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INFLUENCE OF LONG-CHAIN FREE FATTY ACIDS ON THE BINDING OF WARFARIN TO BOVINE SERUM ALBUMIN*

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Abstract—The effects of the concentration of long-chain free fatty acids and bovine serum albumin on the binding characteristics of warfarin with albumin have been studied by absorption and fluorescence spectroscopy using the method of ultrafiltration. The binding affinity of warfarin is higher in a concentrated than in a dilute albumin solution. In a dilute solution of serum albumin (55 μM or less), the binding constant of the warfarin–albumin complex is increased in the presence of 100 μM (or less) of either lauric or oleic acid or a mixture of these two acids, but higher concentrations of the fatty acids decrease such binding. A mixture of saturated and unsaturated fatty acids shows the same effects as those by either fatty acid. Simultaneous measurements of the fluorescence peak intensity and polarization of fluorescence of warfarin bound to albumin in a dilute solution indicate that low and high concentrations of free fatty acids induce different conformational states of the same warfarin-binding sites on the albumin molecule. Such a dualistic behavior of free fatty acids could be best interpreted in terms of allosteric interactions involving heterotropic effects. On the other hand, in a concentrated solution of serum albumin, the binding constant of warfarin is decreased in the presence of both low and high concentrations of free fatty acids. Such a decrease in warfarin binding with concentrated albumin in the presence of free fatty acids is not due simply to a displacement of warfarin by free fatty acids from the warfarin-binding sites, but also could result simultaneously from a further conformational change of warfarin-binding sites caused by the interaction of warfarin and free fatty acids (a negative heterotropic effect).

The effect of various drugs and free fatty acids (FFA) on the binding of the anticoagulant warfarin to albumin is a subject of continuing interest because of the serious clinical implications involved [1–6]. Rudman *et al.* [7] have shown that increasing the molar ratio of FFA to bovine or human serum albumin from 0 to 3.5 produces far less inhibition of drug–albumin binding than increasing this ratio from 3.5 to 7.0. Solomon *et al.* [8] have observed from a similar study with human albumin that lauric, myristic and stearic acids could displace warfarin from albumin by competing with it for the same binding site on the protein. Our previous studies [5, 6] have shown the same phenomenon of competitive inhibition of warfarin–plasma protein binding in rats by the endogenous FFA derived as a result of immobilization stress or fasting. Furthermore, these studies have demonstrated that even physiological changes in the plasma FFA concentration could inhibit such binding. Work by Spector *et al.* [9] concerning the effects of FFA concentration of the binding *in vitro* of two hypolipidemic drugs to human plasma albumin is also in agreement with our previous findings with warfarin [5, 6]. A mechanism of inhibition of drug–protein binding by FFA based on conformational changes in the drug-binding site of the albumin in the presence of FFA has also been proposed [6, 9] but, to our knowledge, no direct experimental evidence of such conformational changes in the protein structure is available at the present time. Furthermore, no quantitative studies involving the effects of variations of the concentration of FFA as well as albumin on the warfarin–albumin binding have been reported yet. Since plasma albumin represents the major transport vehicle for drugs and endogenous FFA, the present paper is concerned with investigating whether or not changes in the FFA as well as albumin concentrations could affect the binding characteristics of warfarin using bovine serum albumin (BSA) as a model protein. The present experiments were, therefore, conducted to determine the influence of various FFA concentrations on the binding affinities of warfarin with BSA as well as the conformational nature of the warfarin-binding sites on the protein molecule.

MATERIALS AND METHODS

Bovine serum albumin of a fatty acid free grade (< 0.005%) and sodium salts of lauric and oleic acids were obtained from Sigma Chemical Co., St. Louis, Mo. They were used without further purification. Warfarin sodium of U.S.P. grade was used. Concentration of BSA was calculated using the value of $E^{1\%}_{1cm} = 6.67$ at 280 nm [10]. Free fatty acids were estimated by the standard colorimetric method [11]. Warfarin concentrations were measured using an extinction coefficient of $1.39 \times 10^4 M^{-1} cm^{-1}$ at 308 nm [12]. Absorption spectra were recorded on a

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Unicam SP 8000 spectrophotometer using quartz cells. All solutions contained 0.1 M phosphate buffer, pH 7.5.

