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# EVIDENCE FOR THE FATTY ACID-INDUCED HETEROGENEITY OF THE N AND B CONFORMATIONS OF HUMAN SERUM ALBUMIN

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**Abstract**—The influence of oleic acid on the interaction between albumin and warfarin, oxyphenbutazone or diazepam has been studied by circular dichroism and equilibrium dialysis. The pH dependences of the molar ellipticity of the drug–albumin complexes and of the free fraction of drug are completely changed by the presence of oleic acid. This phenomenon is attributed to an oleic acid-induced conformational change in both the neutral (N) and the basic (B) conformation of albumin, a change to which the warfarin–oxyphenbutazone binding area and the diazepam binding site is sensitive. The oleic acid-induced conformational states of albumin, the so-called N* and B* conformations, show binding properties that are different from the binding properties of the N and B conformations.

Albumin (in the rest of the text albumin means human serum albumin) is the major transport protein in the blood for many metabolites and drugs that are relatively insoluble in aqueous media. Among the physiological substances that bind with a high affinity to albumin are the long-chain fatty acids. The molar ratio of total fatty acid to albumin ($r_F$) in plasma is usually within the range 0.5–1.5. In several disease states, such as bacterial infection [1], diabetes mellitus [2] and stress [3, 4] and after administration of heparin [5, 6], fatty acid levels are found to increase. In some cases the corresponding $r_F$ values exceed a value of 8. Commercial samples of albumin were found to have $r_F$ values up to 9 [7]. Because the fatty acid content of plasma and commercial albumin samples varies, it is important in drug binding studies to determine whether the capacity of albumin to bind a drug can be influenced by differences in the fatty acid concentrations.

Many studies have already been published about the effect of fatty acids on the free concentration of drug, i.e. the fraction of drug which is thought to induce the pharmacological effect. Spector *et al.* [8] suggested a very plausible model for the fatty acid influence on drug binding. They hypothesized that albumin has fatty acid binding sites which cannot be reached by drugs. Binding of fatty acids to these sites produces conformational changes in the regions where drugs bind. If the fatty acid binding sites are saturated, the fatty acids compete with drugs for the drug binding site. However, the study of Spector *et al.* and almost all the other studies on the interaction between fatty acids and drugs [9–13] have been carried out at one pH value, i.e. pH 7.4. Around pH 7.4 albumin shows a conformational change, the so-called N–B transition [14]. At least the two major drug binding sites, site I and site II, have been found to be sensitive to this N–B transition. Therefore the binding of drugs to site I or site II is pH dependent [14–19].

The N–B transition, and thus the pH dependence of the binding of drugs, can be altered by allosteric mediators [15, 17, 20]. Some of the best known allosteric effectors are the long-chain fatty acids [8, 15, 18, 21]. Consequently the influence of fatty acids on the binding of drugs to albumin must be examined over the pH range of the N–B transition, i.e. from pH 6 to 9, and not only at pH 7.4. In this paper we report on the effect of oleic acid, a long-chain fatty acid, on the pH dependence of the interaction between albumin and certain drugs, namely warfarin, oxyphenbutazone and diazepam. Warfarin and oxyphenbutazone bind to site I on the albumin molecule, whereas diazepam binds to site II.

## MATERIALS AND METHODS

Human serum albumin (infusion solution) was a gift from the Biotest-Serum-Institut GmbH, Frankfurt am Main, F.R.G. This batch was given lot number 820512. The albumin was deionized before use and its concentration determined as described elsewhere [14]. The deionizing procedure yielded a protein solution in which 0.9 mole fatty acid bound per mole albumin, measured as described previously [22]. Sodium warfarin (Brocacef, Maarssen, The Netherlands), diazepam (gift from Hoffmann La Roche, Mijdrecht, The Netherlands), oxyphenbutazone (gift from Ciba Geigy BV, Arnhem, The Netherlands), $^{14}$C-labelled warfarin and $^{14}$C-labelled diazepam (2.04 and 2.11 GBq mmole$^{-1}$ respectively, Amersham Nederland BV, Utrecht, The Netherlands), $^{14}$C-labelled oxyphenbutazone (1.14 GBq mmole$^{-1}$, New England Nuclear, Boston, MA) and oleic acid (sodium salt) (Sigma Chemical Company, St Louis, MO) were used without further purification. All other chemicals were of analytical grade (Merck, Darmstadt, F.R.G. or J. T. Baker, Deventer, The Netherlands). The sodium salt of oleic acid

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was dispersed in demineralized water and then mixed with an albumin solution, which resulted in a clear solution.

