Biochemical Pharmacology, Vol. 35, No. 6, pp. 1005–1009, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

EVIDENCE FOR DISTINCT CONSECUTIVE STEPS IN THE NEUTRAL TO BASE TRANSITION OF HUMAN SERUM ALBUMIN

BETSY J. 'T HART,*† JAAP WILTING* and JOHAN J. DE GIER‡
* Department of Pharmaceutical Chemistry, Subfaculty of Pharmacy, State University of Utrecht, Catharijnesingel 60, 3511 GH Utrecht, The Netherlands, and ‡ Department of Pharmacotherapy, Subfaculty of Pharmacy, State University of Utrecht, Vondellaan 14, 3521 GE Utrecht, The Netherlands

(*Received* 15 *July* 1985; *accepted* 30 *September* 1985)

Abstract—The neutral to base transition in human serum albumin has been studied by means of circular dichroism and equilibrium dialysis using nitrazepam as the non-covalently bound circular dichroic probe. Spectroscopically two transitions were observed over the pH range 5.5–8.5, whereas dialysis data point to only one transition occurring in the latter part of the pH region studied. Both pH dependences could only be correlated if it is assumed that the neutral to base transition consists of at least two distinct consecutive steps.

Human serum albumin is known to have at least two distinct binding sites for a large number of strongly bound drugs, the so-called warfarin site, or site I, and the diazepam site, or site II [1–4].

Several studies [5–7] have shown that the binding of drugs to both site I and site II is pH dependent over the pH range 6–9. This pH dependence [5–7] is attributed to a conformational change of human serum albumin, the so-called neutral to base or N–B transition.

The pH profiles of the binding constant K of drugs such as warfarin [6] and diazepam [5] to albumin and of the molar ellipticity $[\theta]$ of the corresponding complex can be reasonably described by a simple two-state model [5, 6]. The model consists of two states N and B, with pH independent binding constants K_N and K_B and pH independent molar ellipticities $[\theta]_N$ and $[\theta]_B$ of the drug–albumin complex [6]. The pH profile of K cannot be described satisfactorily in all cases with the simple two-state model, particularly around the physiological pH where $dK/d\text{pH}$ has its maximum value [8, this paper]. This may indicate that the simple two-state model needs to be improved and extended. The introduction of distinct consecutive steps into the N–B transition is a possible way of effecting this improvement.

Recently [9, 10] it has been observed that fatty acids introduce heterogeneity into the N and the B conformation such that more than one step is possible in the N–B transition. Support for at least two distinct consecutive steps in the N–B transition has also been found in kinetic studies [11, 12]: at least two intermediates were found in the pH range 6–9. In this paper we present evidence supporting the existence of an intermediate conformation of human serum albumin in the N–B transition. The benzodiazepine, nitrazepam, was used as the non-covalently bound and circular dichroic label.

MATERIALS AND METHODS

Human serum albumin (lot no. 310 071), a 20% solution in water, was a gift from Biotest (Frankfurt, F.R.G.). The albumin solution was deionized as described by Wilting *et al.* [6]. The drug to protein ratio is defined as $r = [\text{drug}]/[\text{protein}]$. The nitrazepam to protein ratio was $r = 6.10^{-4}$ for the equilibrium dialysis and $r = 6.10^{-2}$ for the circular dichroic experiments.

Equilibrium dialysis experiments were performed with a Dianorm equilibrium dialyser (Diachema A.G., Ruschlikon, Switzerland) using membranes and procedure as described previously [5].

The free nitrazepam concentrations (C^*_{free}) obtained after the equilibrium dialysis procedure were determined by gas chromatography, after nitrazepam had been converted into *N*-butyl-nitrazepam [13].

The initial free concentrations (C_{free} = concentration before the equilibrium dialysis procedure) were calculated using the following equation:

$$\frac{C_{\text{tot}} - C_{\text{free}}}{(C_p - C_{\text{tot}} + C_{\text{free}})C_{\text{free}}} = \frac{C_{\text{tot}} - C^*_{\text{free}}(V_1 + V_2)/V_1}{(C_p - C_{\text{tot}} + C^*_{\text{free}}(V_1 + V_2)/V_1)C^*_{\text{free}}}$$

where C_{free}, C^*_{free} is the concentration of the free drug before (respectively after) the equilibrium dialysis procedure; C_{tot} is the total drug concentration; C_p is the protein concentration; V_1 is the volume of the drug–protein compartment; V_2 is the volume of the free drug compartment.

