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Phenylbutazone–Sodium Warfarin Binding Using a Fluorescent Probe Technique

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Abstract □ 1-Anilinonaphthalene-8-sulfonate was used to study the binding of phenylbutazone and sodium warfarin to bovine serum albumin. The protein molecules appeared to have an average of three binding sites for these compounds. The binding constant for phenylbutazone was found to be 2.5 times larger than that for sodium warfarin. Identical binding sites with a different binding affinity between the two drugs indicate a competition for the binding sites between phenylbutazone and sodium warfarin in favor of phenylbutazone. The mechanism of binding for these drugs may be of a hydrophobic nature.

Keyphrases □ Phenylbutazone–warfarin binding—studied using fluorescent probe technique, binding constants, proposed mechanism □ Warfarin–phenylbutazone binding—studied using fluorescent probe technique, binding constants, proposed mechanism □ Fluorescent probe technique—used to study phenylbutazone–warfarin binding, binding constants, proposed mechanisms □ Interactions—phenylbutazone–warfarin–protein binding constants, proposed mechanism

Phenylbutazone has been shown to displace sodium warfarin from plasma protein binding sites. Aggeler *et al.* (1) used dialysis techniques to demonstrate that sodium warfarin is displaced by phenylbutazone. O'Reilly and Levy (2), using *in vivo* studies, showed that the elimination half-life of warfarin is decreased, while its anticoagulant activity is increased, by concurrent administration of phenylbutazone. Other clinical studies (3, 4) also showed a marked increase in prothrombin time of sodium warfarin in the presence of phenylbutazone.

Fluorescence probe techniques were recently employed for the study of drug–protein binding (5) and for elucidation of the competitive binding of substrates and enzymes (6). Therefore, the binding of phenylbutazone and sodium warfarin to bovine serum albumin was studied by the use of 1-anilinonaphthalene-8-sulfonate, and the competition between the two drugs for the binding sites was examined.

EXPERIMENTAL

The general approach and techniques used were essentially the same as those employed by Brand *et al.* (6) in their study of the binding of rose bengal and anilinonaphthalene sulfonates to alcohol dehydrogenase. A few minor modifications were made.

Materials—1-Anilinonaphthalene-8-sulfonate[1] was employed as a fluorescent probe. This compound has minimal fluorescence in aqueous solution but fluoresces strongly when bound to bovine serum albumin. Phenylbutazone[2] and sodium warfarin[3] were used without further purification. Crystalline bovine serum albumin[4] was purchased. Methanol[5] was spectroscopic grade, and all other chemicals were reagent grade or of special purity. Water used in this study was double distilled from glass.

Apparatus—Fluorometric measurements were made with a spectrophotofluorometer[6]. The relative fluorescence intensities of bound probe were obtained directly from fluorometer readings of uncorrected excitation (375 nm.) and emission (475 nm.) wavelengths.

Methods—Fluorescence intensities of the protein–probe complex as a function of probe concentration (1.0–15.0×10^{-6} *M*) were measured at two protein concentrations (0.2 and 2.0 mg. protein/2 ml. solution) in pH 7.2 phosphate buffer. Two milliliters of each protein solution was titrated with successive additions of 2 μl. of 1×10^{-3} *M* probe in methanol. Titrations were performed manually with Hamilton microsyringes. Experiments were carried out at 28°.

Titrations of the protein solutions with probe were repeated in the presence of 1×10^{-4} *M* concentration of both phenylbutazone and sodium warfarin individually. The drugs were added to the solution prior to titration.

Treatment of Data—Enhancement of the fluorescence of the probe upon addition to bovine serum albumin at two concentrations and the subsequent decrease of fluorescence in the presence of the binding competitors, phenylbutazone and sodium warfarin, were used to calculate the binding constants for the probe and competitors. The fraction of probe bound, X, was calculated using the following

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Lot No. SN 46241, Ciba-Geigy Pharmaceuticals, Ardsley, New York, N. Y.
[3] Lot No. 833-1521, Abbott Laboratories, North Chicago, Ill.
[4] Nutritional Biochemicals Corp., Cleveland, Ohio.
[5] Matheson, Coleman & Bell, Norwood, Ohio.
[6] Aminco-Bowman.

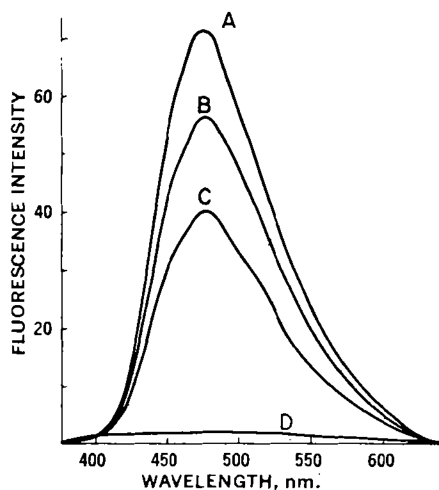

Figure 1—*Fluorescence emission spectra of the probe-protein complex in the presence (A) and in the absence (D) of bovine serum albumin (1.38×10^{-6} M) in pH 7.2 phosphate buffer. Curves B and C are the emission spectra of the probe-protein complex in the presence of sodium warfarin (1×10^{-4} M) and phenylbutazone (1×10^{-4} M), respectively.*