The affinities of warfarin, oxyphenbutazone and diazepam to albumin were studied by means of equilibrium dialysis. The equilibrium dialysis experiments were carried out as described previously [14]. Free concentrations of warfarin, oxyphenbutazone and diazepam were determined by liquid scintillation counting (Packard Tricarb Liquid Scintillation Spectrometer 2425). The dialysis experiments were done with albumin solutions of $6.0 \times 10^{-5}$ M and a total drug-to-protein ratio ($r_D$) of 0.3 at 25°. It should be mentioned that during the equilibrium dialysis experiments $r_D$ changes. Therefore the measured free concentration of ligand is interpolated to a free concentration (and therefore a free fraction) at $r_D$ = 0.30.

Circular dichroic experiments were performed as described previously [14, 16]. The circular dichroic signal of the complexes of albumin with warfarin, oxyphenbutazone and diazepam ($r_D = 0.30$) was measured in phosphate or borate buffers in the pH range 6.0–9.2 (ionic strength = 0.1) at 310, 304 and 330 nm, respectively. The difference between the circular dichroic signal of the drug–albumin mixture and of the albumin alone at a given wavelength is called the observed ellipticity of the drug–albumin mixture. The molar ellipticity ([$\theta$]) of the drug–albumin complex is the observed ellipticity of the drug–albumin mixture related to the concentration drug–albumin complex present in the measured drug–albumin mixture [23]. The concentration of the drug–albumin complex of the various samples was obtained by equilibrium dialysis. The oleic acid–albumin complex did not give a circular dichroic signal at 310, 304 and 330 nm. Since oleic acid does not change the ultraviolet absorption of warfarin, oxyphenbutazone and diazepam at 310, 304 and 330 nm, respectively, it is not likely that oleic acid-induced changes of [$\theta$] of the ligand–albumin complex must be explained by a change in the spectral properties of the bound ligand. Therefore oleic acid-induced changes of [$\theta$] of the ligand–albumin complex will be explained by a change in the spectral properties of the ligand–albumin complex. The circular dichroic experiments were done with albumin solutions of $6.0 \times 10^{-5}$ M, at 25°.

## RESULTS

### *Dialysis experiments*

The results of equilibrium dialysis experiments are usually presented in terms of affinity constants ($K$) of the ligand for the protein. However, the free concentration of drug is directly related to the intensity of the pharmacological effect of the drug [24, 25]. Since in this study the total concentration of drug and that of protein are held constant, the free concentration is linearly proportional to the free fraction (ff) of drug. Therefore we present the results of the dialysis experiments in terms of free fractions. At low free concentration of drug, and therefore at low ff, $r_D$ can be assumed to be equal to the average number of ligand molecules associated with one molecule of albumin. Therefore $K$ can be easily calculated from the ff.

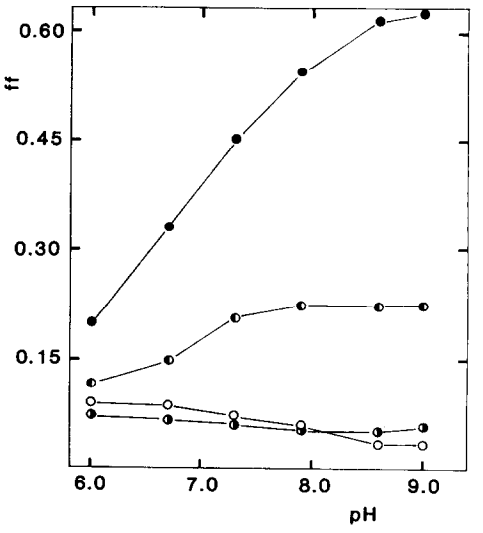


Fig. 1. The pH dependence of the free fraction (ff) of warfarin under various conditions. (○) $r_F = 0.9$; (◑) $r_F = 5.0$; (◐) $r_F = 7$; (●) $r_F = 9.0$. The molar ratio ligand to albumin was 0.3. The 95% confidence interval of the ff is within 4%.

