acid. The CMC describes the lipophilicity of amphiphilic substances (13); the CMC value of chlorpromazine in the presence of acid could be an indirect measure of the lipophilicity of the ion-pair. Figure 4 shows an almost linear correlation between D and the CMC for the different bicarboxylic acids.

Chlorpromazine was selected for this study since it is representative of many tricyclic drugs and its behavior toward carboxylic acids may be important in GI absorption. Ion-pair formation between chlorpromazine and dietary carboxylic acids (such as citric, tartaric, and acetic acid) would give a favorable absorption in GI lumen. Furthermore, these findings could be useful for the development of dosage forms. It was shown (14) that certain drugs can be absorbed in their undissociated state, either directly or by ion-pair or complex formation. The behavior of chlorpromazine in humans might also be ascribed to the interactions of the drug with some of the physiological acids studied.

REFERENCES

(1) E. R. Garrett and P. B. Chemburkar, *J. Pharm. Sci.*, **57**, 949 (1968).
(2) M. Nakano and N. K. Patel, *ibid.*, **59**, 77 (1970).
(3) G. L. Flynn and J. Roseman, *ibid.*, **60**, 1788 (1971).
(4) R. H. Reuning and G. J. Levy, *ibid.*, **58**, 79 (1969).
(5) T. R. Bates, J. Galownia, and W. H. Johns, *Chem. Pharm. Bull.*, **18**, 656 (1970).
(6) G. Levy and E. J. Mroszczak, *J. Pharm. Sci.*, **57**, 235 (1968).
(7) T. Higuchi, A. Michaelis, and A. Hurwitz, *Anal. Chem.*, **39**, 974 (1967).
(8) M. Nakano, *J. Pharm. Sci.*, **60**, 571 (1971).
(9) K. Juni, M. Nakano, and T. Arita, *Chem. Pharm. Bull.*, **25**, 2807 (1977).
(10) M. R. Gasco, M. E. Carlotti, and M. Trotta, *Pharm. Acta Helv.*, **56**, 55 (1981).
(11) A. Tilly, *Acta Pharm. Suec.*, **12**, 89 (1975).
(12) A. L. Green, *J. Pharm. Pharmacol.*, **19**, 89 (1966).
(13) E. Tomlinson, S. S. Davis, and G. I. Mukhayer, in "Solution Chemistry of Surfactants," vol. 2, Mittal, Ed. 1980, pp. 889.
(14) M. J. Cho, and R. Schnabel, *J. Pharm. Sci.*, **64**, 1894 (1975).

Effect of Albumin Conformation on the Binding of Phenylbutazone and Oxyphenbutazone to Human Serum Albumin

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Abstract □ The binding of phenylbutazone (I) and oxyphenbutazone (II) to human serum albumin over pH 6.9–9.3 was studied by difference spectrophotometry and equilibrium dialysis. At each pH tested, there was higher binding affinity of I to human serum albumin than II. Equilibrium dialysis showed that over the pH 7–8.2 range both agents had a single high-affinity site and several sites of lower affinity, with the highest binding constant and number of binding sites at pH 7.4 for both I and II. Both techniques showed that the affinity of both drugs to albumin was higher for the neutral form than for the basic form and this transition occurred in both cases around the neutral region (7–7.4). Both the ionized and unionized forms of I and II participated in the binding. In the neutral region, magnesium ion increased the affinity of both drugs to albumin while chloride ion decreased it slightly.

Keyphrases □ Phenylbutazone—effect of albumin conformation on binding to human serum albumin □ Oxyphenbutazone—effect of albumin conformation on binding to human serum albumin □ Albumin, human serum—effect of conformation on binding of phenylbutazone and oxyphenbutazone □ Binding—of phenylbutazone and oxyphenbutazone, effect of albumin conformation, human serum albumin

Drug–protein binding studies are important for prediction of drug dynamics in the body (1). The affinity of such interaction can possibly be used to correlate therapeutic and toxicological effects, as well as drug distribution and excretion. Most *in vitro* drug–protein interaction studies are conducted in isotonic pH 7.4 buffer. Data reduction in terms of binding constants assumes that for a partially ionized drug each species is bound with equivalent affinity (2).