The "correction" of C^*_{free} to C_{free} was necessary, since due to the relative low affinity of nitrazepam to albumin [14–16] the equilibrium between drug free and drug bound shifts considerably in favour of the drug free during the dialysis procedure. The amount of free drug (175 pmol at pH 7.4 in

---

† To whom correspondence should be addressed.

phosphate) which is passed through the membrane from the protein to the buffer compartment during the equilibrium dialysis procedure cannot be neglected compared to the total amount of drug molecules (710 pmol) in the protein compartment.

The circular dichroic spectra of nitrazepam–albumin complexes were recorded between 360 nm and 300 nm using a dichrograph III (Jobin Yvon, Long Jumeau, France). The slit was programmed for a half-band width of 2 nm (sensitivity = 10^{-6} degree/mm, scanning speed = 3 nm/min, time constant = 10 sec). A rectangular cell of 1 cm path-length was used. The nitrazepam concentration in the CD experiments was 10 mg/l (35.5 μM). The observed ellipticities are the differences between CD spectra of the nitrazepam–albumin mixtures and those of the albumin alone at a given wavelength and are corrected for incomplete binding of the drug to albumin. Molar ellipticities $[\theta]$ were calculated from θ_{obs} using the equation:

$$[\theta] = 100 \times \theta_{obs}/(l \times C) \qquad (1)$$

where l is the path-length in cm and C the molar concentration of nitrazepam–albumin complex.

The results of equilibrium dialysis and circular dichroism were fitted to a two state model, consisting of two conformational states N and B with pH-independent K_N and K_B, $[\theta]_N$ and $[\theta]_B$ values [6]. The albumin as assumed to be completely in the neutral (N) form at pH 5.5 and in the basic (B) form at pH 9.5. The fraction f at some selected pH values of the albumin in the basic (B) form was calculated from the $[\theta]$-pH curve (Fig. 2). The theoretical pH profile of the free concentration of nitrazepam was calculated from their f values and the experimentally found C_{free} values at the extreme pH ($C_{free,N}$ when albumin is in the N form, $C_{free,B}$ when albumin is in the B form) with the equation:

$$\frac{l}{C_{free,pH}} = \frac{l-f}{C_{free,N}} + \frac{f}{C_{free,B}} \qquad (2)$$

The equation is only valid if $K \cdot C_{free} \ll 1$ [6].

RESULTS

Table 1 shows the free nitrazepam concentration in a 4% albumin solution ($r = 6.10^{-4}$) for various compositions of the dialysing fluid at pH 7.4. The free and the bound fractions of nitrazepam and the total nitrazepam concentration are given as well.

It is clear that the binding of nitrazepam to albumin is slightly reduced when chloride is added to the fluid. The subsequent addition of calcium causes a further decrease of the binding from 83% to 75%.

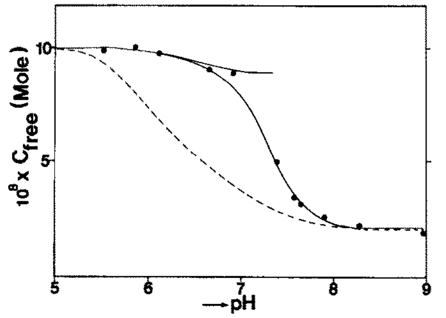

Fig. 1. Free concentrations of nitrazepam (C_{free}) as a function of the pH. The dots represent the experimentally found C_{free}, the dotted line is the theoretical C_{free} assuming one transition and the solid line is the C_{free} assuming two transitions are observed in the pH range 5.5–5–9. Conditions: phosphate $I = 0.1$; [albumin] = 6.10^{-4} M; $r = 5.9\ 10^{-4}$ M; temperature 37°.

