International Journal of Pharmaceutics, 2 (1979) 283–294

SOME FLUORESCENT INVESTIGATIONS OF THE INTERACTION BETWEEN THE ENANTIOMERS OF WARFARIN AND HUMAN SERUM ALBUMIN

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(Received July 19th, 1978)
(Accepted August 7th, 1978)

SUMMARY

The fluorescence of warfarin is greatly enhanced on binding to a single site on human serum albumin. Other sites make only very small contributions to this enhancement. Advantage is taken of this phenomenon to estimate a binding constant for the binding of R, S and racemic warfarins to human serum albumin. Many measurements are made at the clinically significant low drug-to-protein ratios. The binding constant for the S-isomer is higher than that of the R. The abilities of phenylbutazone, ibuprofen, fenoprofen, tolmetin and aspirin to displace warfarin from this site on albumin is investigated at very slow drug-to-protein ratios. Investigations of the binding of warfarin as a function of pH suggest that the affinity is different for the N and B forms of albumin. The binding to both the N and B forms is affected by chloride ions.

INTRODUCTION

The binding of warfarin to human serum albumin (HSA) has been quantitatively investigated by several authors (O'Reilly, 1967, 1969, 1973; Garten and Wosilait, 1972; Solomon and Schrogie, 1967; Sellers and Koch-Weser, 1970; Sunlow et al., 1975; Kober et al., 1978; Chakrabarti, 1978). The first binding constant of warfarin to HSA has been reported to be of the order of 10^5 M^{-1} by these authors. O'Reilly (1969, 1973), using the dialysis technique, found two high affinity sites having binding constants of 2.3×10^5 M^{-1}. However, his investigations presented little data at drug-to-protein ratios less than 1.0; such determinations are necessary for precise estimations of the binding constants.

Chignell (1969a) has observed one binding site of high affinity from investigations of the enhanced fluorescence of warfarin following the binding to HSA, and the quenching of the single tryptophan residue in HSA following the interaction with warfarin. Sunlow and coworkers (1975) have recently reported a binding constant of 2.5×10^5 M^{-1} for a single site from measurements of the enhanced fluorescence of warfarin in the presence of HSA.

However, most of these investigations have been carried out at physiological pH and in buffers made isotonic with sodium chloride. These conditions are considered better for correlation with pharmacological effects but give little insight into the nature of the interaction. O'Reilly (1967, 1969, 1973) investigated the effect of pH, ionic strength and temperature on the interaction of warfarin including its metabolites with HSA. He found that the binding strength rose significantly as the pH was increased. He also concluded that the warfarin–HSA interactions were probably the result of hydrogen bonding and hydrophobic forces. Moreover, he found that a 20-fold variation in buffer ionic strength caused no significant change in the binding constant of warfarin to HSA.

S-warfarin is more potent as an anticoagulant than the R-isomer in both man and the rat (O'Reilly, 1974; Elbe et al., 1966). Yacobi and Levy (1977) have shown that the free fraction of R warfarin in the plasma of both man and rats was significantly larger than that of the S-isomer. Sellers and Koch-Weser (1975), using equilibrium dialysis, found that the binding of S-warfarin to HSA was significantly greater than that of the R-enantiomorph. However, later work (Brown et al., 1977) did not show a significant difference in the binding of the two warfarins to HSA.

Of great importance from a clinical point of view is the displacement of warfarin by anti-inflammatory drugs, including phenylbutazone, from HSA as has been reported (Solomon and Schrogie, 1967; Sellers and Koch-Weser, 1970; Sunlow et al., 1975; Jun et al., 1972; Henry and Wosilait, 1975). Unfortunately, such high concentrations of drugs were used that it is impossible to determine whether or not the primary binding site of warfarin is involved in the apparent displacement. Moreover, in some of the above studies the fluorescence probe technique was used and this could make interpretation of the data ambiguous.

