutazone. The molar ellipticity increased up to a limit of 2.6 moles of drug bound/mole of albumin. This extrinsic Cotton effect is a result of a perturbation of the carbonyl chromophore of phenylbutazone when bound to the albumin. In the present studies, it is the effect of binding on the environment of the spin label attached to the albumin that is being observed. These two different approaches to the study of phenylbutazone-albumin interactions yield different kinds of information about the binding process. in these studies the environment of the spin label is apparently altered at drug to albumin concentration ratios above which the molar ellipticity seems to have a limiting value.

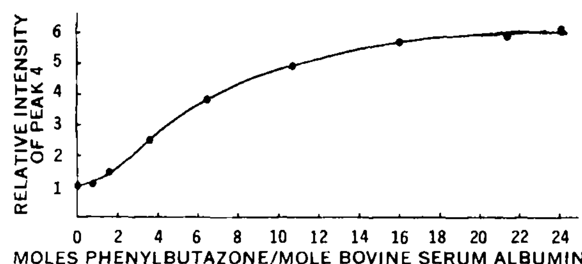

Figure 2—*Plot of the relative spectral intensity of peak 4 against the molar ratio of phenylbutazone–bovine serum albumin.*

It would be instructive to know the extent to which the observed spectral changes correspond to the actual degree of binding of the drug molecule. In addition, some data on the universality of this effect with other strongly binding drugs are desirable. These areas are being investigated further, and complete details of this work will appear shortly.

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