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Dialysis and Circular Dichroism Study of the Binding of Sulfaethidole to Crystalline and Fraction V Bovine Serum Albumin

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Abstract □ Sulfaethidole was found to exhibit similar binding to both crystalline and Fraction V bovine serum albumin. Binding of sulfaethidole to both crystalline and Fraction V bovine serum albumin results in considerable induced optical activity, with no evidence of change in the α-helical structure. Although equilibrium dialysis studies indicate one primary site and three secondary binding sites for sulfaethidole, only the binding at the primary site is detectable from circular dichroism studies.

Keyphrases □ Sulfaethidole binding to crystalline and Fraction V bovine serum albumin—studied by dialysis and circular dichroism □ Dialysis—sulfaethidole binding to bovine serum albumin, determination □ Circular dichroism—sulfaethidole binding to bovine serum albumin, determination

It was recently noted that sulfaethidole exhibits similar binding to both crystalline and Fraction V bovine serum albumin, while salicylate ion shows markedly lower binding to Fraction V than to crystalline bovine serum albumin (1). It was further observed that the addition of 10 mg. % sulfaethidole to 4.55 % bovine serum albumin dispaced salicylate from binding to crystalline bovine serum albumin but markedly enhanced the binding of salicylate to Fraction V bovine serum albumin. It was suggested that sulfaethidole might induce a subtle conformational change in Fraction V bovine serum albumin; studies were, therefore, undertaken to determine if such conformational change could be detected by circular dichroism (CD) measurements.

EXPERIMENTAL[1]

Dialysis Procedure—Ten milliliters of a 5.00 % w/v serum albumin (7.25×10^{-4} *M*) solution in 0.054 *M* phosphate buffer, pH 7.4, adjusted with sodium chloride to be isotonic, was placed in dialysis bags made by knotting the ends of the dialysis tubing. Bags were placed in 120-ml., wide-mouth, screw-capped bottles containing 10 ml. of buffered sulfaethidole solution, and the containers were agitated for 12 hr. at 37°. Prior to analysis, the volumes of inside and outside solutions were carefully measured, and protein concentrations were corrected for volume change (final protein concentration was 4.55 % w/v). Total sulfaethidole concentrations in the protein compartment at equilibrium ranged from approximately 3.2 to 73 mg. % (1.1×10^{-4}–25.7×10^{-4} *M*). Buffer solutions and glassware were sterilized, and the aseptic technique was used in preparing solutions and dialysis bags. Dialysis tubing was boiled in three changes of distilled water and finally in buffer solution.

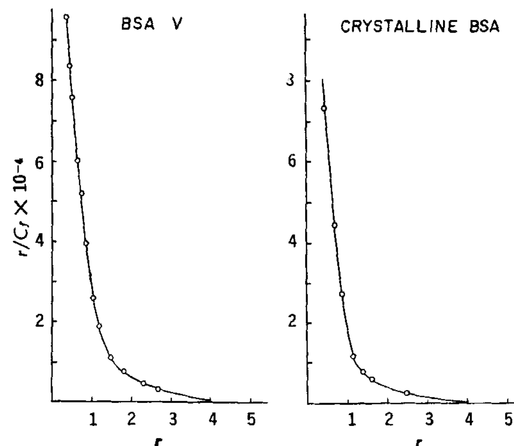

Figure 1—*Scatchard plot for binding of sulfaethidole by bovine serum albumin in 0.054* M *phosphate buffer, pH 7.4, 37°. For bovine serum albumin V, the solid line corresponds to* $n_1 = 1$, $K_1 = 1.5 \times 10^5$, $n_2 = 3$, *and* $K_2 = 1.6 \times 10^3$. *For crystalline bovine serum albumin, the solid line corresponds to* $n_1 = 1$, $K_1 = 1.2 \times 10^5$, $n_2 = 3$, *and* $K_2 = 1.0 \times 10^3$.

Analytical Procedures—Sulfaethidole concentration was determined for both inside and outside solutions following equilibrium, utilizing the Bratton–Marshall (2) procedure. Inside and outside solutions from a sample containing no sulfaethidole were utilized as blanks. Absorbances were determined at 535 nm. using a spectrophotometer[2].

[1] Visking dialysis tubing, 27/32, Union Carbide Corp., Chicago, Ill.; Fraction V bovine serum albumin, Armour Pharmaceutical Co., Chicago, Ill.; and crystalline bovine serum albumin, Nutritional Biochemicals Corp., Cleveland, Ohio, were used as received from the supplier. Sulfaethidole [N^1-(5-ethyl-1,3,4-thiadiazol-2-yl)sulfanilamide] was recrystallized from water, m.p. 185–186°; all other chemicals were reagent grade.