Fluorescence spectroscopy is one of the most versatile and sensitive of the optical techniques for studying the binding of drugs to HSA (Chignell, 1970; Sunlow et al., 1975). Furthermore, this technique is easier and faster than equilibrium dialysis or ultrafiltration when applicable, and possibly more specific for a given binding site. It appears that the enhanced fluorescence of warfarin–HSA mixtures is primarily the result of a single interaction between the protein and the drug. The present study was undertaken to investigate the mechanism of stereoselective binding of warfarin and its enantiomers to HSA using fluorescence. The effect of sodium chloride on the binding of warfarin and its enantiomers to HSA was also investigated. Measurements at very low drug-to-albumin ratios are essential for these investigations as well as for the important competitive binding investigations.

MATERIALS AND METHOD

HSA, crystallized and lyophilized (Lot No. 76C-8145) was obtained from Sigma Chemical Company, St. Louis, Mo. Racemic warfarin, S- and R-warfarin were gifts of Endo Laboratories, Inc., Garden City, N.Y. Phenylbutazone (Ciba-Geigy Company, Summit, N.J.), sodium fenoprofen (Eli Lilly Company, Indianapolis, Ind.), ibuprofen (Upjohn Company, Kalamazoo, Mich.), tolmetin (McNeil Laboratories, Inc., Fort Washington, Pa.), were gifts of the manufacturers. Flufenamic acid was obtained from Aldrich Chemical Company, Inc., Milwaukee, Wisc. Aspirin was obtained from the Sigma

Chemical Company, St. Louis, Mo. All other materials were reagent grade, and deionized water was used throughout.

All solutions were prepared in 0.1 M phosphate buffer of pH 7.4 at 22 ± 1°C. HSA concentrations of 0.2–7.0 X 10^{-5} M (M.W 69,000) were used. Fluorescent measurements were made on a Perkin–Elmer MFP-44A fluorescence spectrophotometer. The fluorometric titrations were carried out as follows: 2.0 ml of the protein solution of an appropriate concentration in a 10 mm pathlength cell were titrated by the successive additions of 2.0 μl volumes of warfarin solution (to give a final drug concentration of 0.1–15 X 10^{-6} M in the cell) and the fluorescent intensity was measured following excitation at 335 nm and emission at 378 nm.

RESULTS AND DISCUSSION

The fluorometric titration of HSA with racemic warfarin is shown in Fig. 1. In the absence of HSA, the fluorescence of warfarin is not significant (curve c). However, the fluorescent intensity was greatly enhanced when HSA was added (curves a and b). At the two high protein concentrations (5 X 10^{-5} and 7.0 X 10^{-5} M), the titration curves were identical, indicating that the drug added was fully bound at both the protein concentrations. Furthermore, these titration results suggest that the fluorescent intensity of the warfarin–HSA complex is constant. At low protein concentration, the drug was only partially bound (curve b). The observed fluorescence in the presence of HSA is a function of the amount bound at the individual sites and the associated fluorescent yield.

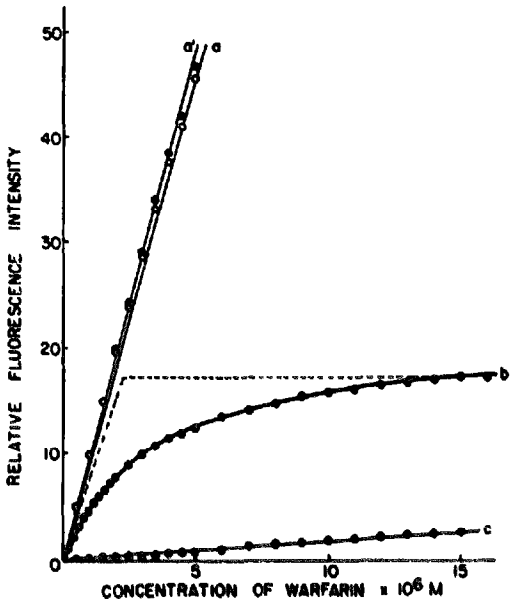

Fig. 1. Plots of relative fluorescent intensities as a function of warfarin concentration for the racemic warfarin–HSA interaction at pH 7.4. HSA; curve a: 5 X 10^{-5} and 7 X 10^{-5} M; curve b: 2 X 10^{-6} M; curve c: 0. The straight line a' is obtained after correcting the fluorescent intensities in curve a for the effect of absorbance (see text).