Binding studies were carried out by both absorption and fluorescence spectroscopy using the technique of ultrafiltration with Centriflo membrane cones (CF-50, from Amicon Corp., Mass.). The method involves the addition of various amounts of warfarin to solutions containing fixed amounts of BSA in the presence or absence of fixed amounts of FFA at 25 and 37°. After incubation for 30 min, the solutions were ultrafiltered and the ultrafiltrates were analyzed for warfarin and FFA. Only 10 per cent of the total sample volume was filtered. According to our experimental conditions, the binding of warfarin to the cones was insignificant.

To study the conformational changes in the protein structure, the polarization of fluorescence of warfarin bound to albumin was measured using excitation and emission wavelengths at 320 and 383 nm respectively. The degree of polarization, P, was calculated as:

$$P = \frac{I_{\parallel} - fI_{\perp}}{I_{\parallel} - fI_{\perp}}$$

where $I_{\parallel}$ and $I_{\perp}$ refer to the intensity of the fluorescent light polarized parallel ($_{\parallel}$) and perpendicular ($\perp$) to the exciting light, and f is a correction factor which accounts for the selective transmission of the monochromators and the selective reflection of the sample tubes. All fluorescence spectra were measured in a Perkin–Elmer MPF-3A fluorescence spectrophotometer. Two film-type polarizers were used for the polarization measurements.

The binding data were analyzed by plotting according to Scatchard [13] using the equation

$$\frac{r}{A} = K(n - r)$$

where r is the moles of warfarin bound/mole of albumin, n is the number of warfarin-binding sites available/albumin molecule, K is the binding (or association) constant for the warfarin–albumin complex, and A is the concentration of free warfarin. Each value of K and n was determined from a best-fitting line using statistical procedure.

RESULTS

Binding parameters of warfarin–albumin complexes in the presence of fatty acids. The experimental results on the measurements of the binding of warfarin to bovine serum albumin at 25 and 37° in the presence of lauric acid, oleic acid, and a mixture of these two fatty acids are summarized in Tables 1 and 2, using 0.4 and 4% albumin respectively. It is seen from Table 1 that in the absence of any fatty acid, warfarin binds to two equivalent non-interacting high affinity sites/BSA with an intrinsic binding (or association) constant of $6.31 \times 10^4\ M^{-1}$ at 25° and $3.06 \times 10^4\ M^{-1}$ at 37°. Such binding constants are found to be increased in the presence of 100 μM of either oleic or lauric acid or a mixture of lauric and oleic acids, both at 25 and 37°. However, the binding of warfarin with albumin is found to be decreased from the control value in the presence of somewhat higher concentrations (500 μM) of fatty acids at both temperatures, although the number of primary drug-binding sites remains the same as that of the control. Furthermore, it is observed that BSA possesses some secondary drug-binding sites of much lower affinity. The binding of warfarin to these secondary sites of albumin is also consistently decreased with increasing fatty acid concentrations (except with 100 μM lauric acid at 25°).

The binding constant of the warfarin–albumin complex using 4% (~580 μM) BSA is higher (Table 2) than that with 0.4% BSA in the absence of any fatty

Table 1. Influence of free fatty acids on the binding parameters of warfarin to dilute bovine serum albumin (~ 58 μM)*