It was reported (3) that conformational changes occur in serum albumin over pH 6–9. Zurawski and Foster (4) established that two conformational states exist in bovine serum albumin over this pH region. They called the form at neutral pH (pH 6–7) the "N" form and the form at higher pH (around pH 9) the "B" (base) form. Thus, the conformational change that occurs is the N to B or B to N transition. It was recently shown (5–7) that the same conformational change occurs in human serum albumin over pH 6–9. The N to B transition is seemingly dependent on pH but may also occur to some extent with ionic strength of the buffer and buffer ion composition. Calcium ion and chloride ion affect this transition (5–10) as well as the binding of drug to the protein.

BACKGROUND

While the macromolecule conformation changes with varying pH, ionization of the drug may also occur over such a pH range. A method was presented recently (2) to distinguish which species of a partially ionized acidic or basic drug bind to the protein and to enable determination of the binding site constant (3) for the interaction. In addition, this method aids in the detection of significant changes in the protein binding site that may result from pH perturbation. Since the blood pH in patients may vary between 6.8 and 7.8 (11), significant changes in drug binding to albumin or other plasma proteins could take place with a change in blood or local change in organ (*e.g.*, liver) pH. This pH range could not be predicted in any one individual; at best these pH differences usually would be only a few tenths of a pH unit (*i.e.*, respiratory acidosis or alkalosis).

The present study used equilibrium dialysis and UV difference spectroscopy to investigate the effect of albumin conformational state on the binding of phenylbutazone and oxyphenbutazone to human serum albumin at various pH values.

EXPERIMENTAL

Materials—The human serum albumin used was previously investigated for purity[1] (12). Phenylbutazone (I) and its metabolite oxyphen-

[1] Armour Pharmaceutical Co., Kankakee, Ill.

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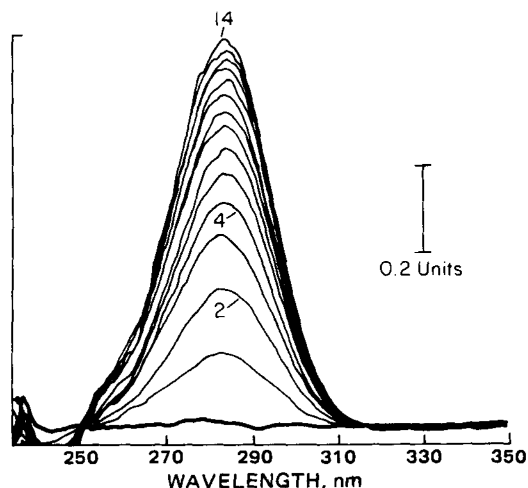

Figure 1—*Difference spectrophotometric titration of 1.45 × 10^{-5}* M *albumin with I at pH 8.2. Each curve represents the addition of 10 µl of I solution (1.5 × 10^{-3}* M*) to 3 ml (initially) of buffer in the reference compartment and to 3 ml of albumin in the sample compartment. The numbers 2, 4, and 14 indicate the number of increments added.*

butazone (II) were obtained commercially[2]. All other chemicals were analytical grade[3,4] and all solutions were prepared in deionized water.

Binding Studies—Binding studies of I and II to human serum albumin were carried out using UV difference spectroscopy and equilibrium dialysis techniques. In all binding studies, the albumin (1.45 × 10^{-5} *M*) was dissolved in phosphate buffer adjusted to various pH values, *i.e.*, 6.8, 7.0, 7.4, 7.8, and 8.2, and pH 9.3 borate buffer. All buffer solutions were made isotonic using sodium chloride or magnesium chloride.

