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The effects of pH, calcium and chloride ions on the binding of benoxaprofen to human serum albumin. Circular dichroic and dialysis measurements

J. Fleitman and J.H. Perrin

College of Pharmacy, University of Florida, Gainesville, FL 32610 (U.S.A.)

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Summary

The binding of the new non-steroidal anti-inflammatory benoxaprofen to human serum albumin has been shown by circular dichroism and dialysis to be dependent on the N → B conformational change of the albumin. Chloride ions appear to displace the drug from its binding site. Although calcium ions increase the B-fraction of albumin they cause a decrease in affinity of the drug for albumin, probably by competing for the imidazole residues at the binding site.

Introduction

Leonard and Foster (Leonard et al., 1963), using optical rotation studies at 313 nm, first discovered a conformational change in HSA in the pH range of 7–9. This wavelength was chosen because it monitors change in tertiary structure, perhaps primarily alterations in aromatic chromophores. This conformational change is now commonly referred to as the N → B transition, the N-form being favored in neutral conditions and the B- at higher pHs.

Histidine, whose imidazole residues have a pK_a of 6.4–7.0, seems to be involved in this transition, and it is hypothesized that 10 imidazolium residues are 'hidden' in the N-form and become available in the B-form (Harmsen et al., 1971). At physiological pHs, albumin can exist in two forms, the N- and the B-form.

In a phosphate buffer at pH 7.4 human serum albumin (HSA) is 50% in the B-form; however, the addition of low concentration of Ca^{2+} ions greatly increases the percentage of the B-form at the physiological pH (Wilting et al., 1980). Chloride

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ions have a much smaller effect on the equilibrium but again favor the B-form.

It is thought that Ca^{2+} ions compete with imidazolium residues for carboxylate binding sites (Pedersen, 1972). These studies also show that calcium ions react reversibly with 12 of the 16 imidazole groups on HSA with the calcium binding increasing over this pH range.

The mechanisms of the N → B transition are not clearly defined. The amount of helical structure involved in the N → B transition is small and is almost unchanged by temperature (Wallevik, 1972). The apparent loss of α-helical structure in the unfolding between pH 7 and 9 is 2.5%. It could also be concluded that the N → B transition causes a pK shift of imidazole groups with protons being released in the neutral regions. The highest pK was found in the low pH conformation suggesting that several histidyl residues are involved in salt bridges (Harmsen et al., 1971). In the presence of calcium ions, the affinity of albumin for protons decreases, resulting in a shift to the neutral state.

Until recently no workers had shown the involvement of the N → B transition in drug–HSA interactions. The first work reported was with warfarin (Wilting et al., 1979, 1980) and the drug has an increased affinity for HSA in the B-form. Calcium ions also increase the affinity of the drug for HSA, presumably by altering the conformation, but the chloride ions, at high concentrations, decrease the affinity by a competitive mechanism.

At the start of this work on benoxaprofen it was expected to make the first report on the effect of the N → B transition on the binding of a drug which goes to the second major binding site (i.e. the non-warfarin site) on HSA; however, recently a paper on the diazepam–HSA interaction has been published which again showed an increased affinity for the drug when HSA is in the B-form; however, Ca^{2+} ions decreased the affinity of the drug for HSA (Wilting et al., 1980b).

A possible physiological importance of the N → B conformation change is exemplified by the pericellular pH in the liver being lower than that in the main blood stream. This means that there is a shift to the N-form of HSA in the liver, resulting in the local release of drugs like warfarin and diazepam. It also means that the free fractions of warfarin available for metabolism and pharmacological action are not accurately estimated from plasma sample determinations. Slight changes in blood pH can also be expected to alter free warfarin concentrations dramatically. A shift in pH from 7.4 to 7.0 almost doubles the free warfarin concentration in the presence of calcium ions. These changes can be expected when a patient has diabetes mellitus or severe renal failure.

In the present work the effect of pH, Ca^{2+} ions, and Cl^- ions on the benoxaprofen–HSA interaction are investigated by dialysis amd circular dichroism.