Compound	K_1 (M^{-1}) × 10^{-4}†	n_1	K_2 (M^{-1}) × 10^{-4}†	n_2
	Temperature = 25°			
Warfarin alone	6.31	2.2	1.75	4.5
Warfarin + oleic acid‡	17.02	2.3	1.15	5.4
Warfarin + oleic acid§	0.80	1.8	0.22	4.3
Warfarin + lauric acid‡	7.9	2.5	1.98	4.4
Warfarin + lauric acid§	1.08	1.7	0.17	6.0
Warfarin + FFA‖ mixture‡	11.6	1.9	1.08	5.0
Warfarin + FFA‖ mixture§	1.06	1.8	0.16	6.0
	Temperature = 37°			
Warfarin alone	3.0	2.4	0.56	8.0
Warfarin + oleic acid‡	7.0	2.2	0.52	6.8
Warfarin + oleic acid§	2.9	1.9	0.25	6.6
Warfarin + lauric acid‡	6.8	2.1	0.50	7.2
Warfarin + lauric acid§	1.7	1.9	0.14	8.7
Warfarin + FFA‖ mixture‡	5.2	2.3	0.47	5.3
Warfarin + FFA‖ mixture§	0.67	1.8	0.13	5.0

* Results are the average of two sets of experiments.

† K_1 and K_2 are the binding constants of the warfarin–albumin complex for the primary (n_1) and secondary (n_2) drug-binding sites respectively.

‡ One hundred μM.

§ Five hundred μM.

‖ Mixture of lauric and oleic acids.

Table 2. Influence of free fatty acids on the binding parameters of warfarin to concentrated bovine serum albumin (~580 μM)*

Compound	Temperature = 25°			
	K_1 (M^{-1}) × 10^{-4}†	n_1	K_2 (M^{-1}) × 10^{-4}†	n_2
Warfarin alone	12.72	2.2	0.33	7.2
Warfarin + oleic acid‡	3.5	2.0	0.32	5.6
Warfarin + oleic acid§	0.58	2.2	0.03	11.9
Warfarin + lauric acid‡	5.4	2.1	0.23	6.7
Warfarin + lauric acid§	0.57	1.9	0.07	5.5
Warfarin + FFA‖ mixture‡	4.66	1.5	0.20	7.2
Warfarin + FFA‖ mixture§	0.55	1.8	0.06	6.0
	Temperature = 37°			
Warfarin alone	7.5	2.4	0.62	5.4
Warfarin + oleic acid‡	3.6	2.0	0.36	5.1
Warfarin + oleic acid§	0.50	1.9	0.15	3.3
Warfarin + lauric acid‡	4.0	2.0	0.32	5.2
Warfarin + lauric acid§	0.60	1.8	0.08	4.6
Warfarin + FFA‖ mixture‡	0.86	2.0	0.08	9.0
Warfarin + FFA‖ mixture§	0.67	1.8	0.04	7.9

* Results are the average of two sets of experiments.

† K_1 and K_2 are the binding constants of the warfarin–albumin complex for the primary (n_1) and secondary (n_2) drug-binding sites respectively.

‡ One thousand μM.

§ Four thousand μM.

‖ Mixture of lauric and oleic acids.

acids, although the number of warfarin-binding sites of high affinity remains at two as before. The affinity of warfarin for the secondary sites in albumin seems to be lowered in the concentrated BSA, both at 25 and 37°. The binding constant of the warfarin–albumin complex is found to be decreased with increasing temperature independently of the concentration of albumin. With 4% BSA, the affinity constant of warfarin is consistently decreased with increasing fatty acid concentrations, the number of primary warfarin-binding sites remaining, however, unchanged. Furthermore, it is quite evident from the present data that the binding parameters obtained in the presence of a mixture of saturated and unsaturated fatty acids (like lauric and oleic acids in the present case) are almost the same as those derived in the presence of either acid, except for the mixture at 37° (Table 2).

Nature of displacement of warfarin from BSA by fatty acids. The inhibition of warfarin binding in the concentrated BSA caused by fatty acids, as reflected from the binding parameters shown above, is due to the displacement of warfarin by the unbound fatty acids from the warfarin-binding site on the BSA molecule. This is evident from the experimental results presented in Fig. 1B and 2. Figure 1B is derived from the following experiment. To the different solutions