The effect of oleic acid on the binding of warfarin, oxyphenbutazone and diazepam is shown in Figs 1, 2 and 3, respectively. Before use the albumin was deionized on a mixed bed ion-exchange column. This method does not remove all the fatty acid from the albumin [22]. In this study the deionizing procedure yielded a protein solution having 0.9 mole fatty acid bound per mole albumin. Although the effect of fatty acids on the N–B transition is being studied we used this albumin sample with $r_F = 0.9$ as our starting material because it may be advisable to work with

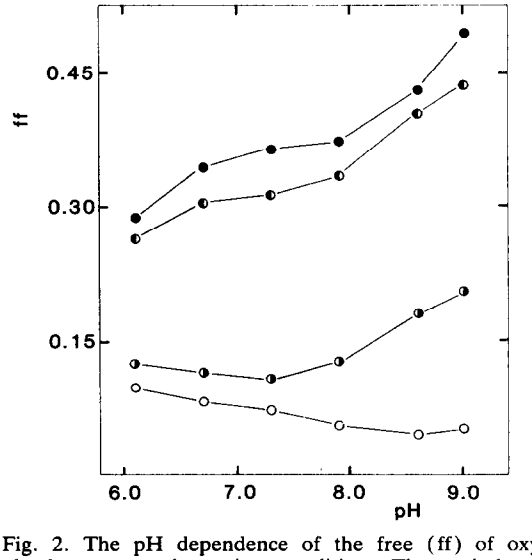


Fig. 2. The pH dependence of the free (ff) of oxyphenbutazone under various conditions. The symbols are explained in the caption to Fig. 1. The 95% confidence interval of the ff is within 4%.

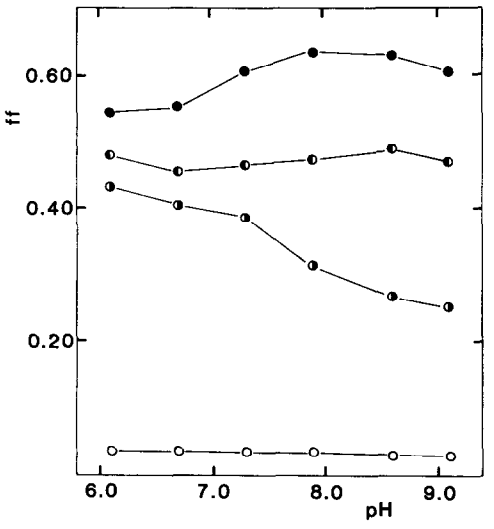


Fig. 3. The pH dependence of the free fraction (ff) of diazepam under various conditions. The symbols are explained in the caption to Fig. 1. The 95% confidence interval of the ff is within 4%.

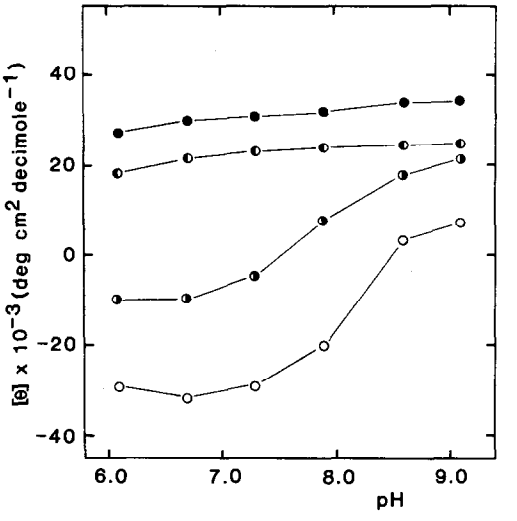


Fig. 4. The pH dependence of the molar ellipticity ($\theta$] of the oxyphenbutazone–albumin complex under various conditions. The symbols are explained in the caption to Fig. 1. The 95% confidence interval of [$\theta$] is about $10^3$ deg cm$^2$decimole$^{-1}$.