Similar results were found for the benzodiazepines, diazepam [5] and *N*-desmethyldiazepam [17].

Several investigators studied a pH dependent conformational change that occurs in serum albumin at approximately physiological pH. This so-called N–B transition affects the binding of several drugs, e.g. warfarin [7], diazepam [5] and *N*-desmethyldiazepam [17]. In the case of warfarin the calcium-induced changes in C_{free} could be explained simply by a shift in the N–B equlibrium in favour of the B form. With diazepam, however, the relation is more complex. At the extreme pH values when albumin is almost completely in the N or B conformation, the free diazepam concentration [5] is still calcium dependent. Since calcium ions bind to albumin De Gier *et al.* [18] suggested that the observed effects must be due to a competition between diazepam and calcium for the same binding site on albumin.

The pH dependence of the free nitrazepam concentration when phosphate is used as a dialysis fluid is shown in Fig. 1. When 0.1 M Cl^- or 0.1 M Cl^- + 2.5 mM Ca^{2+} is used as dialysis fluid pH profiles with similar shape are obtained (not shown).

The C_{free} of nitrazepam decreases from 90 nM to 25 nM over the pH range investigated in the case of 0.1 M phosphate (Fig. 1) and in the case of 0.1 M Cl^-. When 0.1 M Cl^- + 2.5 mM Ca^{2+} is used as the dialysis fluid the C_{free} of nitrazepam varies from 90 nM to 60 nM. At pH 6.0, when nearly all the albumin is in the N conformation, the C_{free} of nitrazepam is therefore not sensitive to the composition of the dialysis fluid. At pH 9.0 however, the C_{free} of nitrazepam when the albumin is mainly in the B conformation, is 25 nM for both 0.1 M phosphate

Table 1. The binding of nitrazepam in albumin solution at pH 7.4, temperature 37° in various compositions of the buffer solution

	C_{free} (nM)	C_{tot} (nM)	% Free	% Bound
Phosphate $I = 0.1$	48 ± 2.3	355	14 ± 1	86 ± 1
0.1 M Cl^-	60 ± 2.8	355	17 ± 1	83 ± 1
0.1 M Cl^- + 2.5 mM Ca^{2+}	88 ± 4.8	355	25 ± 1	75 ± 1

Phosphate buffer composition = 78.4 ml 0.5 M KH_2PO_4 + 60.8 ml 0.5 M NaOH diluted to 1.1.

Table 2. The (apparent) binding constant K of nitrazepam albumin at pH 5.5, 6.9 and 8.9 under various conditions

Condition	K (M^{-1}) × 10^3		
pH	5.5	6.9	8.9
Phosphate $I = 0.1$	5.9 ± 0.2	6.7 ± 0.3	30.0 ± 2.5
0.1 M Cl^-	5.0 ± 0.1	6.6 ± 0.5	25.0 ± 1.1
0.1 M Cl^- + 2.5 mM Ca^{2+}	5.6 ± 0.3	6.7 ± 0.4	10.0 ± 1.1

Phosphate buffer composition = 78.4 ml 0.5 M KH_2PO_4 + 60.8 ml 0.5 M NaOH diluted to 1 l.

and 0.1 M Cl^- but 60 nM for 0.1 M Cl^- + 2.5 mM Ca^{2+}. This suggests that calcium affects the binding of nitrazepam to albumin only when albumin is in the B conformation.