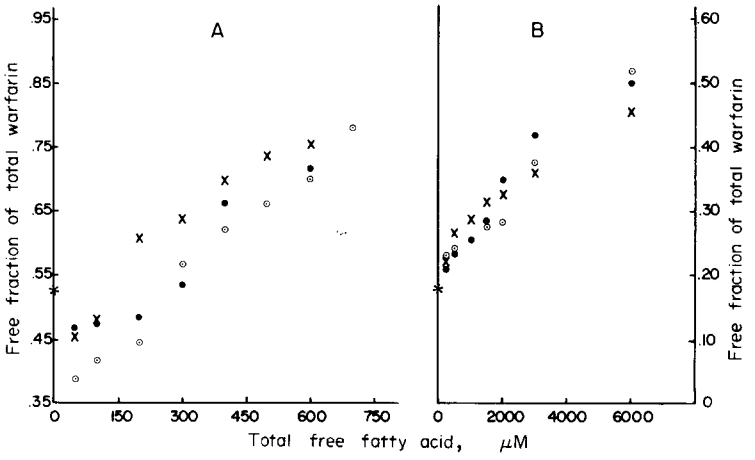

Fig. 1. Effects of oleic (●) and lauric (X) acids and mixture of oleic and lauric acids (⊙) on the amount of unbound warfarin in the warfarin–albumin complex at 25° using (A) 0.4% (~ 58 μM) and (B) 4% (~ 580 μM) bovine serum albumin solutions. An asterisk (*) indicates the corresponding control value. Total concentrations of warfarin are 350 and 3500 μM in (A) and (B) respectively. Details are in the text.

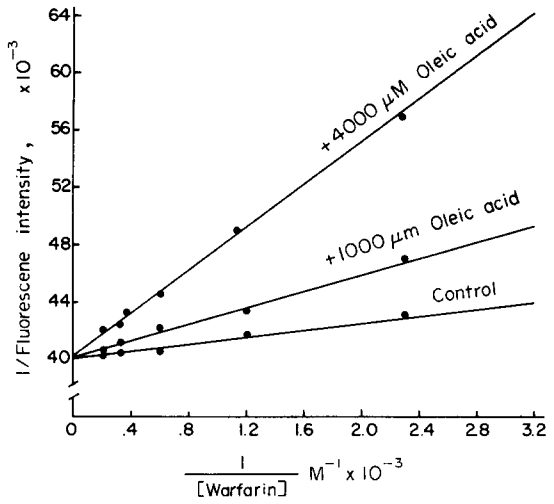

Fig. 2. Typical double reciprocal plot showing the competitive inhibitory effect by oleic acid on the warfarin–albumin binding at 25°. The fluorescence peak intensity of the warfarin–albumin complex was measured on each addition of warfarin to a 4% albumin solution containing either 0 or 3500 μM oleic acid in phosphate buffer, pH 7.5.

containing, in each case, a fixed amount of BSA (580 μM) and a definite but large amount of warfarin (3500 μM) to saturate the warfarin-binding sites, various amounts of fatty acids (250–6000 μM) were added. These solutions together with the appropriate control were incubated at 25 or 37°. After incubation for 30 min, free warfarin was determined from each solution after ultrafiltration. It can be observed, from Fig. 1B, that more and more warfarin was released as the amount of added fatty acids was gradually increased. That such displacement of warfarin by unbound fatty acids is competitive is also evident from a typical double reciprocal plot in Fig. 2, which shows a common intercept. However, these results do not necessarily demonstrate that the unbound fatty acids are competing with both the high affinity binding sites of warfarin in albumin.

On the other hand, the nature of the interactions between warfarin and unbound fatty acids for the protein binding is found to be somewhat different with dilute solutions of BSA (58 μM or less), as illustrated by Fig. 1A. The results were obtained in the same way as for Fig. 1B, except that all the solutions were ten times diluted. It is seen from Fig. 1A that the binding of warfarin is increased (instead of being normally decreased) after the first few additions of fatty acids up to a concentration of 100 μM (corresponding to a molar ratio of FFA/BSA ~ 1.7). This agrees quite well with an increase in the binding constant of the warfarin–albumin complex, as evident from Table 1. However, as the amounts of fatty acids are further increased beyond 100 μM, it is observed that the fraction of free warfarin is increased proportionately leading to a decrease in the warfarin-binding affinity as reflected again in Table 1. An appreciable amount of quenching, by fatty acids, in the range of 35–55 per cent, but not 100 per cent (depending on the concentration of BSA and nature of FFA), of the fluorescence of the warfarin–albumin complex as observed in the present study indicates that the unbound fatty acids cannot completely displace warfarin from its binding sites.