albumin that contains some bound fatty acid [22]. Oleic acid is the most abundant fatty acid in the blood. Since we found that long-chain fatty acids (C16 and C18) influence the drug-albumin binding in a similar way to oleic acid (unpublished results) and since about 80% of the fatty acids in the blood are long-chain fatty acids [26], in the rest of this study the fatty acid bound to the albumin before adding oleic acid will be regarded as if it were oleic acid.

In Fig. 1 the results for warfarin are presented. At the lowest $r_F$ value ($r_F = 0.9$) the ff of warfarin is larger at pH 6.1 than at pH 9.2. At $r_F = 5.0$ oleic acid decreases the ff of warfarin below pH 8, whereas above pH 8 it increases it. The pH dependence of the ff of warfarin has almost disappeared at $r_F = 5.0$. At $r_F = 7.0$ and 9.0 the ff of warfarin is larger at high pH values than at low pH values, whereas at $r_F = 0.9$ the opposite is the case.

Figure 2 shows the results for oxyphenbutazone. At the lowest $r_F$ value ($r_F = 0.9$) the ff of oxyphenbutazone is larger at pH 6.1 than at pH 9.2. In the presence of oleic acid ($r_F = 5.0$, 7.0 and 9.0) the ff of oxyphenbutazone increases at all pH values. At these $r_F$ values the ff of oxyphenbutazone is larger at high than at low pH.

In Fig. 3 the results for diazepam are presented. This figure shows that oleic acid ($r_F = 5.0$, 7.0 and 9.0) increases the free fraction (ff) of diazepam at all pH values. At $r_F = 5.0$ the ff of diazepam is larger at low pH than at high pH, whereas at $r_F = 9.0$ the ff of diazepam is larger at high than at low pH. At $r_f = 0.9$ and $r_F = 7.0$ the ff of diazepam is almost pH independent.

*Circular dichroic experiments*

The effect of oleic acid on the pH dependence of [$\theta$] of the complexes of albumin with warfarin, oxyphenbutazone and diazepam has been studied as well. Figure 4 shows the results for the oxyphenbutazone–albumin complex. At the lowest $r_F$ value ($r_F = 0.9$) [$\theta$] of the oxyphenbutazone–albumin complex increases with the pH. The difference between pH 6.1 and pH 9.2 with respect to [$\theta$] is about $3.5 \times 10^4$ degcm$^2$decimole$^{-1}$. Oleic acid increases [$\theta$] of the oxyphenbutazone–albumin complex at all pH values. At $r_F = 7.0$ and $r_F = 9.0$ the difference between [$\theta$] of the oxyphenbutazone–albumin complex at low and high pH values has almost disappeared.

The effect of oleic acid on the pH dependence of [$\theta$] of the warfarin–albumin complex is similar to the effect of oleic acid on the pH dependence of [$\theta$]

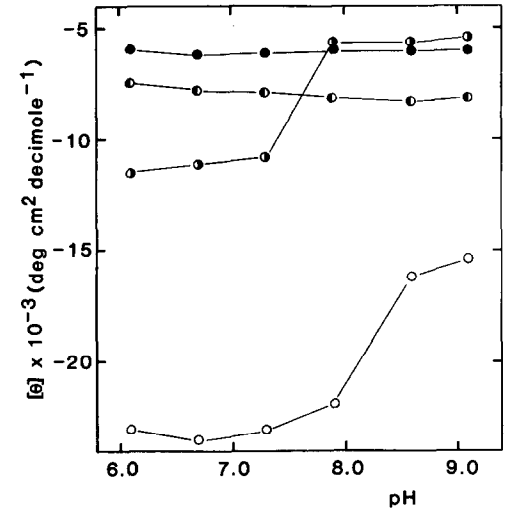


Fig. 5. The pH dependence of the molar ellipticity ([$\theta$]) of the diazepam–albumin complex under various conditions. The symbols are explained in the caption to Fig. 1. The 95% confidence interval of [$\theta$] is about $10^3$ deg cm$^2$ decimole$^{-1}$.

of the oxyphenbutazone–albumin complex. These results are not shown.