Materials and methods

Human serum albumin, fraction V (lot no. 30F-02271) was obtained from Sigma Chemicals (St. Louis, MO). Sodium benoxaprofen (903.5 mg free acid/g) was supplied by Eli Lilly (Indianapolis, IN). Monobasic, dibasic sodium phosphate and

sodium chloride, analytical reagent grade, were obtained from Mallinckrodt (St. Louis, MO). Calcium chloride dihydrate, A.C.S. grade, was obtained from Fisher Scientific (FairLawn, NJ). Dowex 50W-X8 cation exchange resin and 1-X8 anion exchange resin were supplied by J.T. Baker Chemicals (Phillipsburg, NJ).

Albumin was deionized as previously described (Fleitman and Perrin, 1981) and its molecular weight was taken to be 66,500 g/mol. Concentrations of HSA were determined gravimetrically and spectroscopically.

Equilibrium dialysis was performed using the Dianorm equilibrium dialyzer (Diachema, A.G. Ruschlikon, Switzerland) with teflon cells of 10 ml total volume. Free concentrations of benoxaprofen were determined by HPLC as previously described (Fleitman et al., 1980).

Circular dichroic (C.D.) measurements were made using a Jasco model J-500 spectropolarimeter (Jasco International, Tokyo, Japan). The extrinsic benoxaprofen–HSA signal was measured at a slit width of 1 nm, wavelength expansion of 5 nm · cm^{-1}, chart speed 1 cm · min^{-1}, time constant 32 s and a sensitivity of 0.5 or 1.0 m^{0} · cm^{-1}. Pathlengths of 2, 5 and 10 mm were used. Observed ellipticities (θ_{obs}) are the actual C.D. spectra of the benoxaprofen–albumin complex while the induced ellipticity is the observed ellipticity of the complex minus the ellipticity of albumin alone at the corresponding wavelength. The dynode voltage was kept below 0.5 in all experiments. Measurement were made at room temperature (22°C).

Results and discussion

Prior to beginning the study of the pH-dependence of benoxaprofen binding, it was necessary to determine if changes in total phosphate concentration affected the N → B transition. Equilibrium dialysis was used to study the binding of a 1 : 1 ratio of benoxaprofen–HSA in phosphate buffer concentrations of 0.05, 0.10, 0.20 and 0.30 M at pH 7.4 and 25°C. The data of Table I indicate that there would only be a problem if the concentration of the buffer was below 0.1 M. It therefore seemed feasible to use phosphate buffers over the pH range of the N → B transition.

The binding of aromatic propionic acid derivatives has been studied by Perrin

TABLE I

EFFECT OF PHOSPHATE CONCENTRATIONS ON THE FREE CONCENTRATIONS EXPRESSED AS PEAK HEIGHT RATIOS, OF BENOXAPROFEN FROM DIALYSIS AT 25°C AND pH 7.4. [HSA] = 2.5×10^{-4} M = BENOXAPROFEN.

Phosphate buffer conc. (M)	$\bar{X} \pm$ S.D. (N = 5)
0.05	1.50 ± 0.09
0.10	1.34 ± 0.11
0.20	1.31 ± 0.03
0.30	1.26 ± 0.07

(1973) using circular dichroism. It appears that the extrinsic Cotton effects of the benoxaprofen–HSA complex arise as a result of hydrophobic binding of the aromatic rings and hydrogen bonding of the carbonyl and ether oxygen to HSA. No ellipticity is observed from the commercial racemic benoxaprofen, namely [2-(4-chlorophenyl)-4-oxazole]acetic acid, indicated that the molecule failed to exhibit an extrinsic Cotton effect with HSA. It was postulated that the absorption maximum of this compound was at too low a wavelength (281 nm) to distinguish the extrinsic ellipticity of the complexes from the intrinsic ellipticity of the albumin (Mitchell and Rosen, 1978). The extra phenyl ring in benoxaprofen gives it an absorption maximum at 309 nm. This, plus its added planar rigidity, probably gives rise to the measurable Cotton effects of the benoxaprofen–HSA complexes.