[2] Cary 15, Cary Instruments, Monrovia, Calif.

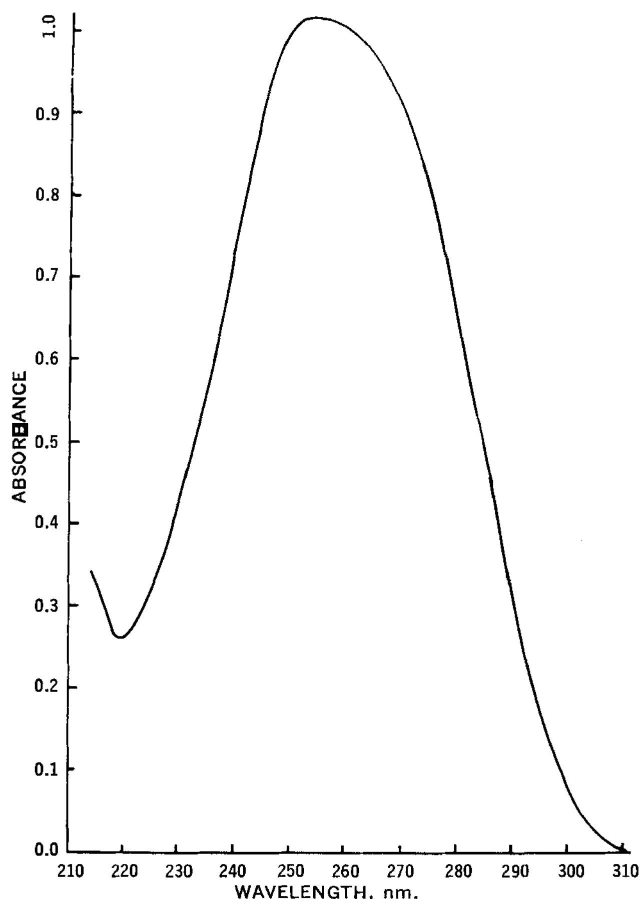

Figure 2—*UV curve for 5.6 × 10^{-5}* M *sulfaethidole in 0.054* M *phosphate buffer, pH 7.4, pathlength 1 cm.*

CD Measurements—The CD spectra of the optically clear solutions were obtained in 2-, 5-, 10-, or 20-mm. cells at 25°, using the 6002 attachment to a spectropolarimeter[3]. The dynode voltage was never allowed to exceed 0.35, and the signal-to-noise ratio was always greater than 50 to 1. The UV curve was obtained in a spectrophotometer[2].

RESULTS

Protein Binding—The concentration of sulfaethidole found in the nonprotein-containing compartment at equilibrium was taken as the concentration of free drug, and the concentration of sulfaethidole determined in the protein compartment was taken as free plus bound drug. Scatchard plots (Fig. 1) were constructed, assuming a molecular weight of 69,000 for the serum albumin. Analysis of the Scatchard plots (3) indicated that both Fraction V and crystalline bovine serum albumin exhibited one primary binding site (n_1) and probably three secondary binding sites (n_2). All data points were in agreement (better than ± 3%) with computer-generated Scatchard plots for the model $r = (n_1K_1C_f)/(1 + K_1C_f) + (n_2K_2C_f)/(1 + K_2C_f)$, if $n_1 = 1$ and $n_2 = 3$, and if for crystalline bovine serum albumin, $K_1 = 1.2 \times 10^5$ and $K_2 = 1.0 \times 10^3$, and for Fraction V bovine serum albumin, $K_1 = 1.5 \times 10^5$ and $K_2 = 1.6 \times 10^3$. No other integer values for n_1 and n_2 provide an acceptable fit of the data for the concentration range investigated.

CD—In the pH 7.4 buffers of these investigations, bovine serum albumin V and crystalline bovine serum albumin showed similar CD spectra down to 200 nm., and the spectra obtained were those associated with the α-helical form. Addition of sulfaethidole did not alter the spectra of either below 250 nm. Additions of salicylate and mixtures of salicylate and sulfaethidole also caused no deviations from the spectra associated wth the α-helical conformation.

