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# CIRCULAR DICHROISM STUDIES ON THE INHIBITING EFFECT OF OLEIC ACID ON THE BINDING OF DIAZEPAM TO HUMAN SERUM ALBUMIN

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**Abstract**—The effect of a free fatty acid (oleic acid) on the binding of a benzodiazepine derivative (diazepam) to human serum albumin (HSA)* has been studied using the technique of circular dichroism. Both qualitative and quantitative results suggest that oleic acid significantly affects the binding of diazepam, even at low molar ratios to albumin (below 1:1). It is suggested that the displacement of bound diazepam occurs primarily through an allosteric mechanism.

Human serum albumin (HSA) is known to bind a great number of compounds of endogenous and exogenous origin [1]. Since the association is reversible, the bound compounds will be in equilibrium with their unbound molecules. HSA can therefore be considered as a buffer medium preventing great fluctuations in the free concentrations of these molecules. This is an important role of the protein, since only the free molecules can exert any physiological effect in the body.

HSA binds many types of agents and it is possible that one agent may influence the binding of another [2]. Free fatty acids (FFA) have been reported to interact with a number of compounds bound to HSA. It has been found that FFA within their physiological concentration range (0.5–2 moles per mole HSA) will displace tryptophan from HSA, resulting in an increase of the free tryptophan plasma level [3]. Rudman *et al.* [4] showed that FFA inhibited the normal binding of a number of drugs to HSA and BSA with a 3.5–7 molar excess of FFA over albumin being necessary to obtain the displacements. Tsutsumi *et al.* [5] studied the binding of a benzo-diazepine derivative to HSA in the presence of rather high molar concentrations of laurate over HSA (4.7:1) and found a significant inhibitory effect on the binding which they suggested was competitive.

It is known that diazepam is bound primarily to only one binding site on HSA [6, 7], probably the same one as the primary site for tryptophan [8]. If this is true, the displacement of diazepam may occur over the physiological concentration range of the FFA. The present paper was instigated to determine if this was the case and to study the interaction mechanism between these ligands when bound to HSA.

* Abbreviations used in this paper: HSA, human serum albumin; BSA, bovine serum albumin; CD, circular dichroism; FFA, free fatty acids; CPIB, chlorophenoxyisobutyrate.

§ This sample of albumin with 0.6 moles FFA/mole HSA is in the following denoted as HSA. The FFA content is included in the molar ratio values given for the oleic acid-HSA complexes.

## MATERIALS AND METHODS

*Monomeric human serum albumin* (HSA) was prepared as described previously [9] from outdated blood. The protein was defatted according to Chen [10]. The remaining fatty acid content was measured by gas chromatography [11] and found to be 0.6 moles/mole HSA.§ The same preparation was used throughout the experiment. The concentration of the protein, dissolved in 0.1 M KCl, 0.005 M sodium phosphate buffer, pH 7.40, was determined on a Schimadzu MPS-5000 spectrophotometer at 280 nm, using $A^{1\%}_{1cm} = 5.80$ [12]. The molecular weight was estimated to be 66,300 from the primary structure presented by Behrens *et al.* [13] and Meloun *et al.* [14].

*Diazepam* was a gift from F. Hoffman-La Roche & Co. AG., Switzerland. It was used without further purification.

*Oleic acid*, gas chromatographic grade, was obtained from Carlo Erba, Italy, and stored in sealed ampoules at 4° until use.

Diazepam and oleic acid were dissolved in ethanol prior to their addition to HSA. The ethanol concentration was kept constant at 1.3% in all test-solutions of HSA, even when diazepam or fatty acid was absent.

*Circular dichroism (CD).* Spectra were obtained at room temperature with a Jasco J-41 A spectropolarimeter. The instrument was calibrated with D-10-camphorsulphonic acid. The results are expressed as molar ellipticity (degrees $cm^2$ $dmole^{-1}$), $\{\Theta\}$, calculated with reference to the HSA concentration, or as difference molar ellipticity, $\Delta\{\Theta\}$.