Benoxaprofen, when bound to HSA, gives rise to an extrinsic Cotton effect with a positive maximum near 303 nm and a negative minimum near 333 nm. The position

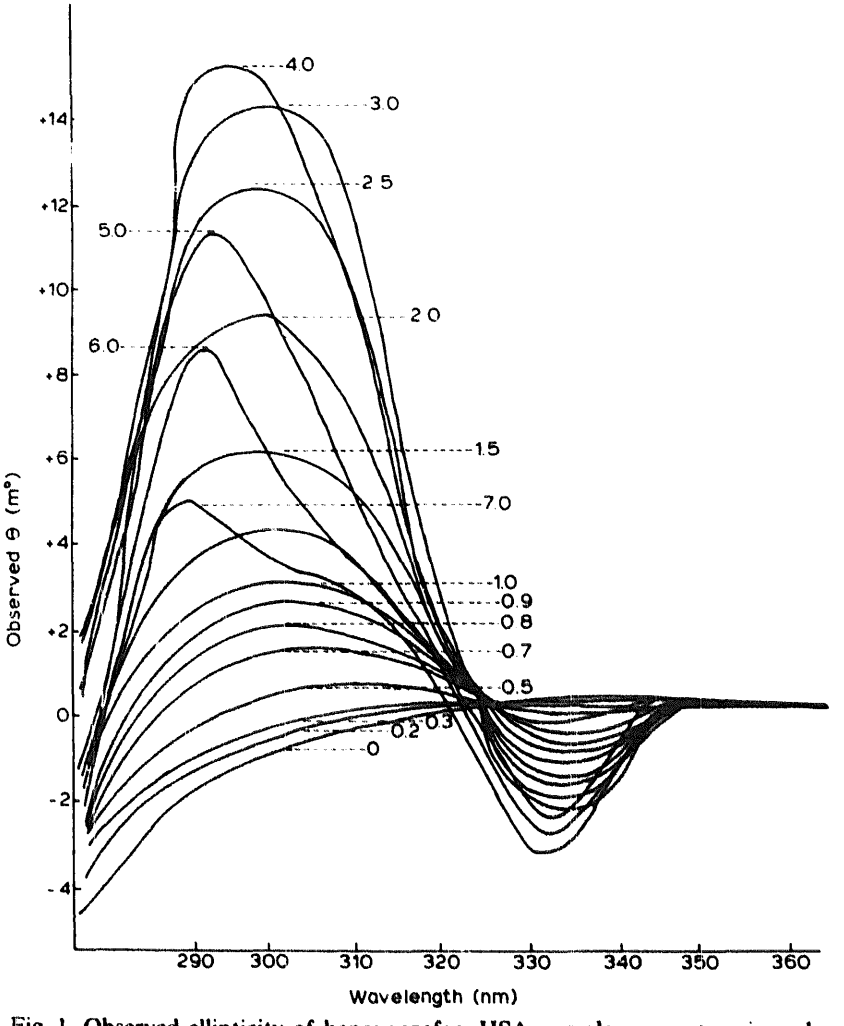

Fig. 1. Observed ellipticity of benoxaprofen–HSA complexes as at various drug-to-protein ratios at pH 7.4 in 0.5 cm cells. [HSA] = 5.0×10^{-5} M. Drug-to-protein ratios are shown on the curves.

of the peaks shift as the HSA becomes saturated as is shown in Fig. 1. The isosbestic point at 325 nm is maintained throughout the titration at low drug-to-protein ratios, suggesting a single binding site of high affinity. When the drug-to-protein ratios exceed 1:1 the isosbestic point is lost and at 3:1 the positive maximum shifts to shorter wavelengths and decreases in magnitude. This phenomenon is easier to see in Fig. 2 where the data at a wavelength of 300 nm are plotted against the drug-to-protein ratio.