Nature of conformational changes in BSA. In an effort to investigate the possibility of ligand-induced conformational changes of the drug-binding sites within BSA, both the fluorescence peak intensity and polarization of fluorescence of warfarin-bound albumin, in the absence and presence of different free fatty acids, were measured. In principle, such measurements using warfarin as a fluorophore could be used as a probe at the same time in the study of hydrophobic regions in the protein and as a means of monitoring conformational changes of the warfarin-binding sites within BSA; thus, changes in environment near the protein-bound fluorophore could be detected. Such experiments were carried out using either concentrated or dilute BSA solutions. Two separate experiments were actually performed.

In the first, a given amount of BSA solution (7 or ~380 μM) was first saturated with an excess amount of warfarin so as to saturate the primary warfarin-binding sites in the albumin. To this solution were then gradually added various amounts of free fatty acids. For each addition of fatty acids, both the fluorescence peak intensity of the warfarin–albumin complex and the polarization of fluorescence (at the peak intensity) were monitored. The results are summarized in Figs. 3 and 4. When warfarin was bound to albumin, the fluorescence peak of warfarin was shifted from 390 to 380 nm, and the fluorescence intensity of warfarin was enhanced, irrespective of the concentration of BSA used. Furthermore, the position of the fluorescence peak due to the warfarin–albumin complex was not affected by the presence of fatty acids. Figure 3, therefore, shows the effects of adding fatty acids on the fluorescence peak intensity and the

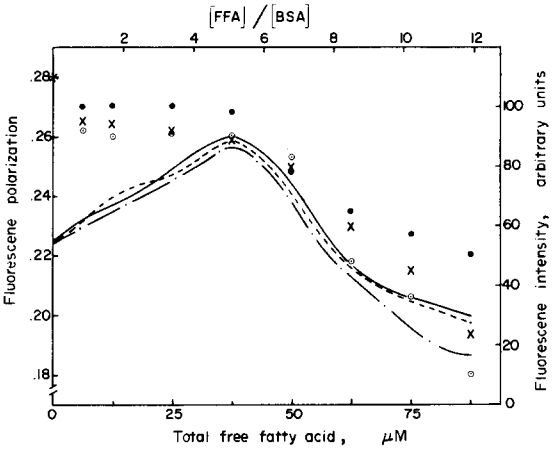

Fig. 3. Effects of free fatty acids on the fluorescence peak intensity and polarization of fluorescence of the warfarin–albumin complex containing 7 μM albumin and 80 μM warfarin at 25° in phosphate buffer, pH 7.5. The symbols for the fluorescence peak intensity and polarization of fluorescence are (——, ●), (—·—, ⊙), and (– – –, X) in the presence of lauric acid, oleic acid and mixture of these two acids respectively. Excitation and emission wavelengths are 320 and 382 nm respectively. Details are in the text.

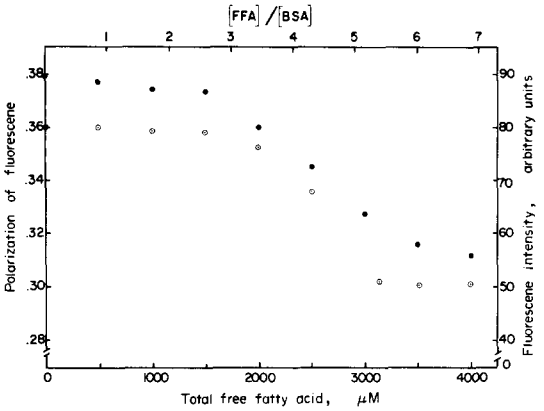

Fig. 4. Effects of oleic acid on the fluorescence peak intensity (●) and polarization of fluorescence (⊙) of the warfarin–albumin complex containing 380 μM albumin and 3500 μM warfarin at 25° in phosphate buffer, pH 7.5. Excitation and emission wavelengths are 320 and 382 nm respectively. Details are in the text.