*Determination of the binding constant of diazepam to HSA* was performed using the CD continuous titration technique [15]. An albumin solution (A) was circulated from a thermostatted (25°) mixing vessel to a cuvette in the spectropolarimeter via a pump system. The ellipticity of the solution was measured at constant wavelength (262 nm). After obtaining an initial base-line, a solution (B) of albumin and diazepam (molar ratio 1:14.6) was continuously added to the mixing vessel. The protein concentrations in A and B were identical. As the HSA–diazepam complex

has a different ellipticity at 262 nm than that of HSA alone, a successively increasing deviation from the base-line will be obtained until a maximum is reached. The above titration was performed until a 4–5 molar excess of diazepam over HSA was obtained in the mixing vessel.

When this technique [15] was used for the calculation of the binding constants of diazepam in the presence of oleic acid, the A and B solutions contained, in addition, equal amounts of the fatty acid.

*CD titrations with oleic acid* were performed in the same manner as above with the exception that to the solution of albumin, or albumin and diazepam (molar ratio 1:1), the same solution containing a 14.6 times molar excess of oleic acid was continuously added.

## RESULTS

The human serum albumin used contained 0.6 moles of free fatty acids per mole protein. This is a high content compared to the values published by Chen [10]. It is difficult, however, to prevent contamination by fatty acids, which may be present in test tubes, vessels etc. Extremely small amounts of these acids (about 2 $\mu$g per mg HSA) will give the molar ratio obtained here. In the present paper, the FFA content in the HSA solution was controlled before and after the experimental procedure in order to determine whether any changes had occurred. The molar ratio between FFA and HSA was found to be the same in both cases.

Figure 1a shows the CD spectra of HSA in the presence and absence of oleic acid. As can be seen, the spectrum of the complex between HSA and oleic acid (1:2.2) almost completely coincides with the pure protein spectrum. At 14.6 times molar excess of oleic acid, a more negative CD spectrum is produced between 250 and 300 nm, and the sharp deviation around 288 nm is less pronounced.

Addition of diazepam to a solution of HSA changes the CD-spectrum (Figs 1b and 2). Extrinsic Cotton effects are produced, which arise from the chromophore of the drug bound in an asymmetric environment on HSA [16]. Unbound diazepam does not show any ellipticity, and the CD spectrum of HSA is probably not affected by the binding of diazepam [7]. Thus the changes of the Cotton effects will be proportional to the concentration of the HSA–diazepam complex, as long as the rotational strength of the complex is constant, and not affected, e.g., by local conformational changes in the protein binding site.

The CD spectrum of the HSA–diazepam complex is very sensitive to the presence of oleic acid (Figs 1b and 2). At a 2.2 molar excess of the fatty acid over HSA, a strong reduction can be seen of the extrinsic Cotton effects described above, and a high molar excess of the fatty acid will completely inhibit their formation. It should be noted that the difference CD spectra in Fig. 2 are obtained by subtracting the spectra in Fig. 1a from the corresponding ones in Fig. 1b, in order to compensate for the effect of oleic acid on the intrinsic Cotton effects of HSA.

A CD-titration was carried out to determine at what molar ratio the fatty acid begins to affect the CD-spectrum of the diazepam–HSA complex. In Fig. 3, line A gives the results obtained when HSA was titrated with a HSA–oleic acid solution (molar ratio 1:14.6) at 262 nm. As expected from the qualitative spectra (Fig. 1a), there was only a minor difference at this wavelength between the final solution, with a molar ratio between HSA and the oleic acid of about 1:4, and the starting, pure HSA solution. When the experiment was repeated, however, with the only difference being that diazepam was added in equimolar amounts to HSA in both the starting and the titrating solutions, a significant change was seen (line B). As there is virtually no difference in the HSA spectrum under these conditions (line A), the reduction seen of line B must depend on a reduction of the extrinsic Cotton effects of the HSA–diazepam complex, which starts as soon as the oleic acid is mixed with the drug-albumin complex.