The Scatchard plot reported in the previous paper indicated that there were many binding sites for benoxaprofen on HSA. The nature of the curve in Fig. 2 is consistent with the hypothesis of the ellipticity arising following interactions with at least 3 binding sites. The first interaction leads to a small positive induced ellipticity, the second to a larger ellipticity of the same sign and the third to a large ellipticity of negative sign. This is similar to that observed for the warfarin–HSA interaction (Wilting et al., 1979, 1980) and the indomethacin–HSA interaction (Ekman et al., 1980). Similar plots at pH 6.5 and 9.2 are shown in Figs. 3 and 4. At pH 6.5 the HSA is predominantly in the N-form and the ellipticities are much larger when HSA is in the N-form. The geometry of the site leading to the generation of the negative ellipticity is completely altered in the B-form resulting in the loss of its negative ellipticity as shown in Fig. 4. These changes might result from the unfolding of the HSA in the B-form (Wallevik, 1972) and the change in the ionization states of the imidazole groups (Harmsen et al., 1971). This conformation may favor a more rigid fit of the benoxaprofen at its third binding site resulting in a positive ellipticity. The

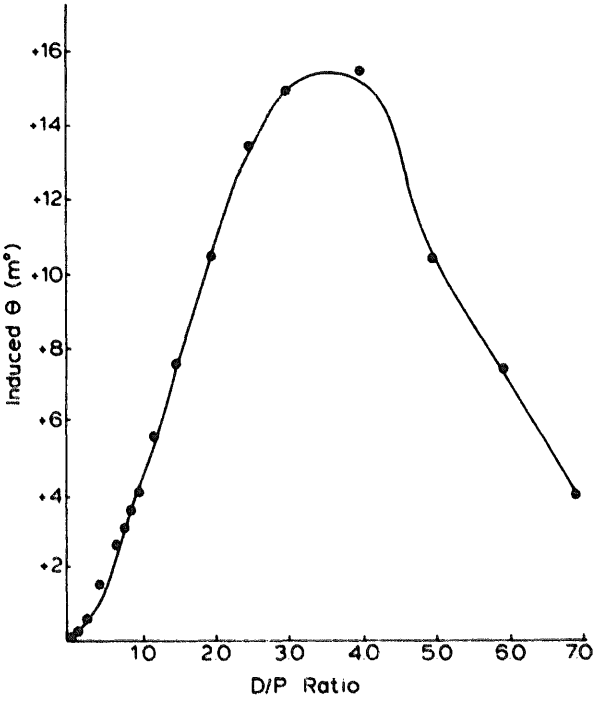

Fig. 2. Induced ellipticity of benoxaprofen–HSA complexes, at pH 7.4 in 0.1 M phosphate buffer [HSA] = 5.0×10^{-5} M. 0.5 cm cell at 300 nm.

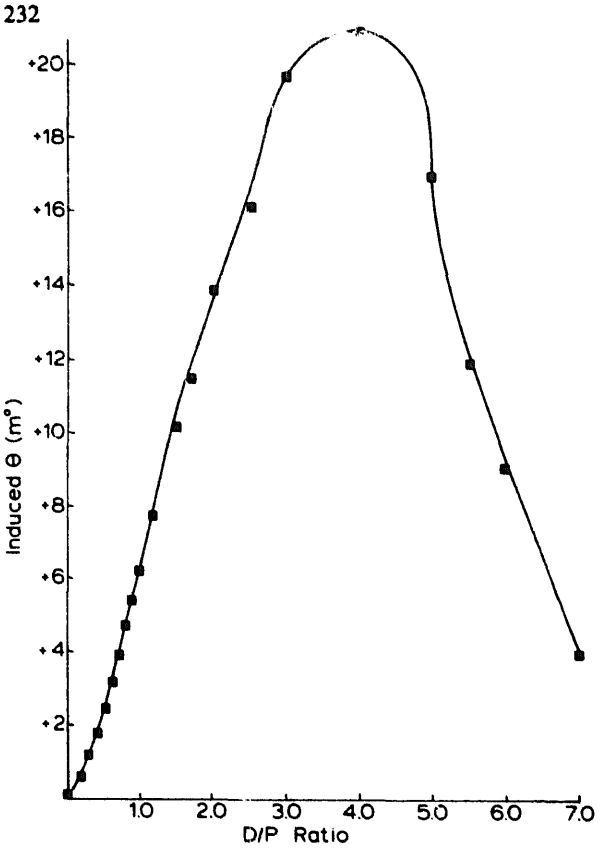

Fig. 3. Induced ellipticity of benoxaprofen–HSA complexes at pH 6.5 in 0.1 M phosphate buffer. [HSA] = 5.0×10^{-5} M. 0.5 cm cell at 300 nm.