The binding constant K of nitrazepam was calculated, on the assumption that there was only one high affinity binding site. Since all experiments were carried out at a very low drug to protein ratio ($r = 6.10^{-4}$) K could be obtained from ν/C_{free}, where ν is the molar ratio of bound drug and albumin. The K values of nitrazepam at certain selected pH values are summarized in Table 2. The binding constants found are in very good agreement with reported values [14–16]. The free concentration was on the average 6.10^{-8} M. This means that $K \cdot C_{\text{free}}$ is about 7.10^{-4}. So equation (2) can be used to calculate C_{free} values between pH 5.5 and 8.5. Equation (2) can only be used if, as explained in Materials and Methods, the [θ]–pH curve is available. Figure 2 shows the pH dependence of [θ] of the nitrazepam–albumin complex for a phosphate buffer as the dialysis fluid. When other compositions of the dialysis fluid are used plots with a similar shape were obtained (not shown). The molar ellipticity [θ] decreases when pH increases from 5.5 and 8.5, whereas [θ] is constant outside this range. An attempt is made to correlate the pH dependences of [θ] and K by using the simple two-state model (see Materials and Methods). The theoretical C_{free}–pH curve can be calculated with equation (2). The C_{free} is obtained from these calculations (dashed line in Fig. 1) do not fit satisfactorily with the experimentally found C_{free} (dots in Fig. 1). Both pH profiles of [θ] and C_{free} could only reasonably be correlated if there were two consecutive transitions between pH 5 and 9: the first from approximately pH 5.5–7 and the second from pH 7–9. The theoretical C_{free}–pH curves for the two transitions are represented as solid lines in Fig. 1. The assumed intermediate in the N–B transition will be denoted by X in the further text. The pH profiles of K and [θ] for the other compositions of the dialysis fluid could also be correlated with one another if such an intermediate X was assumed. In order to be able to say something about the cooperative nature of the intermediate steps in the N–X–B transition, we calculated the Hill coefficient n and the mid-point pH (pH_{50}) of the transitions. For the diazepam–albumin complex it was found that the Hill coefficient n varies from 0.8 to 2.6, dependent of the ionic content of the dialysis solution [5]. However, Table 3 shows that for the overall N–B transition of the nitrazepam–albumin complex n = 0.7–0.8, irrespective of the ionic content of the dialysis fluids. This indicates that nitrazepam suppresses the cooperative nature in the binding of protons to albumin. Similar results were reported for N-desmethyldiazepam [17]. For the N–X transition the Hill coefficients were also close to 1. The Hill coefficients for the X–B transition, however, are significantly above 1. This means that more than one proton is involved in the X–B transition.

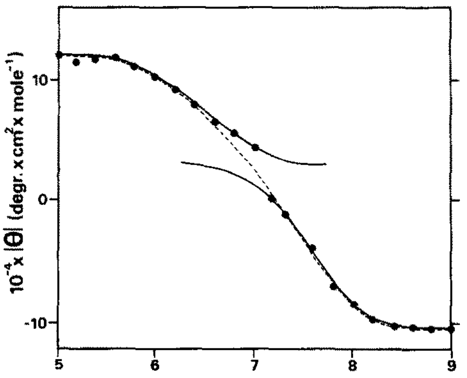

Fig. 2. The molar ellipticity $[\theta]_{330}$ of the nitrazepam–albumin complex as a function of the pH. Condition: $r = 5.9$ 10^{-2} M. Other conditions as in Fig. 1.

DISCUSSION

Earlier evidence has shown that albumin can be regarded as an allosteric protein [19]. Its cooperative nature has its origin in the N–B transition, which is a pH dependent phenomenon.

By analogy with the R–T transition in haemoglobin where oxygen acts as the ligand [20], it is the protons involved in the N–B transition which are responsible for the cooperative behaviour of albumin.

From previous results [19] it is known that about 5 protons are involved in the N–B transition. The N–B transition is a pH dependent process. The N–B equilibrium shifts with increasing pH in favour of the B conformation which is caused by the dissociation of the protons of ionisable groups of the human serum albumin. Therefore the N–B transition can be represented schematically as follows:

$$NH_z \rightarrow \ldots \rightarrow NH_y \rightarrow BH_{y-1} \rightarrow \ldots \rightarrow BH \rightarrow B$$

where only those protons z are given which are involved in the N–B transition $(0 \leqslant y \leqslant z)$. The net charge of the several states is omitted.

Table 3. The Hill coefficient n and the mid-point pH, pH_{50} of the nitrazepam–albumin complex of the "overall" N–B, N–N† and N†–B transition under various conditions of the dialysis solution

	N–B		N–N†		N†–B	
	n	pH_{50}	n	pH_{50}	n	pH_{50}
Phosphate $I = 0.1$	0.8	7.1	1.1	6.6	2.0	7.6
0.1 M Cl^-	0.7	7.1	0.9	6.4	1.9	7.8
0.1 M Cl^- + 2.5 mM Ca^{2+}	0.7	7.2	1.1	6.4	1.8	7.8

It can be stated that the conformational change from the N to the B form is triggered by the release of the proton in the step $NH_x \rightarrow BH_{x-1}$.

