Fluorescence Techniques in Drug–Plasma Protein Binding

Keyphrases □ Drug–protein binding—determination, fluorescent probe indicators □ Protein–drug binding—determination, fluorescent probe indicators □ Fluorometry—determination, drug–protein binding

Sir:

Various experimental methods have been employed for the study of drug–protein interactions (1). These include equilibrium dialysis, ultrafiltration, ultracentrifugation, solubility measurement, and, more recently, nuclear magnetic relaxation measurements and fluorescence techniques. Although fluorescence techniques have proved useful for following enzyme–substrate interactions (2), they have not been adequately studied as a tool for elucidating drug–plasma protein interactions. The fluorescence techniques reported here employ fluorescent probes as indicators of binding. The usefulness of this technique for the study of drug–protein binding comes from the fact that fluorescent probes are practically nonfluorescent in aqueous solutions but fluoresce strongly when bound to protein molecules (3). The enhancement of fluorescence intensity of a probe as a result of its interaction with protein is taken as an indication of the extent of binding. If a drug competes at the binding site or sites on a protein molecule, the decrease in fluorescence is commensurate with the decrease in bound probe molecules. The difference in fluorescence with and without a drug can then be related to a measurement of binding of the drug molecules to proteins (2).

Fluorescence intensities of the bound probes were measured at two protein concentrations (0.2 and 1.0 mg. protein/2 ml. solution). Two milliliters of each protein solution was titrated with the addition of successive 2 μl. of a 1×10^{-3} *M* solution of the probes in methanol. The fluorescence titrations were carried out manually with Hamilton microsyringes. Experiments were carried out at room temperature and at pH 7.4.

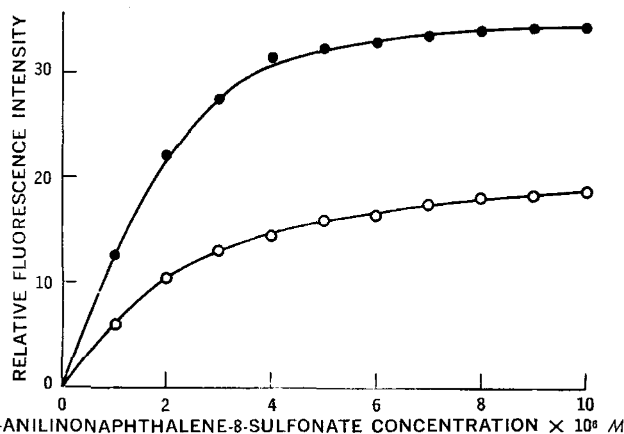
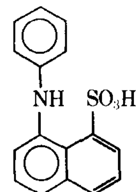


Figure 1—*Fluorescence titration of bovine serum albumin (0.2 mg. bovine serum albumin/2 ml. solution) with 1-anilinonaphthalene-8-sulfonate. The upper curve (●) represents the fluorescence intensity of 1-anilinonaphthalene-8-sulfonate complex. The lower curve shows the decreased intensity of the 1-anilinonaphthalene-8-sulfonate-bovine serum albumin complex due to bound phenylbutazone.*

Fluorometric measurements were made with a Perkin-Elmer model 203 fluorometer. The relative fluorescence intensities were obtained directly from fluorometer readings at uncorrected excitation and emission wavelengths for the probes.

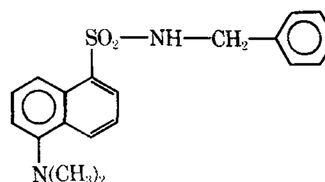
The probes used in this study were as follows:

Probe I—1-Anilinonaphthalene-8-sulfonate



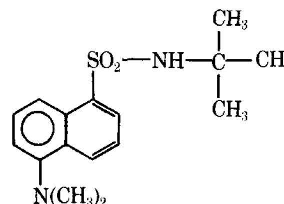
excitation, 350 nm.; emission, 465 nm.

Probe II—*N*-Benzyl-(5-dimethylaminonaphthalene)-1-sulfonamide



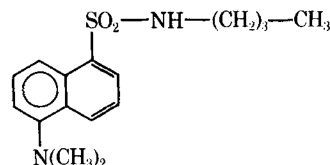
excitation, 320 nm.; emission, 495 nm.

Probe III—*N*-*tert*-Butyl-(5-dimethylaminonaphthalene)-1-sulfonamide



excitation, 320 nm.; emission, 495 nm.

Probe IV—*N*-*n*-Butyl-(5-dimethylaminonaphthalene)-1-sulfonamide



excitation, 325 nm.; emission, 500 nm.

Bovine serum albumin, Fraction V and crystalline, was purchased[1]. Acetylated bovine serum albumin was prepared according to the procedure of Fraenkel-Conrat *et al.* (5). Percentage acetylation was estimated to be 2.5% of the terminal amino groups[2].