The fraction of the drug bound, X, is usually determined by using the equation (Weber and Young, 1964):

$$X = \frac{F_p - F_0}{F_b - F_0} \tag{1}$$

where F_p and F_0 are the fluorescent intensities of a given concentration of drug in a solution of low protein concentration and in solution without any protein, respectively, and F_b is the fluorescent intensity of the same concentration of completely bound drug. Such treatment yields the true value of X, provided the fluorescent intensity of the bound drug is a linear function of the concentration. The linearity of F_b with the concentration of the drug–protein complex can only be taken when the absorbance of the complex at the wavelength of excitation is low according to the fluorescence–absorbance relationship equation (Naik et al., 1975):

$$F_b - F_0 = \phi I_0 \left[2.3A - \frac{(2.3A)^2}{2!} + \frac{(2.3A)^3}{3!} + \frac{(2.3A)^4}{4!} + \ldots\right] \tag{2}$$

where ϕ is the quantum yield of the emitting species, I_0 is the intensity of the exciting radiation, and A is the absorbance. When the absorbance is low, the higher power terms in the equation become negligible, but when the absorbance is high, the second or even the third term must be considered. After correcting the observed fluorescence with the second term, a straight line (line a′ in Fig. 1) was obtained, verifying that the deviation from linearity of curve a was indeed due to absorbance.

When the titrations are carried out at low protein concentrations (curve b), the drug is only partially bound. In curve b, the absorbance, A, is so small that the second term in Eqn. 2 may be neglected because the absorbance is the difference between the absorbance of the bound warfarin, i.e. that responsible for the fluoresence and the absorbance of unbound drug. For purposes of equilibrium calculations, the fluorescent intensity read from curve b can be compared directly to the corresponding reading of the intensities on line a′. Fig. 2 shows the induced fluorescent intensity at pH 8.5 to be significantly greater than that at pH 6.6. This suggests that the fluorescent yield or binding increases with pH. The fluorescent titrations for warfarin isomers are shown in Fig. 3. The straight lines in Figs. 2 and 3 were obtained after correcting for the absorbance effect.

The plateau in each titration curve obtained at low protein concentration (Figs. 1–3) indicates the saturation of HSA binding sites. Intersection of the plateau with the tangent to the curve at zero drug concentration gives the total number of binding sites, N, to be 1.1, indicating that the enhanced fluorescence is mainly due to the binding at a single site on HSA (Fig. 1).

To further establish the value of N, a Job's plot (Job, 1928) was constructed for the warfarin and its enantiomer–HSA systems by keeping the total concentration of warfarin and HSA at 1.0×10^{-5} M. These plots are shown in Fig. 4. The inflection point gives a value of 0.51 which corresponds to N = 1.05. Therefore, it is reasonably certain that the maximum number of binding sites contributing to the fluorescence of the warfarin–HSA interaction is one. However, for all of the Job plots it should be noted that the fluorescent intensity at high drug-to-protein ratios is somewhat greater than at the lower ratios. This obviously indicates that more than one site is contributing to the induced fluores-