In order to determine whether this reduction depends on a change of the rotational strength of the HSA–diazepam complex, or on a diminished

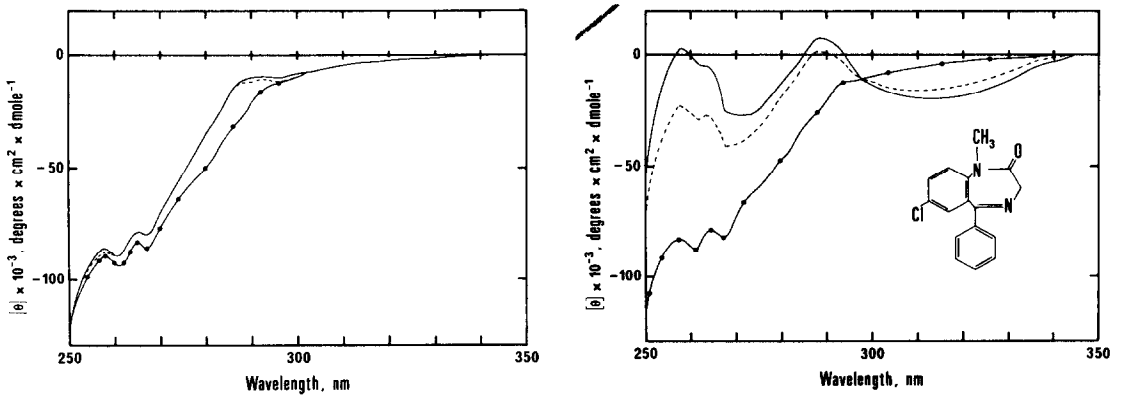


Fig. 1. (a) Circular dichroic spectra of human serum albumin (——), and of human serum albumin and oleic acid in 1:2.2 (– – –), and 1:14.6 molar ratios (●–●–●) respectively§. The protein concentration was $1.5 \times 10^{-5}$ M in 0.1 M KCl, 0.005 M sodium phosphate, pH 7.4. (b) Circular dichroic spectra of equimolar amounts of human serum albumin and diazepam in the absence (——), and presence of oleic acid in 2.2 (– – –) and 14.6 (●–●–●) molar excess§. Inserted in the figure is the chemical structure of diazepam. The protein concentration was $1.5 \times 10^{-5}$ M in 0.1 M KCl, 0.005 M sodium phosphate, pH 7.4.

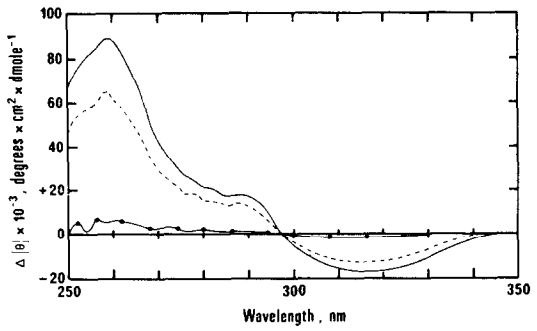


Fig. 2. Calculated difference CD spectra of the complex of human serum albumin and diazepam (molar ratio 1:1) in the absence (——), and presence of oleic acid in 2.2 (– – –) and 14.6 (●–●–●) molar excess§. These spectra were obtained from the corresponding spectra in Figs 1a and b.

binding to the protein, a number of quantitative studies were performed in the presence of different amounts of oleic acid. The results are shown in Fig. 4, where the apparent association constants of the HSA–diazepam complex have been plotted against the molar ratio of oleic acid and HSA. The quantitative determinations show that even small amounts of oleic acid will reduce the association constant of the drug to HSA. It can be noted in this context that extrapolation to the *y*-axis will give a value close to that found, when an essentially fatty acid free sample of HSA was used [7].

The 14.6 times molar excess of oleic acid over HSA used in the titrating solutions does not irreversibly affect the drug-binding properties of the protein, since a dilution of a mixture of oleate, HSA and diazepam (molar ratios 14.6:1:1) with a HSA–diazepam solution (molar ratio 1:1) to give a 2.2:1:1 molar ratio, produces the same qualitative CD-spectrum as a directly mixed solution with the same proportions between these molecules.

## DISCUSSION

The effect of a long-chain fatty acid on the binding of a benzodiazepine derivative to HSA has been studied with one analogue from each class, oleic acid and diazepam. Most probably, the results obtained with these substances could be transferred to interactions between long-chain fatty acids and benzodiazepines in general since benzodiazepines are bound to HSA primarily through one common binding site [6, 7], and oleic acid, one of the major free fatty acids occurring in blood plasma, is bound with high affinity to HSA in the same way as other long-chain fatty acids [17].