polarization of fluorescence when warfarin is bound to a very dilute solution of BSA (7 μM). It is observed, from Fig. 3, that the fluorescence peak intensity increases initially after the first few additions of small amounts of fatty acids until it reaches a maximum at about 38 μM of fatty acids (corresponding to a molar ratio of FFA to BSA ~ 5). On further gradual increase of fatty acid concentrations, the fluorescence peak intensity tends to decrease. The polarization of fluorescence of albumin-bound warfarin is changed slightly after the first addition of a small amount of fatty acids and it remains at this level until the concentration of fatty acids is increased beyond 38 μM. When the concentration of fatty acids exceeds 38 μM, the polarization of fluorescence tends to decrease to a final value of about 0.19 from the control value of 0.28.

The results of a similar experiment using a concentrated solution of BSA (~380 μM) in the presence of various amounts of oleic acid are shown in Fig. 4. The fluorescence peak intensity of the complex decreases continuously from the very first addition of a small amount of oleic acid until it levels off to a value of about 55. The polarization of fluorescence of the albumin complex, however, remained unchanged (0.36) in the presence of low concentrations of oleic acid (corresponding to a molar ratio of FFA to BSA ⩽ 2), and decreased to a final value of about 0.30 with higher concentrations of oleic acid (corresponding to a molar ratio of FFA to BSA ~ 7). With lauric acid or a mixture of lauric and oleic acids, exactly the same behavior as that mentioned above was observed and, therefore, is not illustrated in Fig. 4.

A second experiment was performed to further investigate the nature of the conformational changes in BSA as follows. To a given solution of BSA (dilute or concentrated) containing a fixed but large amount of fatty acids (to saturate the primary fatty acid-binding sites), various amounts of warfarin were added. After each addition of warfarin, both the fluorescence intensity and the polarization of fluorescence at the complex maximum were monitored. The results using oleic acid are presented in Fig. 5 for a very dilute BSA solution (7 μM). It is seen, from the figure, that the fluorescence peak intensity of the albumin-bound warfarin is at first increased rapidly from that of the control (without warfarin) after the first addition of a small amount of warfarin. Then, the fluorescence intensity is gradually increased on further additions of warfarin until it levels off at a concentration of about 45 μM warfarin. On the other hand, the polarization value of the albumin rapidly rises from the control value of 0.25 (the control value was determined using fluorescence due to tryptophan residue in the albumin) to about 0.30 on complexation with a small amount of warfarin initially. Then, the polarization of warfarin-bound albumin remains essentially at the same level up to a warfarin concentration of about 18 μM. On further increasing the concentration of warfarin, the degree of polarization tends to decrease to a final value of about 0.25. However, only a very small change in the degree of polarization of warfarin-bound albumin is observed in the system containing either no, or a small amount (12.5 μM) of, oleic acid. A similar behavior was observed with lauric acid or a mixture of oleic and lauric acids and is, therefore, not shown in Fig. 5.

With a higher concentration of BSA (380 μM) and a definite but large amount of oleic acid (either 1000 or 4000 μM), the fluorescence peak intensity of the bound warfarin follows qualitatively the same behavior as that observed with a dilute solution of BSA. However, the fluorescence titration curve of warfarin is hyperbolic in a concentrated BSA solution, whereas it is somewhat sigmoidal in the dilute solution. A small change (0.28 to 0.25) in the value of polarization is observed in a concentrated BSA solution with increasing warfarin concentrations in the presence of higher amounts of free fatty acids (corresponding to a molar ratio of FFA to albumin of about three or more).