Difference Spectrophotometry—The tandem technique (13) was employed in the split-beam mode[5]. Identical 10-µl increments[6] of either I or II (1.50 × 10^{-3} *M*) were added to the buffer cell in the reference beam and to the albumin cell in the sample beam to give final concentrations of I or II of 4.97 × 10^{-6}–6.4 × 10^{-5} *M*, representing drug to protein ratios of 0.34–4.4. To maintain a constant albumin concentration in each beam after drug addition, a volume of albumin equal to that of the drug added, but twice the concentration of the albumin already in the cell, was added to the protein cell. Drug concentration was kept constant by the addition of an identical volume of buffer to the buffer cell in the reference compartment. Resulting difference spectra were subsequently recorded.

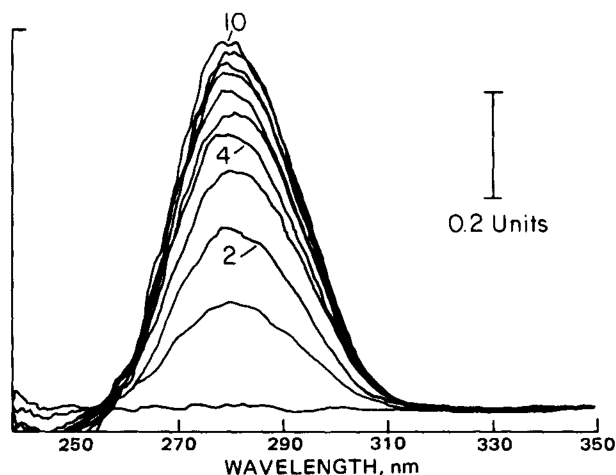

Figure 2—*Difference spectrophotometric titration of 1.45 × 10^{-5}* M *albumin with II at pH 7. Each curve represents the addition of 10 µl of II solution (1.5 × 10^{-3}) to 3 ml (initially) of buffer in the reference compartment and to 3 ml of albumin in the sample compartment. The numbers 2, 4, and 10 indicate the number of increments added.*

[2] Ciba-Geigy, Summit, N.J.
[3] J. T. Baker Chemical Co., Phillipsburg, N.J.
[4] Fisher Scientific Co., Fair Lawn, N.J.
[5] Model 118, Cary spectrophotometer, Varian Instruments, Palo Alto, Calif.
[6] Hamilton Co., Reno, Nev.