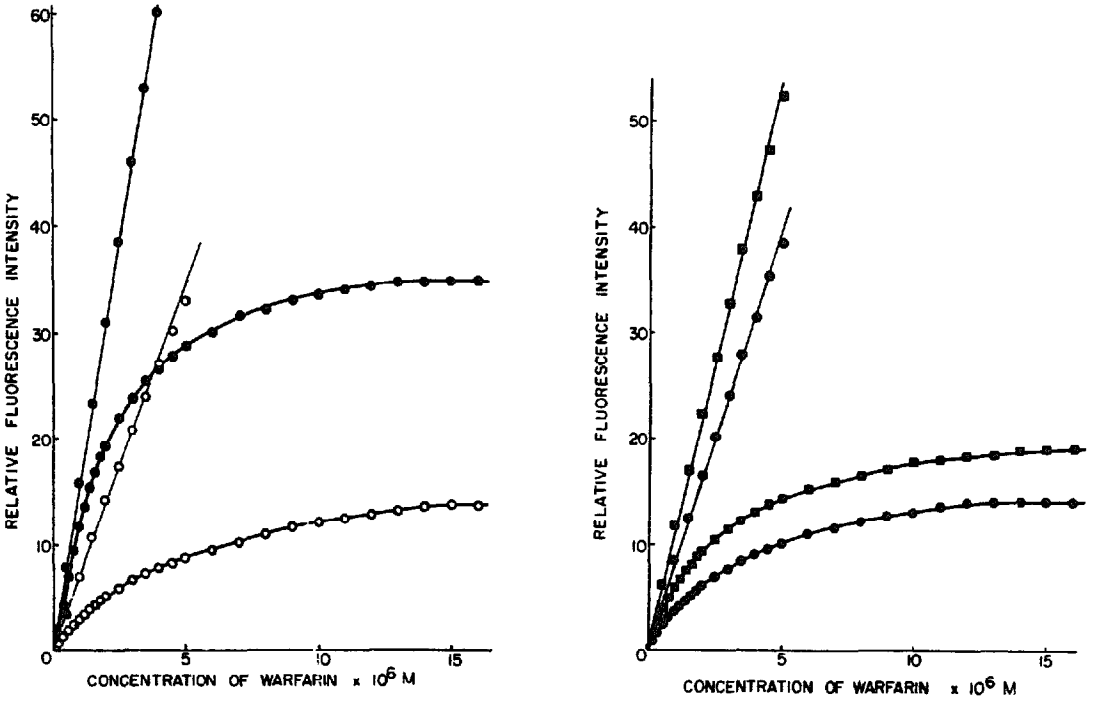

Fig. 2. Plots of relative fluorescent intensities as a function of warfarin concentration for the racemic warfarin–HSA interaction at pH 6.6 and 8.5. HSA concentrations as in Fig. 1. Curve: o———o, pH 6.6, •———•, pH 8.5.

Fig. 3. Plots of relative fluorescent intensities as a function of warfarin concentration for the warfarin–HSA interaction at pH 7.4. HSA concentrations as in Fig. 1. Curve: •———•, R-warfarin; ▪———▪, S-warfarin.

cence at high drug-to-HSA ratios. The results of titration curves and Job plots suggest that the binding constants for the first site for warfarin and its isomers are significantly larger than those for the second site, or that the fluorescent yield for the first site is greater than that for the second binding site.

If the assumption is made that the fluorescent yield is the same from all binding sites, then from estimates of the free and bound drug made as described above, a Scatchard plot can be constructed as shown in Fig. 5. The Scatchard plots are curved, indicating a heterogeneity in binding sites and the data is analyzed assuming two classes of binding sites:

$$r = \frac{n_1 K_1 A}{1 + K_1 A} + \frac{n_2 K_2 A_2}{1 + K_2 A} \tag{3}$$

The theoretical line in Fig. 5 is drawn using binding parameters calculated by a newly developed computer method (Perrin et al., 1974). The data obtained are summarized in Tables 1 and 2 together with the results obtained by an iterative least squares technique assuming a 1 : 1 complex and using the data at low drug-to-protein ratios. The primary binding constants obtained by the two methods are in agreement. This clearly indicates

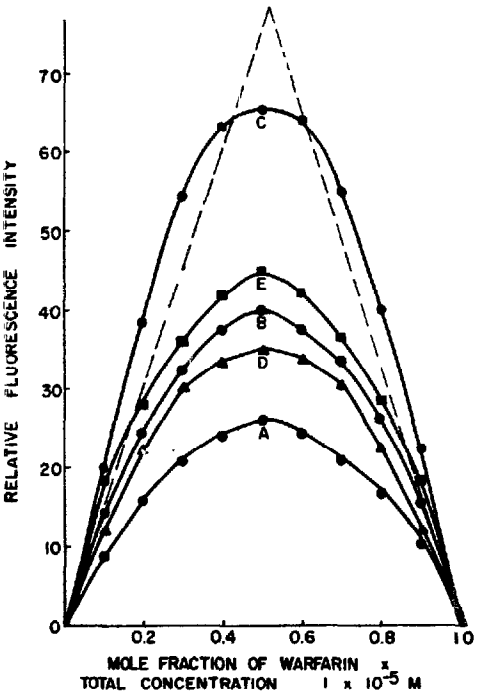

Fig. 4. Job's plots of relative fluorescent intensities for the warfarin and its enantiomers–HSA interactions. The final total concentration is fixed at 1.0×10^{-5} M. A: racemic warfarin at pH 6.6. B: racemic warfarin at pH 7.4. C: racemic warfarin at pH 8.5. D: R-warfarin at pH 7.4. E: S-warfarin at pH 7.4.