It has to be admitted, however, that experiments on polarization performed with concentrated solutions are not strictly quantitative because of a possibi-

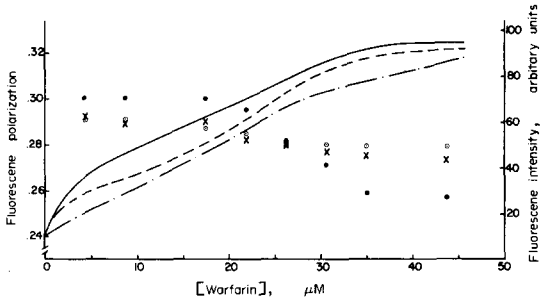

Fig. 5. Fluorescence peak intensity (——) and polarization of fluorescence (⊙) of the warfarin–albumin complex containing 7 μM bovine serum albumin in phosphate buffer, pH 7.5, as a function of warfarin concentration at 25°. In the presence of low (12.5 μM) and high (50 μM) concentrations of oleic acid, the respective peak intensity and polarization symbols are (——, X) and (—·—, ●).

lity of a certain degree of concentration depolarization.

DISCUSSION

The present studies with higher concentrations (nearly physiological) of serum albumin reveal that the warfarin binding could be decreased even within the physiological range of FFA concentrations corresponding to a molar ratio of FFA to albumin of less than two. This is in agreement with some previous works [9, 14–19] involving other drugs also, as well as with our own results [5, 6] on warfarin binding with rat plasma proteins. However, the present binding studies with very dilute solutions of serum albumin (55 μM or less) show some unexpected behavior at low concentrations of fatty acid anions in relation to the binding characteristics of warfarin. In other words, low concentrations of fatty acids do not release warfarin from a dilute solution of the albumin, but instead increase the warfarin–albumin binding. The mechanism of such a behavior will be discussed later.

Solomon *et al.* [8], in studying the warfarin binding to human albumin, did not, however, investigate the effects of fatty acids on such dilute solutions of albumin as presently reported. Chignell [12], O'Reilly [20] and other workers [21] did not study the effects of FFA on the warfarin binding to human serum albumin. On the other hand, the findings of Santos and Spector [14, 15] on the effects of fatty acids on the binding of 1-anilino-8-naphthalene sulfonate (ANS) to bovine and human serum albumin do not fully agree with our results using warfarin. Indeed, these authors observed inhibition of the ANS binding to BSA independently of the concentrations of palmitic acid used. No satisfactory explanation for such a difference using different fluorescent probes can, however, be formulated at the moment.

It is known [22] that the bovine serum albumin possesses at least two kinds of sites capable of binding to hydrophobic structures. One site is responsible for the specific binding of FFA and the second type of site is specific for substrates or other organic ligands or drugs. Furthermore, it has been well established [22] that the binding affinities of FFA for the albumin are much higher (100–1000 times) than those of most of the drugs. Both FFA and warfarin, therefore, should normally attach at first to their respective binding sites in the albumin, as dictated by the specific hydrophobic regions on the protein molecule. The present results do not, of course, allow any detailed conclusion about the geometry of these two different sites or the location with respect to each other. A marked enhancement as well as a blue shift in the fluorescence maximum of warfarin on complexation suggests the hydrophobic character of the warfarin-binding sites in the serum albumin.

It is of considerable interest to look again at the effects of low and high concentrations of fatty acids on the binding characteristics of warfarin with dilute BSA solutions in which the albumin is present mainly in the monomeric form (Figs. 1A, 3 and 5). When a fatty acid binds to its own site in the defatted albumin, it changes the structure of the protein molecule [23–25]. This, in turn, could affect the conformation of the warfarin-binding sites as well. Considering the results in Figs. 3 and 5, it is, therefore, likely that the very tight binding of low concentrations of a fatty acid to its own site results in a change of either the whole albumin structure or the conformation of the warfarin-binding sites leading to an increase in the quantum yield of warfarin bound to its tight sites as well as an increase in the binding constant. The rearrangement in the protein structure may not be drastic, however, as suggested by the present fluorescence spectral behavior (which remained the same in shape and structure). It is possible, therefore, that during the change of one structure of BSA to the other, only the local environment involving warfarin-binding sites is altered.