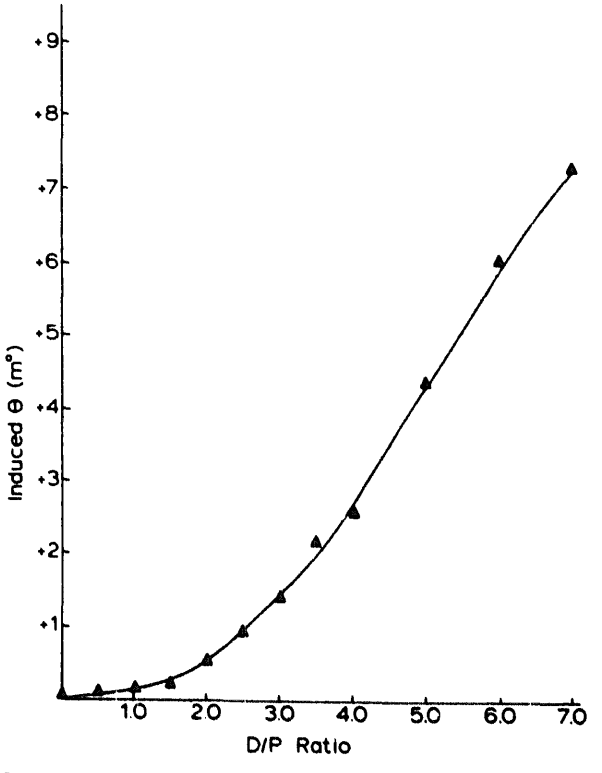

Fig. 4. Induced ellipticity of benoxaprofen–HSA complex at pH 9.2 in 0.1 M phosphate buffer. [HSA] = 5.0×10^{-5} M. 0.2 cm cell at 300 nm.