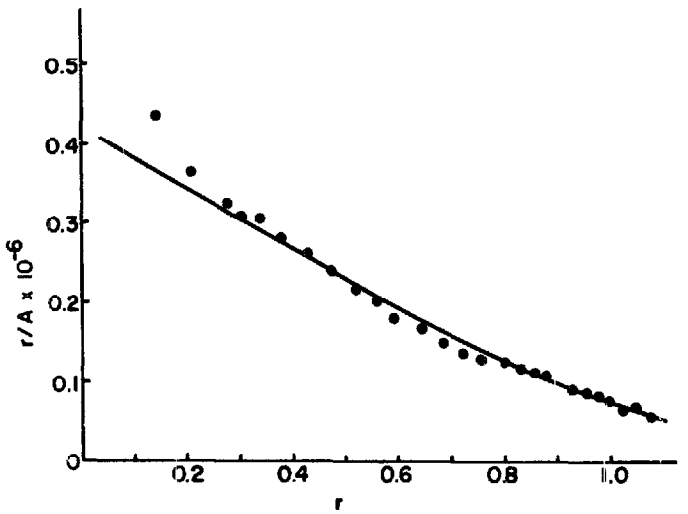

Fig. 5. Scatchard plots of warfarin–HSA interaction. The solid line was computed using the binding parameters $n_1 = 1.0$, $K_1 = 4.1 \times 10^5$, $n_2 = 1.0$, $K_2 = 1.3 \times 10^4$.

TABLE 1

BINDING PARAMETERS FOR RACEMIC WARFARIN–HSA COMPLEXES AT VARIOUS pH

pH	N_1	K_1	N_2	K_2	$K_{1:1}$
6.6	1	4.1×10^5	1	1.3×10^4	4.8×10^5
7.4	1	5.4×10^5	1	1.1×10^4	5.9×10^5
8.5	1	2.3×10^5	1	1.9×10^4	2.4×10^5

that the second binding constant is much smaller than the first binding constant.

The primary binding constant at pH 7.4 for racemic warfarin is in reasonable agreement with the literature value. However, this value is somewhat higher than those reported by Sunlow et al. (1975) and Kober et al. (1978), and it is somewhat smaller than that reported by Meyer et al., and Guttman (1970). DIfferences can be due to buffer composition, variations in albumins, as well as differences arising from the different experimental techniques employed. The binding constants increase with pH in agreement with the observation of O'Reilly (1969), who found that the primary binding constant increased as the pH was increased from 6 to 9. This suggests that the N → B transition may be involved in the binding of warfarin to HSA (Wilting et al., to be published). The binding constant for the S-isomer is slightly greater than that for the R-isomer, as has been previously reported (Sellers and Koch-Weser, 1970; Yacobi and Levy, 1977), these binding constants quantitatively explain the values obtained for the free fractions of the enantiomorphs found in cardiovascular patients (Yacobi and Levy, 1977). The present technique clearly indicates that there is one high affinity site and other weaker binding sites on the protein over a wide pH range. However, in other studies there are claims for the existence of two tight sites. The reason for these discrepancies are not apparent, except that too few data points were collected using the separation techniques at low drug-to-albumin ratios.

Fig. 6 shows the effect of pH on the fluorescence induced by the binding of warfarin and its enantiomers. The drug-to-HSA ratio of 0.1 is used because a single binding site on HSA contributes to the induced fluorescence at this low ratio, even if two sites of similar affinities are involved. The fluorescent intensity of warfarin–HSA complexes increases significantly on raising the pH, whereas the fluorescence of warfarin and HSA when

TABLE 2

BINDING PARAMETERS OF BINDING OF RACEMIC WARFARIN AND ITS ENANTIOMERS TO HSA AT pH 7.4

Warfarin	N_1	K_1	N_2	K_2	$K_{1:1}$
R(+)	1	4.4×10^5	1	1.0×10^4	4.8×10^5
S(–)	1	5.9×10^5	1	0.8×10^4	6.7×10^5
RS(±)	1	5.4×10^5	1	1.1×10^4	5.9×10^5