Figure 5 shows the results for the diazepam–albumin complex. At the lowest $r_F$ value ($r_F = 0.9$) $[\theta]$ of the diazepam–albumin complex increases with the pH. The difference between pH 6.1 and pH 9.2 with respect to $[\theta]$ is about $8 \times 10^3$ degcm$^2$decimole$^{-1}$. Oleic acid increases $[\theta]$ of the diazepam–albumin complex at all pH values. At $r_F = 5.0$ there is still a large difference between the $[\theta]$ values at low and high pH. At $r_F = 7.0$ and $r_F = 9.0$ there is no longer any significant difference between $[\theta]$ at low and high pH. At low pH values $[\theta]$ of the diazepam–albumin complex increases with $r_F$. At high pH values, however, $[\theta]$ increases between $r_F = 0.9$ and $r_F$ 5.0, decreases between $r_F = 5.0$ and $r_F = 7.0$ and increases again between $r_F = 7.0$ and $r_F$ 9.0.

## DISCUSSION

### *The effect of oleic acid on the pH dependence of the oxyphenbutazone–albumin and the warfarin–albumin interaction*

The effect of oleic acid on the oxyphenbutazone–albumin and the warfarin–albumin interaction is shown in two different ways. Figures 1 and 2 show the influence of oleic acid on the pH dependence of the ff of warfarin and oxyphenbutazone respectively, whereas Fig. 4 shows the influence of oleic acid on the pH dependence of $[\theta]$ of the oxyphenbutazone–albumin complex. An attempt will be made to explain the influence of oleic acid on the ff and on $[\theta]$ in terms of the same mechanism. From Fig. 4 it was seen that $[\theta]$ of the oxyphenbutazone–albumin complex at $r_F = 0.9$ shows a significant difference at low and at high pH values. Recently it was shown that this pH dependence was due to the N–B transition of the oxyphenbutazone–albumin complex [17]. This means that at $r_F = 0.9$ the molar ratio B/N is different at low and high pH values. At $r_F = 7.0$, however, the pH dependence of $[\theta]$ of the oxyphenbutazone–albumin complex has almost disappeared (Fig. 4). Allosteric effectors such as the long-chain fatty acids can influence the N $\rightleftarrows$ B equilibrium. Therefore the pH dependence of $[\theta]$ at $r_F = 7.0$ might be explained by a constant molar ratio B/N over the pH range 6–9. The fact that in the absence of oleic acid $[\theta]$ of the oxyphenbutazone–albumin complex at high pH values is larger than $[\theta]$ at low pH values means that oleic acid may shift the N–B transition towards the B conformation. If so, then the ff of oxyphenbutazone must be less pH dependent in the presence of oleic acid than in its absence. From Fig. 2, however, it was seen that the ff of oxyphenbutazone is even more pH-dependent in the presence than in the absence of oleic acid. Furthermore, it was seen that the pH dependence was reversed in the presence of oleic acid. Therefore the effect of oleic acid ($r_F = 7.0$) on the binding of oxyphenbutazone cannot be fully explained by a shift in the N–B transition. The effects of oleic acid on $[\theta]$ and the ff of the warfarin–albumin interaction are similar to those of the oxyphenbutazone–albumin interaction, and therefore the effect of oleic acid on the binding of warfarin cannot be explained by a shift in the N–B transition either.

Figure 4 shows an increase in $[\theta]$ of the oxyphenbutazone–albumin complex with $r_F$ at all pH values. As discussed above, this increase cannot be fully due to a shift in the N $\rightleftarrows$ B equilibrium. Alterations in the molar CD signal of a ligand–protein complex might be due to other processes such as translocation of the bound marker or perturbation of the binding site of the marker [27–29]. Translocation of the bound oxyphenbutazone would mean that the oxyphenbutazone is displaced from its primary binding site to its secondary binding sites. From unpublished results it is known that $[\theta]$ of the complex of oxyphenbutazone with its secondary binding sites is different at pH 6.1 and pH 9.2. This means that translocation alone cannot make $[\theta]$ of the oxyphenbutazone–albumin complex pH independent (see Fig. 4, $r_F = 7.0$).