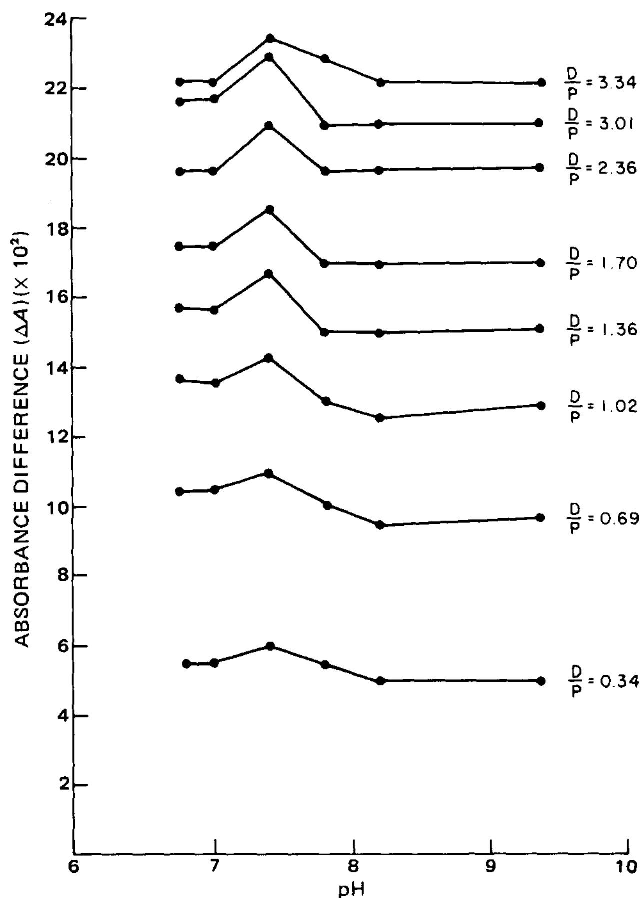

Figure 3—*Effect of pH on the absorbance differences (ΔA) of I–albumin complex.*

Equilibrium Dialysis—Equilibrium dialysis experiments were performed with an equilibrium dialyzer[7] using cell compartments of 10 ml total volume. Hydrated cellulose membranes[8] were prepared and used as suggested by the manufacturer. The concentrations of I or II used ranged from 7.6 × 10^{-6} to 1.5 × 10^{-3} *M*. Equilibrium was achieved within 4 hr for the lowest concentration and took as long as 15 hr for the highest concentration. Determination of the free drug was carried out spectrophotometrically; both I and II had a maximum at 264 nm in phosphate buffer. Standard calibration curves were constructed for each drug at each pH. Control experiments were conducted using buffer solution instead of protein.

Effect of Chloride and Magnesium Ions—The binding of I and II to albumin was also studied in phosphate buffer containing no sodium chloride and in phosphate buffer containing 1 × 10^{-3} *M* magnesium chloride. The equilibrium dialysis technique was used and phosphate buffers of pH 7.4 and 7.8 were selected for I and II, respectively.

RESULTS AND DISCUSSION

Both I and II were shown to bind to human serum albumin at different pH by the technique of difference spectroscopy and equilibrium dialysis. Examples of difference absorption spectra obtained for the association of I and II with albumin are shown in Figs. 1 and 2. In the case of I, the spectra were characterized by positive peaks at 282 ± 2 nm and negative troughs at 244 ± 2 nm. In the case of II, the spectra were characterized by a positive peak at 280 ± 2 nm and negative peak at 244 ± 2 nm. Families of curves were generated by incremental addition of I or II to albumin. At each pH examined, the absorbance difference spectra (ΔA) of I was larger than II. This indicates that I has a higher affinity for albumin than II (since their molar absorptivities are equivalent).

Absorbance difference curves at different pH were generated by plotting the change in absorbance measured as the difference in intensity of the peak (280 or 282 nm) and trough (244 nm) for I and II, respectively,

[7] Dianorm, Diachema AG, Ruschlikon, Switzerland.
[8] Diachema AG, Ref. 10.14, m.w. cut-off 5000, and diameter 63.

Table I—Primary Association Constant of Phenylbutazone (I) and Oxyphenbutazone (II) and Number of Sites Calculated at Different pH from Scatchard Plots

pH	K_1, liters/mole I	K_1, liters/mole II	N_1 I	N_1 II	$N_2 (N_t - N_1)$ I	$N_2 (N_t - N_1)$ II	K_2 I	K_2 II
7.0		$2.20 \pm 0.15 \times 10^5$		1.42		6.2		$4.76 \pm 0.84 \times 10^3$
7.4	$5.06 \pm 0.86 \times 10^5$	$3.53 \pm 0.74 \times 10^5$	1.25	1.30	8.1	8.0	$5.63 \pm 0.61 \times 10^3$	$4.20 \pm 0.19 \times 10^3$
7.8	$2.96 \pm 0.57 \times 10^5$	$1.97 \pm 0.64 \times 10^5$	1.30	1.25	3.5	2.7	$7.08 \pm 1.03 \times 10^3$	$9.70 \pm 0.84 \times 10^3$
8.2	$1.92 \pm 0.74 \times 10^5$	$1.62 \pm 0.31 \times 10^5$	1.55	1.45	3.5	4.0	$5.60 \pm 5.1 \times 10^3$	$7.67 \pm 0.59 \times 10^3$

versus pH (Figs. 3 and 4). These figures indicate that a change in pH influences the difference spectra produced as a result of the drug–protein interaction. In the case of I (Fig. 3) the curves indicate a maxima in the difference spectra at pH 7.4. The absorbance difference is slightly higher in the slightly acidic region over that observed in alkali.