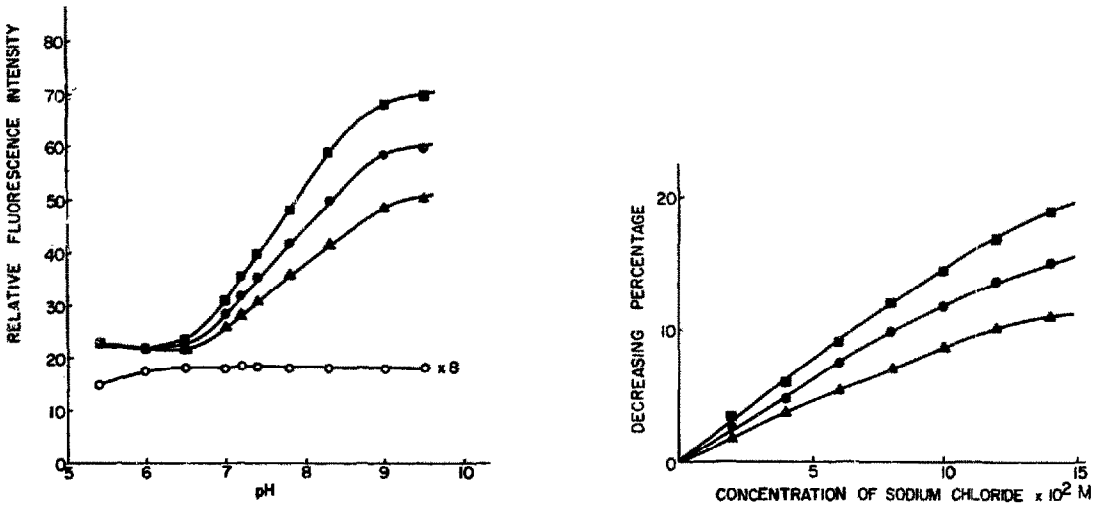

Fig. 6. Effect of pH on the fluorescent intensities for the binding of warfarin and its enantiomers to HSA. HSA: 1.0×10^{-5} M; warfarin: 1×10^{-6} M. ●, racemic warfarin; ▲, R-warfarin; ■, S-warfarin.

Fig. 7. Effect of sodium chloride on the fluorescent intensities of the binding of warfarin and its enantiomers to HSA at pH 7.4. Concentration as in Fig. 6. ●, racemic warfarin; ▲, R-warfarin; ■, S-warfarin.

alone is not significantly changed on raising the pH from 6.6 to 9.0. The pH profile of the induced fluorescence for warfarin–HSA systems clearly indicates that the first binding constant increases in the pH region associated with the N → B transition (Leonard et al., 1963; Harmsen et al., 1971). Müller and Wollert (1974, 1976) have recently suggested that this N → B transition is involved in the binding of benzodiazepines to HSA.

The effect of sodium chloride on the fluorescence of warfarin–HSA complexes is shown in Fig. 7. The reduced fluorescence of warfarin and its enantiomers may be caused by the direct competition of sodium chloride for the same binding site or by a conformational change in the albumin being induced on the addition of sodium chloride. It should be noted that the binding of the S-isomer is more affected by the addition of sodium chloride than that of the R-isomer. Sodium phosphate (0.01 ~ 0.15 M) has no measurable effect on the fluorescence of warfarin–HSA complexes. This suggests that chloride ions are responsible for the above observations. Brown and Crooks (1974) reported the binding of tolbutamide to HSA in phosphate buffer to be considerably reduced in the presence of sodium chloride (0.04 ~ 0.15 M).