The other possible phenomenon which can alter the molar CD signal of a ligand–protein complex is perturbation of the binding site of the marker molecule. This means that oleic acid induces a conformational change in the albumin molecule, as a result of which the oxyphenbutazone binding site alters. Since warfarin and oxyphenbutazone share the same binding site on the albumin [30–34] the oleic acid-induced conformational change must also alter the warfarin binding site. This is confirmed by the fact that oleic acid changes $[\theta]$ of both the warfarin– and the oxyphenbutazone–albumin complex in a similar way and that oleic acid also reverses the pH dependence of warfarin (Fig. 1), just as with oxyphenbutazone.

It should be noted that besides inducing this conformational change oleic acid also may shift the N–B transition or may translocate the site I ligand to binding sites of lower affinity.

To support our conclusions based on the circular dichroic experiments we measured the absorbance of the oxyphenbutazone–albumin complex ($r_D = 0.30$) at pH 6 and pH 9 in the presence ($r_F = 7.0$) and in the absence of oleic acid ($r_F = 0.9$). It was seen that the difference in absorbance of the oxyphenbutazone–albumin complex between pH 6 and pH 9 disappeared when oleic acid was added to the albumin.

### *The effect of oleic acid on the pH dependence of the diazepam–albumin interaction*

Diazepam binds to the albumin on a binding site different from the warfarin–oxyphenbutazone binding area. According to the literature [35], the long-chain fatty acids do not bind to site I or site II, but exert different allosteric effects on these two sites [18, 35–37]. It has been seen already that the long-chain fatty acids did increase the fluorescence and binding of probes at site I, whereas they did not at site II [18]. The influence of fatty acids on the circular dichroism signal of complexes of site II-probes with albumin at pH 7.4 has been investigated by Sjödin [38]. He suggested that diazepam was displaced by oleic acid through an allosteric mechanism. Figures 4 and 5 show that oleic acid influences the pH dependence of $[\theta]$ of the diazepam–albumin complex and $[\theta]$ of the oxyphenbutazone–albumin complex in a similar way. At $r_F = 7.0$ and $r_F = 9.0$ the pH dependence of $[\theta]$ disappears, just as in the case of oxyphenbutazone. The possibility that oleic acid

shifts the N ⇄ B equilibrium of the diazepam–albumin complex can be ruled out in the same way as in the case of oxyphenbutazone. The possibility that oleic acid displaces the diazepam from its high affinity sites to its low affinity sites (translocation) can be ruled out because $[\theta]$ of the diazepam–albumin complex at high pH not only increases or decreases with $r_F$, but increases between $r_F = 0.9$ and 5.0, decreases between $r_F = 5.0$ and 7.0 and increases again between $r_F = 7.0$ and 9.0 (see Fig. 5). Therefore oleic acid induces a conformational change in the albumin to which the binding of site II-probes is sensitive. Consequently site I and site II are sensitive to an oleic acid induced conformational change just as they are sensitive to the proton-induced N–B transition.

*The oleic acid conformational change and its consequences for binding studies*

As discussed above, oleic acid induces a conformational change in the albumin, to which site I and site II are sensitive. The binding of warfarin, oxyphenbutazone and diazepam to this new conformation is also pH dependent, just as it was in the absence of oleic acid. From this observation it is natural to assume that this oleic acid-induced conformational state occurs in an N and a B conformation which are different from the N and B conformation in the absence of fatty acid. This means that both the N and the B conformation can undergo a conformational change induced by oleic acid. In the rest of the text these oleic acid-induced conformations are called the N* and B* conformations.