*The effect of oleic acid on albumin conformation.* Between 250 and 300 nm native HSA produces a negative CD-spectrum. Two negative maxima are obtained around 261 and 267 nm. These probably originate from phenylalanine residues in the protein [18, 19]. When oleic acid is added in a 2.2 molar excess over HSA, only minor effects can be seen on the CD-spectrum (Fig. 1a).

However, according to Soeteway *et al.* [20] small conformational changes occur in BSA when it binds long-chain fatty acids over the physiological range (0.5–2 moles fatty acids per mole albumin). The binding increases the volume of BSA but decreases its axial ratio and dipole moment. This is probably also true for HSA, and might be due to configurational adaptability as suggested by Karush, who studied the binding of small ligands to BSA and HSA [21, 22]. According to his ideas and to the induced fit theory of Koshland [23], the ligands modify the structure of the protein and create their binding sites, which are not fully preexisting.

At the higher concentration of oleic acid used, the changes in the CD-spectrum are most significant around 280–290 nm (Fig. 1a). In this wavelength region the main contributions arise from tyrosine and tryptophan residues in the protein [18]. Steinhardt *et al.* [24] have with spectroscopic techniques found that the first 2 moles of fatty acid (dodecanoic and oleic acid were studied) perturb the tyrosine absorbance spectrum of HSA. The tryptophan spectrum, however, was not affected until more than 2–3 moles of the fatty acid was bound to HSA. It is thus possible that the increased negative Cotton effects at 280–290 nm arising upon the complexation of higher amounts of oleic acid to HSA may be due to perturbations of the single tryptophan in the protein.

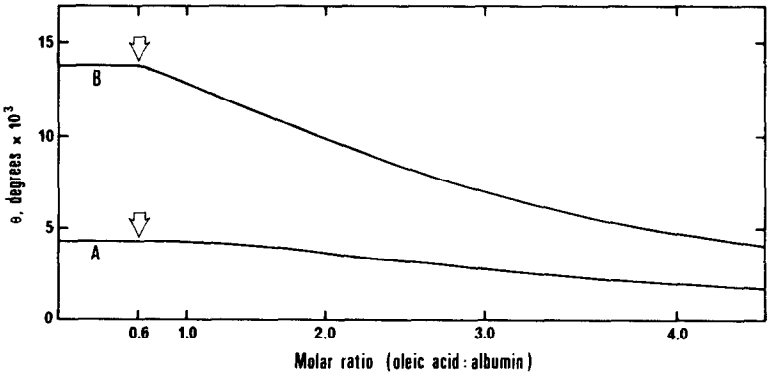


Fig. 3. The ellipticity change at 262 nm when 5 ml of a human serum albumin–oleic acid solution (molar ratio 1:14.6) was continuously added to 10.5 ml of a solution of human serum albumin (A), and a human serum albumin–diazepam solution (molar ratio 1:1) B, respectively.§ The protein concentration in all samples was $1.5 \times 10^{-5}$ M in 0.1 M KCl with 0.005 M sodium phosphate, pH 7.4. The arrows indicate the starting points of the titrations.

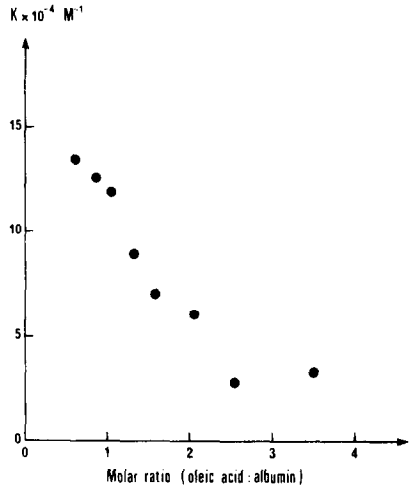


Fig. 4. Binding constants of the human serum albumin–diazepam complex in the presence of varying amounts of oleic acid§. In the absence of oleic acid the binding constant has a value of $18 \times 10^4 M^{-1}$ (ref. 7).