Fig. 8 shows the pH profile for the effect of sodium chloride on the fluorescent intensity of racemic warfarin–HSA complexes. The decrease in percentage is defined as $(F_0 - F) \times 100/F_0$; where F_0 and F are the fluorescent intensities in the absence and presence of sodium chloride, respectively. The effect of sodium chloride decreases with increased pH. The reduced fluorescence in the presence of sodium chloride can be explained on the basis of displacement. The binding constant for warfarin–HSA com-

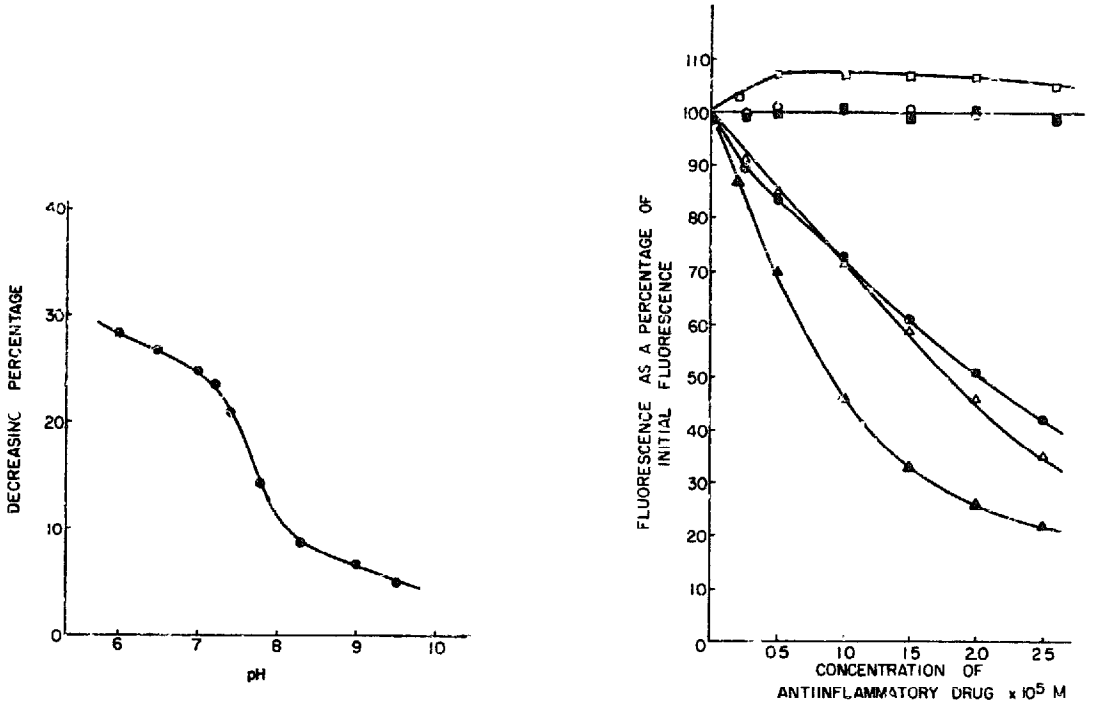

Fig. 8. pH dependence of the effect of sodium chloride on the fluorescent intensities of the binding of warfarin and its enantiomers to HSA. HSA 1.0×10^{-5} M, warfarin 1.0×10^{-6} M, sodium chloride 1.5×10^{-1} M.

Fig. 9. Effect of anti-inflammatory drugs on the fluorescent intensities for the warfarin–HSA complex at pH 7.4. Concentrations as in Fig. 6. ▲, phenylbutazone; △, flufenamic acid; ●, tolmetin; ○, fenoprofen; ■, aspirin; □, ibuprofen.

plexes increases with pH (Table 1 and Fig. 6), but the binding constant for sodium chloride decreases with pH (Scatchard and Yap, 1964). However, it seems more likely that chloride ions alter the type of the interaction or the nature of the binding site by an indirect effect on both the N and B forms and this effect decreases as the pH is increased.