equation (6):

$$X = \frac{(I_o/I_f) - 1}{(I_b/I_f) - 1} \quad \text{(Eq. 1)}$$

where I_o and I_f refer to the fluorescence intensities of a given concentration of probe in solutions of lower protein concentration and in solutions without protein, respectively; and I_b refers to the fluorescence intensity of the same concentration of probe in solutions of high protein concentration. Therefore, I_b gives the fluorescence intensity of the probe in the presence of excess binding sites. After the value X is found for each point along the titration curve,

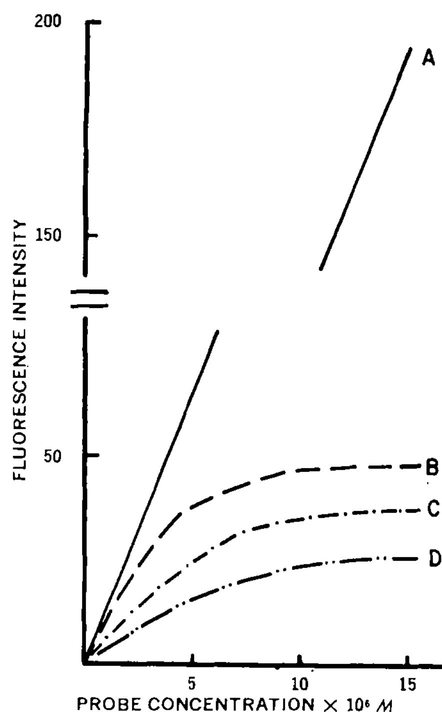

Figure 2—*Fluorescence titration curves of bovine serum albumin with the probe at higher (A) and lower (B) protein concentrations. Curves C and D are the titration curves of lower protein concentration with the probe in the presence of sodium warfarin and phenylbutazone, respectively.*

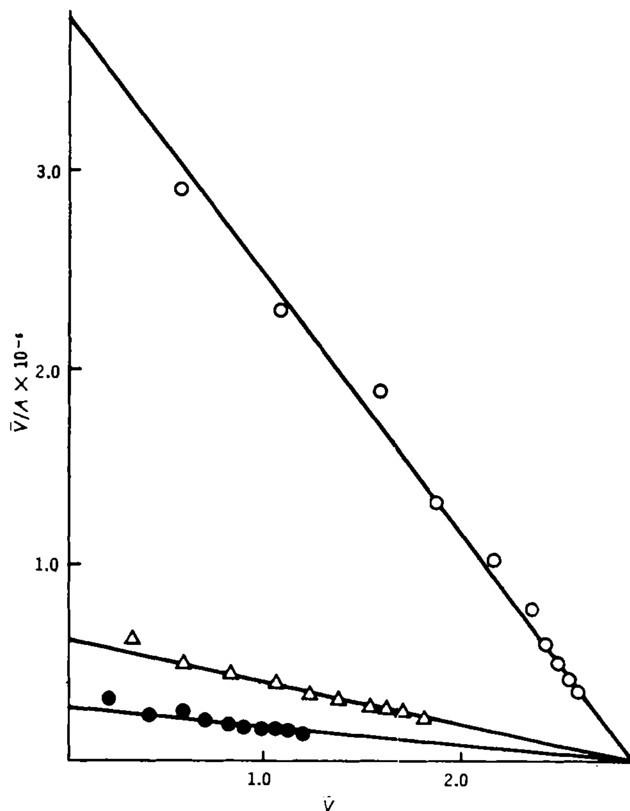

Figure 3—*Scatchard plots for the probe and protein complex (○) and in the presence of sodium warfarin (Δ) and phenylbutazone (●) at 28°.*

the Scatchard equation (7) may be applied to calculate the association constant of the probe:

$$\bar{V}/A = nK_a - \bar{V}K_a \quad \text{(Eq. 2)}$$

where $\bar{V}$ is the number of moles of bound probe per mole of protein, A is the concentration of free probe, n is the number of binding sites on the protein molecule, and K_a is the association constant of the probe to the protein. The $\bar{V}$ is determined by multiplying the value for X by the ratio of the total probe concentration to the total protein concentration in solution. When $\bar{V}/A$ is plotted against $\bar{V}$, a straight line is obtained with a slope equal to $-K_a$. The ordinate and abscissa intercepts of this line give nK_a and n, respectively.