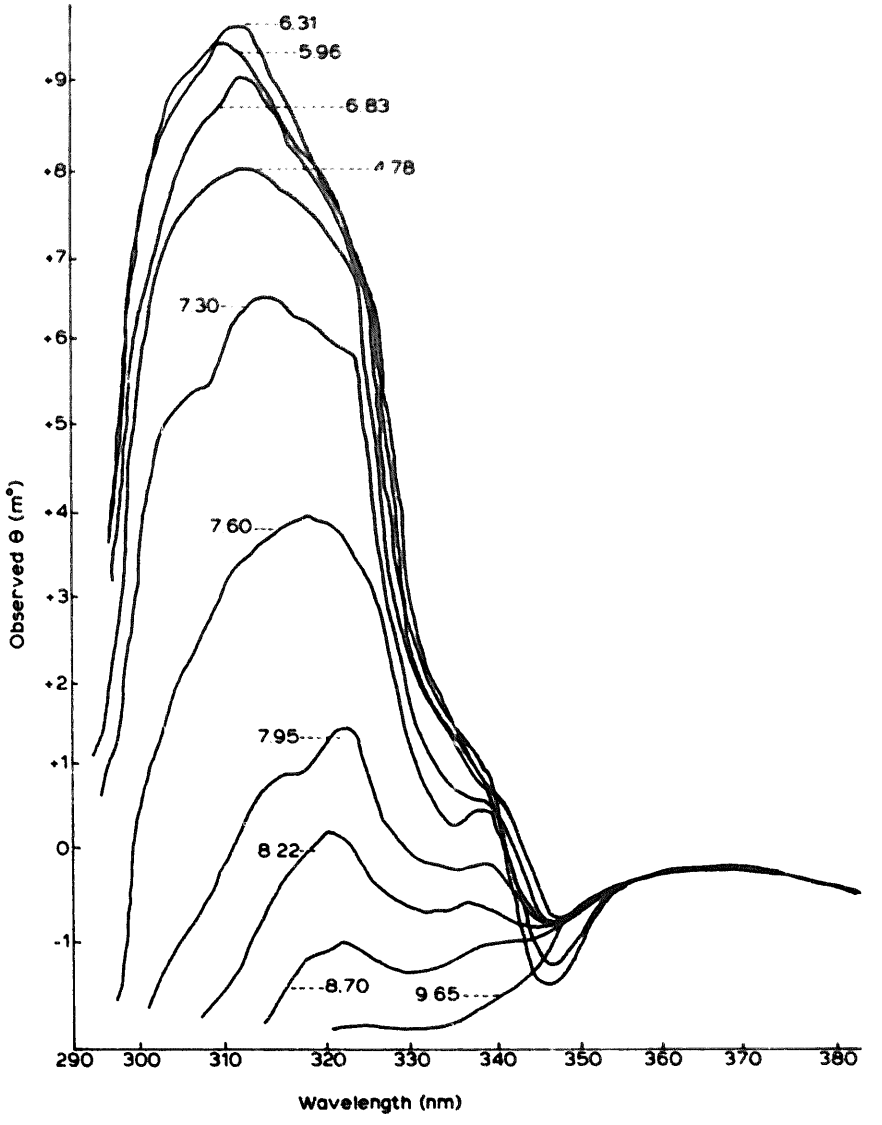

Fig. 5. Observed ellipticity as a function of pH in the presence of 0.1 M phosphate buffer [HSA]. 5.0×10^{-5} M = [benoxaprofen]. 1.0 cm cell.

effect of pH on the ellipticity of 1:1 benoxaprofen–HSA complexes is shown in Figs. 5 and 6. The size of the signal is not corrected for the contribution of the HSA which is constant under these experiments conditions. The changes in ellipticity as a function of pH cannot be due to the changes in the states of ionization of the drug because its pK_a has been reported to be between 3 and 4 (Chatfield and Woodage, 1978), and the drug is fully ionized over the pH region of these investigations. The size of the signal decreases as the pH is increased; however, this is not due to a decreased affinity of the drug for HSA in the B-form as is shown by the dialysis data of Table 2. This decrease in ellipticity of the complexes when HSA is in the B-form is contrary to the observations made with warfarin and diazepam, but again must be

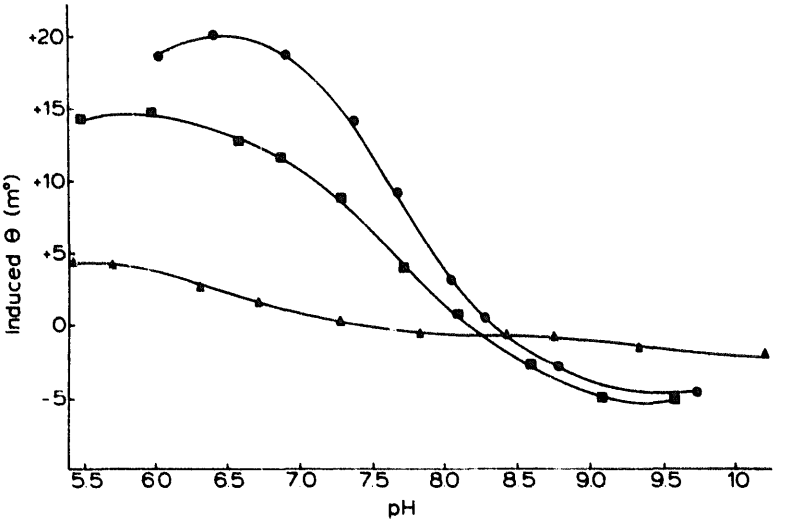

Fig. 6. Effect of chloride and calcium ions on the induced ellipticity at 305 nm. [HSA] = 5×10^{-5} M benoxaprofen. ● = 0.1 M phosphate; ▲ = 2.5×10^{-3} M $CaCl_2$; ■ = 0.1 M naCl.