A number of drugs were studied as potential antagonists of warfarin on HSA. In order to objectively determine the relative potency of anti-inflammatory drugs in this regard, and the specificity of the binding sites, several non-steroidal anti-inflammatory drugs were investigated. The ratio of warfarin to HSA was 0.1 and the ratio of competitors to HSA ranged from 0.1 to 3.0. Fig. 9 shows the changes in fluorescence of warfarin bound to HSA on the addition of drugs up to a drug to HSA ratio of 2.5. Phenylbutazone, tolmetin and flufenamic acid give significant displacement of warfarin at lower concentrations. In clinical conditions, it is likely that only the first binding site of warfarin is of significance, and phenylbutazone has been reported to effectively displace coumarin anticoagulants from albumin (Aggeler et al., 1967). The present data also clearly indicate that the two drugs do indeed share the same binding site. Flufenamic acid has been reported to have three very high affinity sites and 8 sites of lower affinity for HSA (Chignell, 1969b; Kaneo et al., 1976). Binding constants for sites with high affinity are greater than those

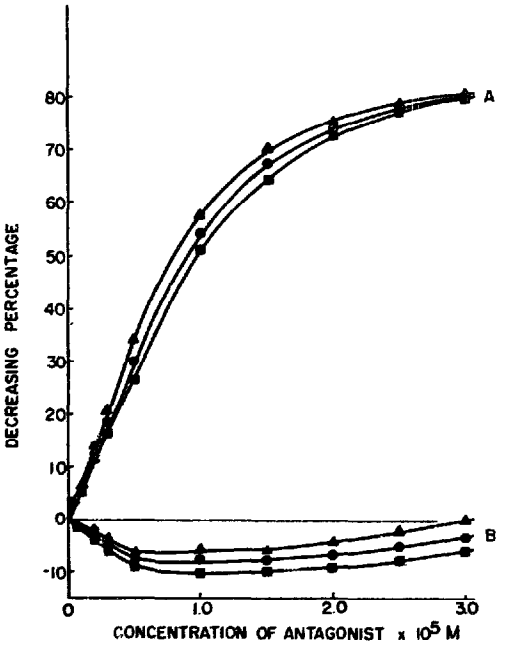

Fig. 10. Effect of phenylbutazone and ibuprofen on the fluorescent intensities for the binding of warfarin and its enantiomers to HSA at pH 7.4. Concentrations as in Fig. 6. Curves A: phenylbutazone; curves B: ibuprofen. ●, racemic warfarin; ▲, R-warfarin; ■, S-warfarin.

for phenylbutazone. On the other hand, the displacement data indicate that displacement by phenylbutazone is larger than that by flufenamic acid, suggesting that the second or third high affinity site of flufenamic acid may be the first site for warfarin. Tolmetin, a new anti-inflammatory drug, also displaces warfarin from its first binding sites, suggesting that the two drugs do share the binding site. Aspirin and fenoprofen did not displace any warfarin under the present experimental conditions; aspirin has been shown to have a much smaller binding constant than does warfarin (Perrin and Nelson, 1973). Fenoprofen has been reported to have about the same binding constant (Vallner et al., 1976; Otagiri et al., 1978) as warfarin, and so they probably do not share the same first binding site on HSA. The addition of ibuprofen gave a small enhancement of fluorescence of warfarin. This effect is likely to be mediated by a drug-induced change in the conformation of the albumin molecule. The present results are in agreement with the pharmacokinetic data; warfarin is not affected by co-administration of ibuprofen (Concalnes, 1973; Penner and Albrecht, 1975). Although subsequent in vitro work has shown that at very high concentrations ibuprofen can cause an increase in the free fraction of warfarin in human serum (Slattery and Levy, 1977). On the other hand, phenylbutazone causes some displacement of warfarin and changes in its pharmacokinetic behavior (Lewis et al., 1974). Furthermore, the present data also supports the suggestion of Sunlow et al. (1975) that human serum albumin has two specific binding sites.

Fig. 10 shows the effect of phenylbutazone and ibuprofen on the binding of warfarin and its enantiomers to HSA. At low concentrations phenylbutazone displaces more of the

R-isomer than the racemic, but less of the S than the racemic molecule. The magnitude of the reduced fluorescence depends upon the binding constants for the warfarin enantiomers. This clearly indicates that the three warfarins share the same binding sites. On the contrary, the induced fluorescence is enhanced in the presence of ibuprofen; ibuprofen may cause conformational change of albumin and the S-isomer may be more sensitive to this conformational change. This effect of ibuprofen on warfarin and its enantiomers is in good agreement with that of sodium chloride. It should be noted that S-warfarin is more sensitive to this conformational change of albumin than the R-isomer. This can be of relevance in a clinical situation.

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