This difference may indicate that I is bound more to albumin in the neutral region. In the case of II (Fig. 4), the effect of changing pH on the difference spectra was more pronounced. At all drug–albumin ratios used, the absorbance difference was larger in the neutral or slightly acidic region than in the alkaline region. An abrupt decrease occurred around pH 7–7.4. This decrease was more pronounced at higher drug–albumin ratios. The inflection points of these curves was at pH 7.8 indicative of the greatest change. This higher value of ΔA obtained in the neutral region indicates that II has a higher affinity to the N form of the albumin.

Data obtained from the equilibrium dialysis studies were plotted according to Scatchard (14) (Figs. 5 and 6). Each plot is curved and, therefore, the data were analyzed in terms of two classes of binding sites using the computer program of Perrin *et al.* (15). Results of the Scatchard plot analysis are given in Table I. There is a single high affinity binding site for both I and II and several sites of lower interaction potential (Table I). It should be pointed out that the range of I and II concentrations used in the binding studies varied considerably, from 7×10^{-6} to 1×10^{-3} *M*; previous literature reports use a narrower concentration range.

Considering the effect of pH on the association constants of I and II, Table I shows that *K* for both drugs decreases with increasing pH. In addition, at pH 7.4 the largest number of sites seems to be present. At each pH used, the association constant of I was larger than that of II.

Other workers calculated similar albumin association constants for both I and II. Solmon *et al.* (16) reported that albumin has only one binding site for I with an affinity constant of $1.17 \times 10^5\ M^{-1}$. However, the concentration of I used by these workers was always less than that of albumin. Chignell (17) showed that albumin had three binding sites for I, one binding site of high affinity ($K = 1 \times 10^5\ M^{-1}$), and two others with lower affinity ($K = 4 \times 10^4\ M^{-1}$). For II he reported two association constants $k_1 = 2.28 \times 10^5\ M^{-1}$ and $k_2 = 3.7 \times 10^4\ M^{-1}$. Two association constants were also reported (18) for I, $k_1 = 2.37 \times 10^{-5}\ M^{-1}$ $k_2 = 4.56 \times 10^4\ M^{-1}$, and for II, $k_2 = 2.32 \times 10^5\ M^{-1}$ and $k_3 = 1.16 \times 10^4\ M^{-1}$. The report indicated that *K* for II was very large and the affinity constants (K_2 and K_3) calculated assumed $n_1 = 1$; the first molar equivalent of II was so strongly bound as to be virtually removed from solution.

The pKa of I and II are 4.5 and 4.7, respectively (19). To determine whether the ionized and/or unionized species of these two drugs is bound to albumin, the method recently reported (2) for the determination of the site binding constant of each species of a partially ionized drug was applied to the equilibrium dialysis data. The method appeared applicable over the pH range examined for each compound and indicated that both species of I and II were responsible for the binding. The site binding constants for the ionized species were 1.40×10^5 and 1.16×10^5 for I and II, respectively. The site binding constants for the unionized species of I and II were much greater, approaching 2.93×10^8 and 1.18×10^8, respectively. However, the overall affinity or binding constant would be expected to be only somewhat greater than ionized drug since only a very small proportion of each drug is present in the unionized form, consequently adding little to the overall constant. Alternatively, it may be possible that a conformational change in the protein occurs and is re-