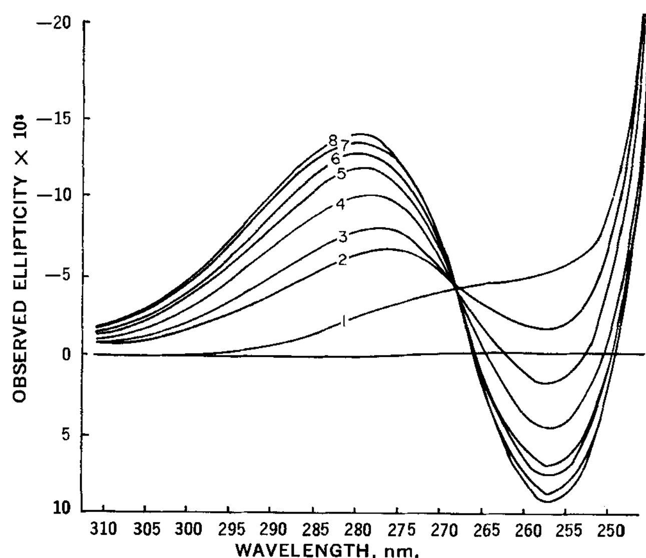

Figure 3—*CD curves for the binding of sulfaethidole to bovine serum albumin V, using 2-mm. cells, at the following D/P ratios: 1 = no drug, 2 = 0.29, 3 = 0.49, 4 = 0.78, 5 = 1.17, 6 = 1.46, 7 = 1.9, and 8 = 2.4.*

However, sulfaethidole alone showed considerable induced optical activity at higher wavelengths with both bovine serum albumin V and crystalline bovine serum albumin. Because of the large absorbances at the wavelengths of investigation, the effect of salicylate on these induced activities could not be determined; however, salicylate alone did not show any measurable induced activity.

The UV curve of sulfaethidole in buffer (Fig. 2) clearly results from at least two overlapping electronic excitations within the sulfaethidole molecule and gives a peak at 254 nm. These electronic transitions result in two induced CD peaks on binding to both albumins, a positive peak at approximately 257 nm. and a negative peak at approximately 279 nm. (Fig. 3).

The curves in Fig. 3 are those obtained with bovine serum albumin V; crystalline bovine serum albumin gave, within experimental error, identical curves. Figure 3 shows the optical activity induced at wavelengths above 250 nm. for various drug/protein (D/P) ratios, as well as the small contribution of the bovine serum albumin V at these wavelengths.

For these investigations of induced optical activity, the protein concentrations were fixed at 1.45 × 10^{-5} mole/l. as in previous studies (4), and drug-to-protein ratios of up to 10 to 1 (Figs. 3–5) were used. In Figs. 4 and 5, the induced ellipticities at both observed peaks are plotted at various D/P ratios for the two fractions, the

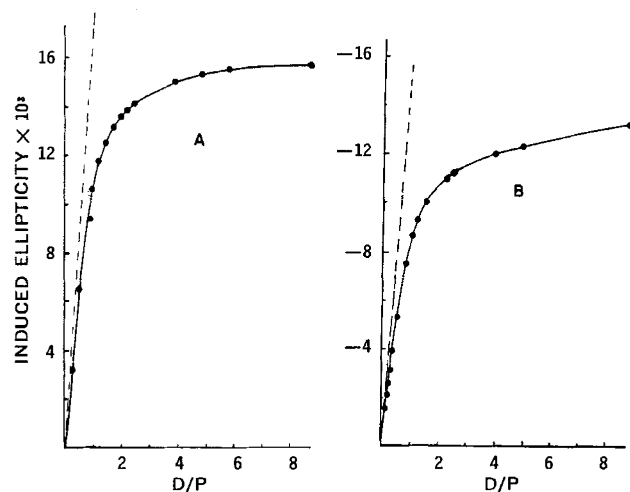

Figure 4—*Bovine serum albumin V—plot of induced ellipticity in 5-mm. cells for various D/P ratios. Bovine serum albumin concentration = 1.45 × 10^{-5}* M. *Key: A, 256.8 nm.; and B, 278.8 nm.*

[3] Cary 60.

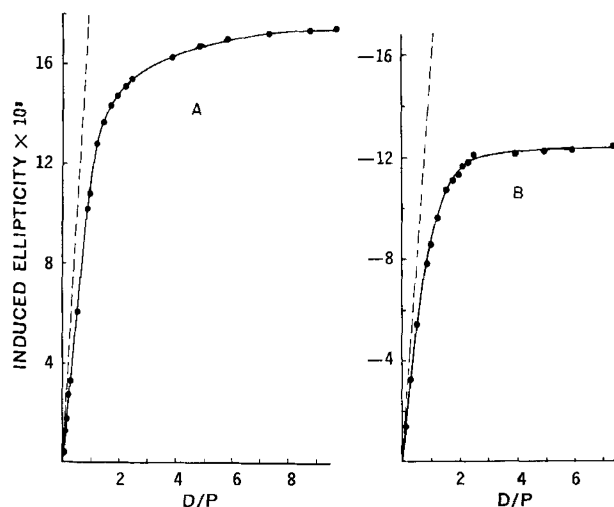

Figure 5—*Crystalline bovine serum albumin—plot of induced ellipticity in 5-mm. cells for various D/P ratios. Bovine serum albumin concentration = 1.45 × 10⁻⁵ M. Key: A, 256.8 nm.; and B, 278.8 nm.*