Figure 1 shows the application of this technique for the binding of phenylbutazone to bovine serum albumin, and binding is indicated by the decreased

[1] Nutritional Biochemicals Corp., Cleveland, Ohio.
[2] By Galbraith Laboratories, Inc., Knoxville, Tenn.

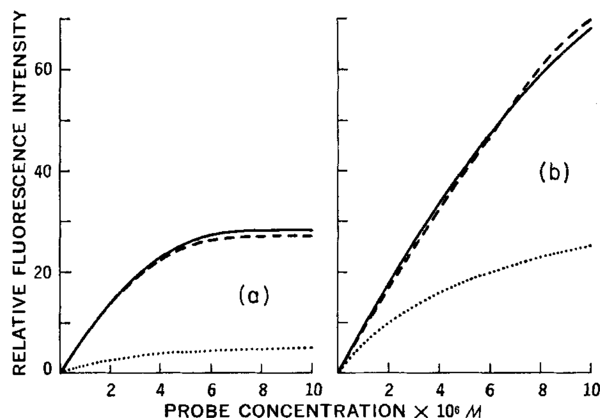


Figure 2—*Fluorescence titration of crystalline bovine serum albumin (—), Fraction V bovine serum albumin (- - -), and acetylated bovine serum albumin (. . .) with 1-anilinonaphthalene-8-sulfonate. Key: (a) lower bovine serum albumin concentration; and (b), higher bovine serum albumin concentration.*

fluorescence intensities of a probe in the presence of the drug. Phenylbutazone is known to bind to bovine serum albumin; with this technique binding is further demonstrated by displacement of the bound probe by the drug.

During these studies, using this technique, it was found that the binding of the probes to Fraction V and crystalline bovine serum albumin appeared to be nearly identical. This observation supports recent findings (4) of identical binding properties of the two bovine serum albumin fractions for sulfaethidole. Figures 2 and 3 show the relative fluorescence intensities of probes bound to bovine serum albumin fractions. All curves labeled "a" indicate a saturation of binding sites of the proteins at relatively low fluorescence intensities, and this was due to the low concentration of protein (0.2 mg. protein/2 ml. solution). The "b" curves show that more binding sites were available (1.0 mg. protein/2 ml. solution), and this was indicated by a saturation at higher intensities. The "a" and "b" curves showed no significant difference in the binding of the probes to Fraction V and crystalline bovine serum albumin fractions.

To establish the validity of the experiment, a comparison was made of the binding of the probes for acetylated bovine serum albumin and the bovine serum albumin fractions. A significant difference was found between the binding of 1-anilinonaphthalene-8-sulfonate to acetylated bovine serum albumin and the crystalline and/or Fraction V bovine serum albumin (Fig. 2). The difference in binding affinity for 1-anilinonaphthalene-8-sulfonate between acetylated bovine serum albumin and the bovine serum albumin fractions could be due to the increased net negative charges on the acetylated bovine serum albumin molecule as a result of acetylation of the terminal amino groups of bovine serum albumin (5). This could cause a reduced

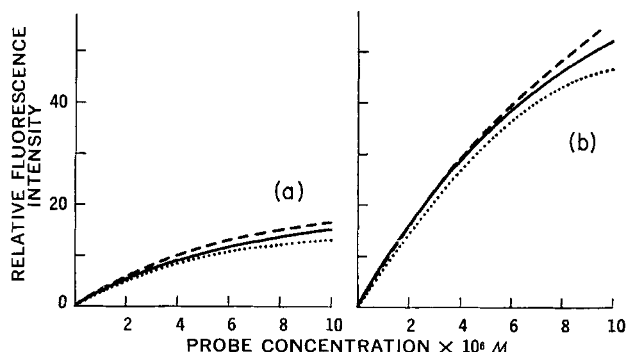


Figure 3—*Fluorescence titration of crystalline bovine serum albumin (—), Fraction V bovine serum albumin (- - -), and acetylated bovine serum albumin (. . .) with Probe II. Key: (a), lower bovine serum albumin concentration; and (b), higher bovine serum albumin concentration.*

affinity for the more negatively charged 1-anilinonaphthalene-8-sulfonate for acetylated bovine serum albumin when compared to the three amide probes (see structures of probes) which would be expected to be more hydrophobic than 1-anilinonaphthalene-8-sulfonate. This finding seems to indicate that 1-anilinonaphthalene-8-sulfonate tends to interact with terminal amino groups of the bovine serum albumin, while the other probes bind preferably at the more hydrophobic environments of the bovine serum albumin molecules.

The explanation as to why the more negatively charged acetylated bovine serum albumin has less binding affinity for the three amide probes than do the intact bovine serum albumin fractions (see typical Fig. 3) is not immediately apparent from experimental data. An electrostatic interaction between the proteins and the probes may be a factor. Additional work in these laboratories is aimed at extending the significance of these observations to understand better the drug–protein and drug–drug interactions through elucidation of the nature of the binding using these techniques.

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