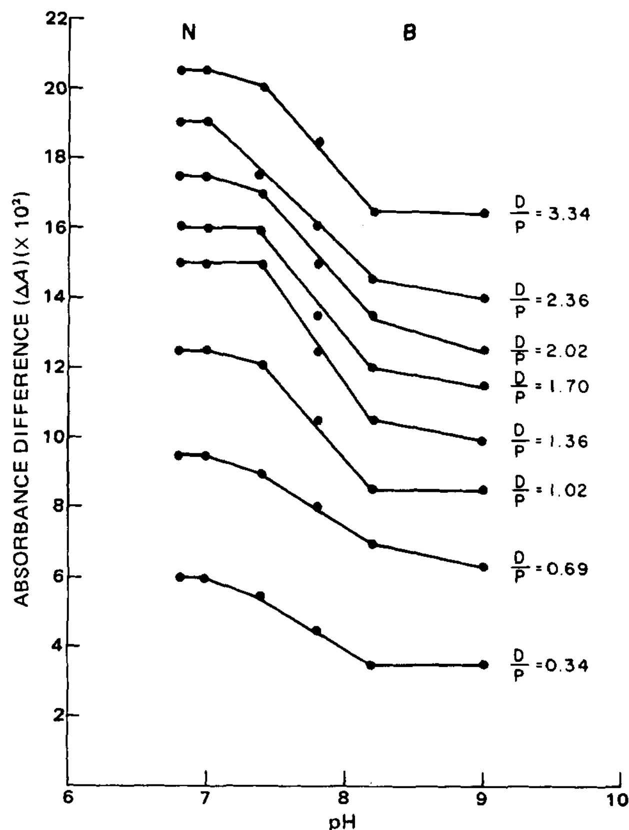

Figure 4—*Effect of pH on the absorbance differences (ΔA) of II-albumin complex.*

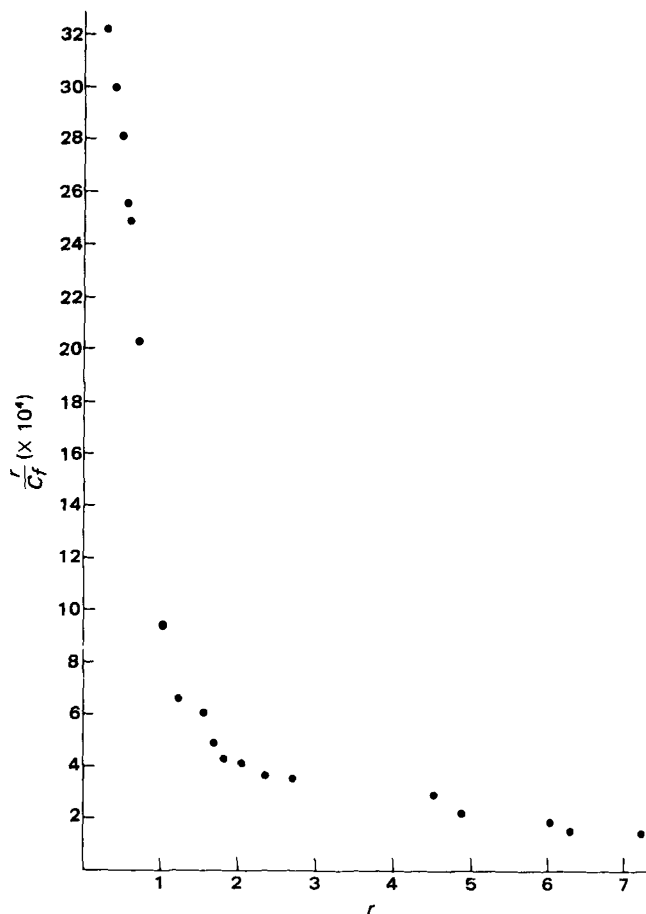

Figure 5—*Scatchard plot of dialysis data for the interaction of I with albumin at pH 7.4. Key:* r, *moles of drug bound per mole of albumin; and* C_f, *free drug concentration.*