With a simple two-state model it is possible to describe reasonably the pH dependent binding of some drugs to albumin [5–7]. It follows that the binding constant K of the drug and the molar ellipticity $[\theta]$ of the drug–albumin complex are only functions of the conformational state. Thus although the net charge of albumin varies with pH, K and $[\theta]$ are independent of the number of bound protons to albumin. Therefore we concluded that the simple two state model of the N–B transition contained the following implicit assumptions:

$$K_{NH_n} = \ldots = K_{NH_x}$$
$$[\theta]_{NH_n} = \ldots = [\theta]_{NH_x}$$
$$K_{BH_{x-1}} = \ldots = K_{BH} = K_B$$
$$[\theta]_{BH_{x-1}} = \ldots = [\theta]_{BH} = [\theta]_B$$

From previous studies of the N–B transition it is clear that the simple two-state model can lead to reasonable results, but that the results obtained around the physiological pH, where the dK/dpH has its maximum value [8] are rather poor. It is therefore more likely that the various N and B states do not have the same K and $[\theta]$. This may be the case particularly when protons are released in the direct vicinity of the binding site.

As shown in the Results section, the C_{free}–pH curve for the binding of nitrazepam to albumin cannot be described with the simple two-state model. This cannot be attributed to a change in ionization state of nitrazepam over the pH range investigated, since the pK_a values of this drug are 3.2 and 10.8 [21]. Thus the drug molecule is uncharged over the pH range 6–9. There must be at least two distinct consecutive transitions, one between 5.5 and 7 and the other between 7 and 9, for a good fit of the experimentally obtained C_{free}. When the pH profiles of C_{free} and $[\theta]$ (Figs. 1 and 2) are compared, it should be noticed that over the pH range 5.5–7, where C_{free} does not change markedly, $[\theta]$ decreases, whereas over the pH range 7–9 both C_{free} and $[\theta]$ decrease. From this it can be concluded that there must be at least one intermediate conformation X for which the $[\theta]$ value is intermediate between the $[\theta]$ values of the extreme conformations. It is proposed that X can be N† or B†, which means that X has N-like or B-like properties.

The experimental results of the binding of nitrazepam to albumin (C_{free}) show that the N conformation and X have the same binding characteristics. The $[\theta]$ of the N and X conformation, however, have different values. It is known [5, 7] that the $[\theta]$ of the drug–albumin complex is very sensitive to environmental influences and therefore can have a variety of values, particularly when the protein is in the N conformation. Moreover the $[\theta]$ value of the drug–albumin complexes show a larger variation than K of the corresponding association [5, 7, 17]. Furthermore it is known [6] that the binding properties change when an "overall" conformational change occurs. These arguments support the view that the intermediate is an N† and that therefore the conformational changes can be described by N–N†–B.

We already mentioned that the N and N† should have a different number of bound protons, which means that they should have a different net charge on the surface area. On the basis of our results nothing can be said about the different number of protons bound to N and N†. Since the Hill coefficient for the N†–B transition is about 2, more than 2 protons must be involved in this transition. It is likely that the proton(s) released in the N–N† transition are bound in the direct vicinity of the binding site.

The existence of intermediates in the N–B transition in which the number of bound protons differs from those in the N and B conformation, was also detected by Jacobsen and Faerch [11, 12] using a stopped-flow technique.

Dröge *et al.* [10] and Wanwimolruk and Birkett [9] found evidence for a fatty acid induced heterogeneity in the N–B transition, which they denoted as an N*–B* transition in the presence of fatty acids. Dröge also showed that in the N–N* and B–B* transitions protons are involved [10], which make these processes also pH dependent.

Whether or not the nature of the fatty acid induced conformotional state N* is similar to that of N† we shall now discuss.