From Figs 4 and 5 it can be seen that the effect of oleic acid on $[\theta]$ of the oxyphenbutazone–albumin complex and the diazepam–albumin complex is not saturated at $r_F = 9.0$. Figures 1 and 3 show that between $r_F = 7.0$ and $r_F = 9.0$ the shape of the pH dependences of the ff of warfarin and diazepam still changes. From these observations it can be concluded that the N–N* and B–B* transitions involve nine or more oleic acid molecules. It is possible that in going from $r_F = 0$ to $r_F = 9$ we are dealing with several N* and B* conformations. This latter idea is supported by the observation that $[\theta]$ of the diazepam–albumin complex at high pH increases between $r_F = 0.9$ and $r_F = 5.0$, decreases between $r_F = 5.0$ and $r_F = 7.0$ and increases again between $r_F = 7.0$ and $r_F = 9.0$. This means that we are dealing with at least three B* and therefore three N* conformations.

Earlier investigations already pointed to a fatty acid induced conformational change of the drug binding site [8, 21, 35–37] and recently Wanwimolruk and Birkett [18] have shown that this fatty acid-induced conformational change is different from the N–B transition. According to the latter investigators the fatty acid-induced conformational transition and the N–B transition have similar and additive effects at site I, i.e. the warfarin–oxyphenbutazone binding area. The results reported in this paper do not point to an oleic acid-induced shift of the N ⇄ B equilibrium. However, since oleic acid induces conformational changes in the N and B conformations, the N ⇄ B equilibrium will be disturbed.

From Figs 1, 2 and 3 it can be seen that the difference in the affinity of warfarin, oxyphenbutazone and diazepam to the N* and B* conformations is contrary to the difference in the affinity of the ligands to the N and B conformation. Although the effect of oleic acid on the interaction of albumin with the two ligands studied can be described qualitatively using the same mechanism, the effects differ from each other quantitatively. It can be seen from Figs 1 and 2 that the reversal of the pH dependence of the oxyphenbutazone binding occurs at a lower $r_F$ than the reversal of the pH dependence of the warfarin binding. The reversal of the pH dependence of the ff of diazepam occurs at a much higher $r_F$ value than in the case of warfarin and oxyphenbutazone. Furthermore, in the case of diazepam relatively low $r_F$ values ($r_F = 5$) increase the difference between the ff of the ligand at low and high pH values, whereas in the case of warfarin and oxyphenbutazone this was not seen.

The results reported in this study explain some of the contradictions found in the literature. The warfarin–albumin complexes and the diazepam–albumin complexes show a smaller difference between $[\theta]$ at low and high pH values than was found by other investigators [14, 16]. The latter investigators, however, used albumin samples with 0.2 moles fatty acid bound to one mole albumin, whereas we used an albumin sample with a starting $r_F$ value of 0.9. From the above results it will be clear that a difference in $r_F$ can cause large differences between the pH dependences of $[\theta]$ of a ligand–albumin complex having different $r_F$ values.

Elbary *et al.* [19] estimated the pH dependence of the affinity of oxyphenbutazone to albumin. From their results it was concluded that oxyphenbutazone shows a larger affinity for the N conformation than for the B conformation of albumin. Figure 2, however, shows that the affinity of oxyphenbutazone to albumin ($r_F = 0.9$) is larger for the B conformation than for the N conformation of albumin. At $r_F = 6$ and $r_F = 8$, however, where the albumin exists in the N* or B* conformation, it can be seen that the affinity of warfarin and oxyphenbutazone is larger for the N* than for the B* conformation. This reversal of the pH dependence might explain the contradiction between the findings of Elbary *et al.* [19] and our results. Elbary *et al.* used an albumin sample whose fatty acid content is not mentioned. Since commercial preparations of albumin were found to have $r_F$ values between 0 and 9, this means that the $r_F$ of the albumin sample we used may have been quite different from the $r_F$ of the albumin sample used by Elbary *et al.* Therefore it is possible that Elbary found a higher affinity of oxphyenbutazone for albumin at high pH than at low pH, whereas we found the opposite result in the absence of oleic acid.

It will be clear from this paper that fatty acids introduce heterogeneity into the N and B conformations of albumin. This fatty acid-induced heterogeneity means that one should exercise great caution when comparing results obtained with albumin samples with different ratios of fatty acid.

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