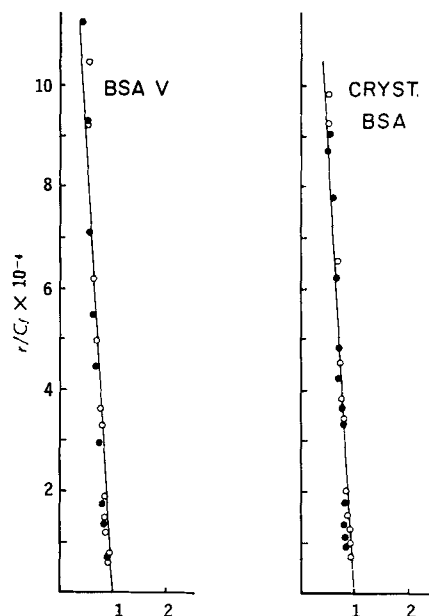

Figure 6—*Scatchard plots from CD data at 25°. Key: ●, data for 256.8 nm.; and ○, data for 278.8 nm. The solid line is the least-squares line; for bovine serum albumin V, it corresponds to* n = *1 and* K = *2.2 × 10⁵; for crystalline bovine serum albumin, it corresponds to* n = *1 and* K = *2.1 × 10⁵.*

induced ellipticity being defined as the observed ellipticity minus the small fixed contribution of the protein at the same wavelength. Figures 4 and 5 indicate a remarkable similarity in the optical activity induced by the two bovine serum albumins. The magnitudes of the induced activities for similar D/P concentrations at both the positive and negative peaks are similar for both albumins.

These data were analyzed according to the method of Rosen (5), in which it is assumed that the tangent to the curve as drug concentration approaches zero can be used to indicate the concentration of bound drug at all drug concentrations. The tangent is indicated as a dotted line in Figs. 4 and 5. By utilizing this procedure, concentrations of free and bound drug were calculated from the CD data; these data are presented as Scatchard plots in Fig. 6. Binding data from the CD and dialysis studies are not in agreement. While the dialysis data illustrated in Fig. 1 show one primary site and three secondary sites, binding to only the primary site is detectable from the CD study. Although the dialysis studies were conducted at 37° and the CD studies at 25°, the affinity constants obtained from the CD studies are in reasonable agreement with the values for the primary site affinity constants obtained from the dialysis study. Least-squares analysis of the CD binding data give an affinity constant of 2.1×10^5 for crystalline bovine serum albumin and 2.2×10^5 for Fraction V bovine serum albumin.

Although there is an indeterminate error associated with constructing a tangent and, therefore, in affinity constants calculated by this method, no tangent that might be constructed provides data indicative of other than a single binding site. It is, therefore, suggested that although sulfaethidole clearly exhibits both primary and secondary binding sites, only the binding at the primary site may be detected by CD studies. CD studies provided no evidence for the secondary interactions, even though such studies were extended to higher drug-to-protein ratios than were used in the dialysis studies. The concentrations of albumins used in the CD experiments were one-fiftieth of those used in the dialysis experiments, a situation necessitated by the high UV absorption of the protein and drug and the possibility of turbidity when higher concentrations of albumin are used. The two peaks in the induced CD curves probably result from excitations within both the sulfanilamide and the thiadiazole portions of the sulfaethidole molecule, and the similarity of the shapes of the curves for both the positive and negative deviations in Figs. 4 and 5 suggests a nonspecific nature of the binding as far as the sulfaethidole molecule is concerned. The rigid nature of the complex necessary for induced optical activity in drug–protein interactions in aqueous systems (4, 6, 7) seems to indicate a predominantly hydrophobic phenomenon.

The results from both the CD and dialysis studies suggest very little difference in the binding of sulfaethidole to bovine serum albumin V or crystalline bovine serum albumin. The CD data indicate little or no change in conformation of the α-helical proteins on binding of the sulfaethidole; however, any overall minor, but locally significant, conformational changes in the protein which may result on binding would probably not be observed by the CD technique.

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