When Scatchard plots were made from the binding data obtained for the probe in the presence of the binding site competitors, phenylbutazone and sodium warfarin, straight lines were obtained with decreased slopes but with identical abscissa intercepts, indicating competition between the probe and the drugs for the same binding sites. The decrease in probe binding and the subsequent decrease in fluorescence of the probe protein complex at lower protein concentration can be used to calculate the binding constant of phenylbutazone and warfarin. Klotz *et al.* (8) derived equations describing simple competition between two compounds for identical protein binding sites:

$$K_b = \frac{n(P_o)K_a(A) - K_a(A)(PA) - (PA)}{(B_t)K_a(A) - (P_o)K_a(A) + K_a(A)(PA) + (PA)} \times \frac{K_a(A)}{(PA)} \quad \text{(Eq. 3)}$$

where:

K_b = association constant for competitor
K_a = association constant for probe
A = concentration of free probe
PA = concentration of bound probe
n = number of binding sites
P_o = lower protein concentration
B_t = total concentration of competitor

RESULTS AND DISCUSSION

Evidence that the probe binds to bovine serum albumin is shown by protein-induced fluorescence changes. Figure 1 shows fluorescence emission spectra of the probe in the presence and absence of bovine serum albumin. The fluorescence intensity of the probe (2×10^{-6} *M*) in pH 7.2 buffer was not significant (curve D); but when the protein was added (0.2 mg./2 ml.), the intensity was greatly enhanced (curve A). Curves B and C show the fluorescence emission spectra of the probe in the presence of sodium warfarin and phenylbutazone, respectively. A decrease in fluorescence of the probe-protein complex in the presence of sodium warfarin or phenylbutazone is an indication of the competition between the probe and the drugs for the binding sites on the protein (5, 6). A diminution of fluorescence could only be related to a reduction in the number of available binding sites for the probe in the presence of the drugs when no quenching effect or denaturation of binding sites due to the drugs was observed.

Figure 2 shows the fluorescence titration curves of bovine serum albumin with the probe. Curve A of Fig. 2 is linear when fluorescence is plotted *versus* increasing probe concentration at 2 mg./2 ml. protein solution. This indicates the presence of excess binding sites at all probe concentrations examined. Curve B indicates an early saturation of binding sites of the protein brought about by a lower protein concentration (0.2 mg./2 ml.). Curves C and D are the titration curves for the probe at the same low protein concentration in the presence of sodium warfarin (1×10^{-4} *M*) and phenylbutazone (1×10^{-4} *M*), respectively. A decrease in fluorescence of the protein-probe complex is seen. When the drugs are added to protein solution prior to the titration, binding sites are initially occupied by drug molecules, with subsequent competition for the binding sites between drug and probe molecules following titration with the probe. Competition can be quantified through calculation of the binding constants for the probe and drugs using the equation derived by Klotz (8) (see *Treatment of Data*).

Figure 3 shows Scatchard plots made from the binding data taken for 1-anilinonaphthalene-8-sulfonate alone and one each for the probe with sodium warfarin and with phenylbutazone. The intercepts on the abscissa are identical for the three compounds, but the slope is decreased in the presence of both phenylbutazone and sodium warfarin. This indicates a competition between the probe and the drugs for the binding sites measured. By using these techniques, the binding constants for sodium warfarin and phenylbutazone with bovine serum albumin were found to be 4.9×10^4 and $12.2 \times 10^4/M$, respectively. The protein molecules appeared to have three binding sites (an average of 2.9) for these compounds under the described experimental conditions.

Observations that can be made from this experiment are that the number of binding sites appears to be identical for the three compounds and that there is a 2.5-fold difference in the binding constant of phenylbutazone compared to that of sodium warfarin (12.2×10^4 *versus* $4.9 \times 10^4/M$). Considering the identical binding sites with a different binding affinity between the two drugs, competition for the binding sites between phenylbutazone and warfarin is expected to occur in favor of phenylbutazone. Additional evidence for displacement of binding sites of warfarin by phenylbutazone is that there is no significant additive decreasing effect in fluorescence of the bound probe in the presence of the two drugs. If the two drugs bind at different independent binding sites to which the probe binds, an additional decrease in fluorescence would be observed. This, however, may not indicate that phenylbutazone has completely displaced all warfarin molecules bound to protein. Information on the fraction of displacement by phenylbutazone may be very useful but it is not available at this time. This observation is in agreement with previous studies (1, 2) showing that phenylbutazone competitively displaces sodium warfarin from protein binding sites.

An additional observation is that the mechanism of binding for both phenylbutazone and warfarin may be of a hydrophobic nature. This observation is made in light of the hydrophobic binding characteristics of the probe used (9).

There is one discrepancy between this and previous studies (10, 11), and it lies in the numerical values reported for the binding constants. This may be explained on the basis that previous studies measured only one protein binding site compared with three in this study. In addition, it is possible that the binding sites from this study and from previous studies for which the constants are calculated may not be identical.

The information obtained in these laboratories, combined with other studies (5, 6), readily shows that the fluorescence probe technique may indeed prove to be a useful tool in the evaluation of competitive drug interactions for the binding sites. However, the usefulness of the probe technique may be limited in that the binding studies are related only to the number of sites to which the probe binds.

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