Considering what has been mentioned above, a decrease in the fluorescence of warfarin-bound albumin in the presence of higher concentrations of free fatty acids is, therefore, likely to be due to a decrease in the quantum yield of warfarin to its tight sites. Such a decrease in the fluorescence intensity, accompanied by a simultaneous decrease in the polarization of fluorescence, strongly suggests a further change in the conformation of the warfarin-binding sites in the protein structure induced by higher concentrations of free fatty acids. It is, therefore, quite apparent from the present results that low and high concentrations of free fatty acids induce different conformational states of the same warfarin-binding sites on the albumin molecule. However, considering the results in Fig. 5, it is also conceivable that the steady state conformational changes of the warfarin-binding sites leading to an inhibition of warfarin binding could be brought about by mutual interactions between warfarin and unbound fatty acids and not just by unbound fatty acids alone.

On the basis of the above discussion, it is quite possible that, in the present system with dilute solutions, an allosteric mechanism is operating involving "heterotropic" effects, i.e. indirect interactions between distinct specific binding sites of free fatty acids and warfarin. These interactions, in order to be allowed, are believed to be mediated by some kind of molecular transition (allosteric transition) [26] which is induced or stabilized in the protein when it binds an "allosteric ligand." Such a phenomenon is possibly realized when considering the results presented in Figs. 3 and 5. In other words, a positive heterotropic (i.e. cooperative) effect is observed at low molar ratios of fatty acids and albumin, while a negative heterotropic (or antagonistic) effect appears to be present at high molar ratios of fatty acids and albumin. Such a phenomenon is further exemplified in Fig. 5, which shows how the sigmoidal shape of the fluorescence titration curve at lower concentrations of FFA is changed with increasing concentrations of fatty acids.

Although it is not definitely clear from the present experimental results why such a cooperative effect is not observed in concentrated solutions of BSA, any one of the following reasons could possible explain such a discrepancy. First, in a concentrated solution, serum albumin exists in dimeric and/or polymeric forms consisting of two or more subunits or protomers. The formation of specific associations involving multiple bonds between subunits is likely to imply

a certain amount of rearrangement of the tertiary structure of the monomers resulting in some functional alterations in the structure of the active site or sites in each protomer.

Second, the spatial geometry or the molecular symmetry of dimeric and/or polymeric albumin molecules is such that the allosteric ligand (FFA) in one subunit could be in closer proximity (than in a purely monomeric albumin molecule) to the warfarin-binding site in another subunit, due to subunit–subunit interactions resulting eventually in a rather direct interference between the distinct but different binding sites. Consequently, a negative cooperativity or competitive inhibition of warfarin binding even in the presence of very low molar ratios of FFA to albumin could be possible

Third, there may be no difference between the conformations of the two states involved in the allosteric transition in the dimeric or polymeric albumin molecule. Alternatively even if there were a difference in the conformation of the two allosteric states, there might not be any differential affinity of the allosteric ligand for one state over the other.

Changes in the structure of albumins have been shown as a result of pH-changes and of binding to detergents and anionic azo dyes [27–29]. Weder and Bicker [30] have shown that desipramine binding to BSA is cooperative in character and they presented some spectral evidence that this was as a result of changes in the albumin structure.

It is, however, impossible to speculate at this time whether the present results are applicable in general to all types of albumins or other proteins, other drugs and/or other fatty acids or other endogenous substances, or whether they apply only to specific molecular structures and/or specific functional groups on any molecular structure. Some aspects of these questions are currently under investigation in our laboratory.

In conclusion, the present data suggest that the interactions between drug and endogenous substances at the level of protein binding need not be necessarily considered always in terms of purely competitive displacement, but may also arise from conformationally mediated interactions between distinct binding sites. Hence, the ability of free fatty acids to regulate the binding of a drug with plasma protein depends not only upon the strength with which a particular drug binds to albumin, but also on the nature of the conformational changes of the distinct binding sites associated with such interactions.

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