Dröge *et al.* [10] observed that $[\theta]$ of the N* and B* complexes were larger than the $[\theta]_N$ and $[\theta]_B$. They found that the reversal of the binding constants K_{N^*} and K_{B^*} were also larger than the reversal of K_N and K_B. This is in contrast with N†, which binds nitrazepam with the same binding constant as N.

At first glance the foregoing results point to a difference in the nature of N* and N†: which can mean a different amount of fatty acids is bound [22]. However, for several batches of albumin samples a maximum in the pH profiles of $[\theta]$ was found [8]. Therefore a similarity in the nature of N* and N† cannot be ruled out.

The distinct consecutive steps found in the N–B transition can be summarized. Thus: N†, N* and the

N conformation all have a different number of bound protons. On the basis of our results no similarity between $N^{\dagger}$ and N^{*} is expected. Both the $N\text{–}N^{\dagger}\text{–}B$ and $N\text{–}N^{*}\text{–}B$ transitions are pH dependent processes.

Acknowledgements—The authors would like to thank Mrs S. M. McNab for correcting the English text. They also would like to express their gratitude to the Biotest-Serum-Institut (Frankfurt am Main, F.R.G.) for the gifts of human serum albumin.

REFERENCES

1. R. Brodersen, T. Sjödin and I. Sjöholm, *J. biol. Chem.* **252**, 5067 (1977).
2. K. J. Fehske, W. E. Müller and U. Wollert, *Biochim. biophys. Acta* **577**, 346 (1979).
3. G. Sudlow, D. J. Birkett and D. N. Wade, *Molec. Pharmac.* **12**, 1052 (1976).
4. W. E. Müller and U. Wollert, *Pharmacol.* **19**, 59 (1979).
5. J. Wilting, B. J. 't Hart and J. J. de Gier, *Biochim. biophys. Acta* **626**, 291 (1980).
6. J. Wilting, W. F. van der Giesen, L. H. M. Janssen, M. M. Weideman, M. Otagiri and J. H. Perrin, *J. biol. Chem.* **255**, 3032 (1980).
7. J. Wilting, M. M. Weideman, A. C. J. Roomer and J. H. Perrin, *Biochim. biophys. Acta* **579**, 469 (1979).
8. W. F. van der Giesen and J. Wilting, *Biochem. Pharmac.* **33**, 1679 (1984).
9. S. Wanwimolruk and D. J. Birkett, *Biochim. biophys. Acta* **709**, 247 (1982).
10. J. H. M. Dröge, L. H. M. Janssen and J. Wilting, submitted for publication.
11. J. Jacobsen and T. Faerch, *Biochim. biophys. Acta* **623**, 199 (1980).
12. J. Jacobsen and T. Faerch, *Biochim. biophys. Acta* **670**, 124 (1981).
13. J. J. de Gier and B. J. 't Hart, *J. Chromatogr.* **163**, 304 (1979).
14. W. Müller and U. Wollert, *Naunyn-Schmiedeberg's Arch. Pharmac.* **280**, 229 (1973).
15. W. Müller and U. Wollert, *Naunyn-Schmiedeberg's Arch. Pharmac.* **283**, 67 (1974).
16. T. Sjödin, N. Roosdorp and I. Sjöholm, *Biochem. Pharmac.* **25**, 2131 (1976).
17. J. Wilting, B. J. 't Hart and J. J. de Gier, submitted for publication.
18. J. J. de Gier, B. J. 't Hart and J. Wilting, *Meth. and Find. exp. clin. Pharmac.* **5**, 49 (1983).
19. L. H. M. Janssen, M. T. Wilgenburg and J. Wilting, *Biochim. biophys. Acta* **669**, 244 (1981).
20. J. Monod, J. Wyman and J. P. Changeux, *J. mol. Biol.* **12**, 88 (1965).
21. J. M. Clifford and W. F. Smyth, *Analyst* **99**, 241 (1974).
22. B. J. M. Harmsen, S. H. de Bruin, L. H. M. Janssen, J. F. Rodrigues de Miranda and G. A. J. van Os, *Biochem.* **10**, 3217 (1971).