*The effect of oleic acid on the diazepam-albumin complex.* Most benzodiazepine derivatives give strong extrinsic Cotton effects when bound to HSA [7, 16]. Thus the CD-spectrum of the complex of diazepam and HSA differs from that of HSA alone (Fig. 1b). The difference spectrum (Fig. 2) can roughly be characterized as biphasic with a positive and a negative extrinsic Cotton effect below and above 300 nm, respectively. The positive effects have been attributed to $\pi \rightarrow \pi^*$ transitions of the aromatic A-ring in diazepam [7, 25], and the broad negative effect with a maximum around 315–320 nm is typical for those benzodiazepines, having an oxygen on their second carbon atom [7]. When oleic acid is added, the extrinsic Cotton effects are diminished. Their qualitative characteristics remain, however, until they disappear at high molar excess of oleic acid (Fig. 2).

As judged from the titration experiment (Fig. 3), the fatty acid appears to affect the CD spectrum of HSA and diazepam already at very low concentrations. This is also verified in the quantitative studies (Fig. 4), showing that oleic acid and HSA at molar ratios below 1:1 will reduce the apparent association constant for the HSA–diazepam complex.

*Comparison with earlier reports.* Other investigators have not observed that the presence of less than 1–2 moles of the fatty acids will displace ligands bound to albumin. However, Spector *et al.* [17] have suggested that the presence of 1–2 moles of palmitate per mole HSA is able to qualitatively affect the binding of chlorophenoxy-isobutyrate (CPIB) through an allosteric mechanism. CPIB shares at least one of its binding sites on the protein surface not only with a number of drugs but also with free tryptophan [26, 27]. The binding of this amino acid is competitively inhibited by diazepam [8]. It is therefore reasonable to conclude that CPIB and diazepam have one binding site in common, in addition their binding constants are of approximately equal strength. The HSA–diazepam complex, however, is both qualitatively and quantitatively affected by the presence of a long-chain fatty acid at molar ratios below 1:1. This difference may be due to the sensitivity of the techniques used, but it may also be possible that the absence of strong secondary binding sites for diazepam [6, 7] makes this substance more sensitive to displacement.

*Interaction mechanisms.* The displacement of diazepam from HSA proceeds linearly with increasing amounts of oleic acid until a 3 molar excess of the fatty acid over HSA is obtained (Fig. 3). This is confirmed by the measurements of the binding constants, which also seem to decrease proportionally to the oleic acid concentration (Fig. 4). As a linearity is found, it is probable that diazepam is displaced by oleic acid bound to a certain site on HSA. Since the displacement starts as soon as oleic acid is added to the diazepam–HSA complex, it must be the strongest bound oleic acid molecules(s) that exert(s) this effect. The association constant for the diazepam–HSA complex is $10^3$ to $10^4$ times lower than the corresponding constants for the four strongest bound oleic acid molecules [7, 28]. Therefore, the interaction between diazepam and oleic acid for their binding to HSA is probably not competitive, since this would give a more pronounced effect on the HSA-diazepam complex, resulting in considerably steeper slopes of the lines in Figs 3 and 4. An allosteric type of interaction seems more reasonable, and the findings of Spector *et al.* [17], Soeteway *et al.* [20] and Karush [21, 22], discussed above, support this.

The configurational changes produced in HSA when it binds ligands, however, probably only affect limited regions of the protein molecule. For example, no quantitative differences can be seen in the binding of bilirubin to its primary site on HSA until about 4 moles of oleate or palmitate per mole HSA are present [29]. It has also been shown that bilirubin, which has a primary binding constant approximately equal to that of oleic acid, neither qualitatively nor quantitatively interferes with the binding of benzodiazepines (e.g., diazepam) to HSA. The binding of these drugs is independent of the bilirubin binding at low molar ratios to HSA [30].

As the tertiary structure of HSA is still unknown, it is impossible to make any definite conclusions about the relative positions of the binding sites for oleic acid, diazepam and bilirubin. It is obvious, however, that the presence of small amounts of oleic acid influence the binding of diazepam to HSA, but the connections between the primary binding sites of these ligands and that of bilirubin are very weak.

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