TABLE 2

EFFECT OF CHLORIDE AND CALCIUM IONS ON THE PRIMARY BINDING CONSTANT FOR THE BENOXAPROFEN–HSA INTERACTION AT 25°C. DIALYSIS DATA [HSA] = 2.0×10^{-4} M = BENOXAPROFEN

	pH	K_1 (l/mol)
0.1 M phosphate buffer	6.00	1.38×10^6
	6.50	1.46×10^6
	7.00	1.70×10^6
	7.50	2.66×10^6
	8.00	3.82×10^6
	8.50	3.88×10^6
	9.00	3.34×10^6
0.1 M NaCl	6.35	6.75×10^5
	6.85	6.29×10^5
	7.30	6.19×10^5
	7.80	8.81×10^5
	8.40	1.07×10^5
	9.10	1.33×10^5
0.1 M Nacl and 2.5×10^{-3} M $CaCl_2 \cdot 2\,H_2O$	6.30	3.72×10^5
	6.59	4.17×10^5
	6.81	4.98×10^5
	7.35	6.08×10^5
	7.74	6.16×10^5
	8.12	6.07×10^5
	9.13	5.61×10^5

due to a change in the geometry of the binding site following the N → B transition. Cl ions decrease the ellipticity of the complexes over the pH range of the N → B transition as shown in Fig. 6. The affinity of the drug for HSA is also decreased over this pH range (Fig. 7 and Table 2). The large concentration of Cl^- ions probably compete for the benoxaprofen binding site in the sane way as they did for the warfarin binding site (Wilting et al., 1980), and the decrease in ellipticity is primarily due to the decreased binding of benoxaprofen rather than a conformational change.

Ca^{2+} ions cause large decreases in the ellipticity of the complexes (Fig. 6) and substantially decrease the affinity of the drug for HSA (Fig. 7 and Table 2). The N → B change, appears to be 50% completed at pH 6.9 in the presence of Ca^{2+} ions confirming that the ions increase the percentage of the B-form. The decreased affinity of benoxaprofen for HSA in the presence of Ca^{2+} ions seem to be due to the competition between Ca^{2+} ions and the drug for the imidazole residues at the binding sites on HSA (Pedersen, 1972).

When albumin is in the N-form, the effects of chloride and calcium are emphasized, indicating that in this conformation HSA is most sensitive to the effects of these ions. Ca^{2+} ions also decrease the concentration of diazepam–HSA complexes (Wilting et al., 1980b), but for warfarin–HSA complexes the presence of Ca^{2+} causes small increase in affinity due to the effect on the N → B change. The

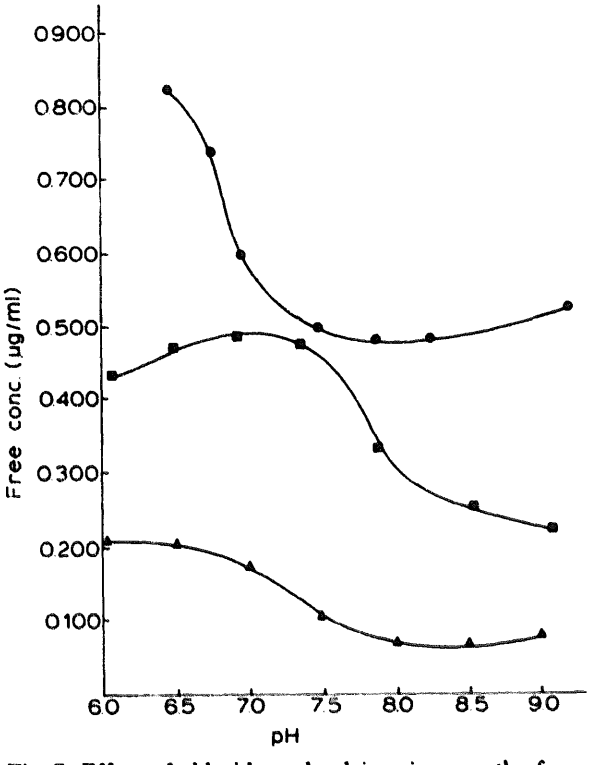

Fig. 7. Effect of chloride and calcium ions on the free concentrations of benoxaprofen as a function of pH. Dialysis data at 25°C. [HSA] = 2.0×10^{-4} M = benoxaprofen. ● = 2.5×10^{-4} M = $CaCl_2$; ■ = 0.1 M NaCl; ▲ = 0.1 M phosphate.

imidazole site does not appear to be involved in the warfarin binding. The difference in behavior of the complexes of warfarin and benoxaprofen with HSA in the presence of Ca^{2+} ions may be a useful general method for distinguishing between their two distinct binding sites.

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