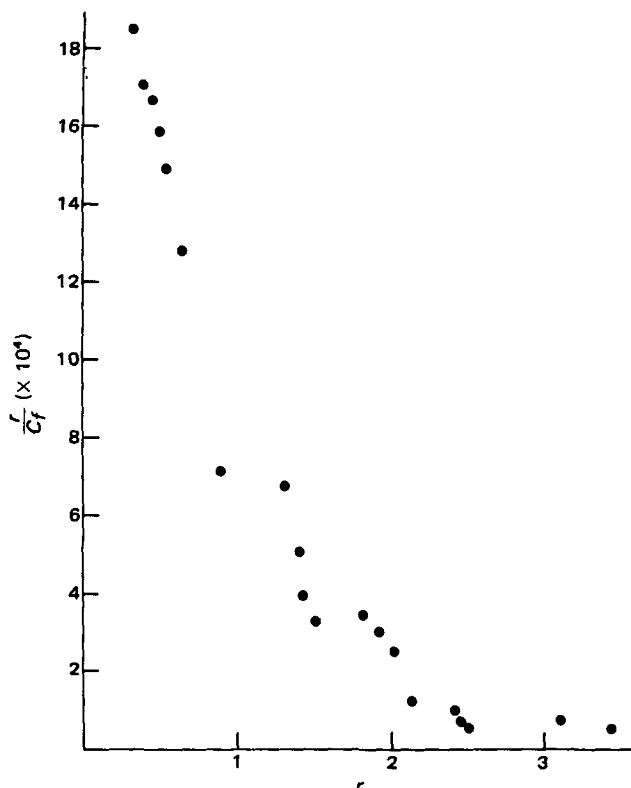

Figure 6—*Scatchard plot of dialysis data for the interaction of II with albumin at pH 7.8. Key:* r, *moles of drug bound per mole of albumin; and* C_f, *free drug concentration.*

sponsible for marked differences in affinity between various drug forms.

The effect of chloride and magnesium ions on the binding of I and II to albumin was opposite in nature. Chloride ions reduced the amount of drug bound. This was most evident in the neutral pH region for both drugs. The mechanism is possibly one of displacement of drug from albumin as postulated earlier (6, 10). Magnesium ion increased the binding of both drugs in the same pH region. The increase was as much as 10% in the presence of magnesium at pH 7.4. Cations, especially divalent cations, have been found to affect the N to B transition of the protein (6, 7) and this is possibly the reason for the increased binding. The effects of both chloride and magnesium ions were greater on the high affinity sites than on the sites in the second class of binding.

The UV difference spectroscopy and equilibrium dialysis data indicated that albumin has a higher affinity for I than II at each pH examined. Oxyphenbutazone is less hydrophobic (more polar) than phenylbutazone, and the importance of hydrophobic interactions in the binding of drugs to protein was noted previously (17, 20, 21).

With either method of examining binding it is evident that pH affects the affinity of the interaction. It is also apparent that the binding affinity of each drug is higher in the neutral region (N form of protein) than in the alkaline region (B form). This fact, coupled with the results of absorbance difference *versus* pH which show a marked transition (inflection) in the neutral pH region, indicate that the N to B transition of albumin may be responsible for the changes in the affinity of the complex with pH.

REFERENCES

(1) B. K. Martin, *Nature*, **207**, 274 (1975).
(2) D. L. Parsons and J. J. Vallner, *Acta Pharm. Suec.*, **17**, 12 (1980).
(3) W. J. Leonard, K. K. Vijai, and J. F. Foster, *J. Biol. Chem.*, **238**, 1984 (1973).
(4) V. R. Zurawski and J. F. Foster, *Biochemistry*, **13**, 3465 (1974).
(5) J. Wilting, M. M. Weideman, A. C. Roomer, and J. H. Perrin, *Biochim. Biophys. Acta*, **579**, 469 (1979).
(6) J. Wilting, W. F. Van Der Giesen, L. H. M. Janssen, M. Weideman, M. Otagiri, and J. H. Perrin, *Biol. Chem.*, **255**, 3032 (1980).
(7) J. Jacobsen and T. Faerch, *Biochim. Biophys. Acta*, **623**, 199 (1980).
(8) S. Katz and I. M. Klotz, *Arch. Biochem. Biophys.*, **44**, 351 (1953).
(9) B. H. M. Harmsen, S. H. de Bruin, L. H. M. Janssen, J. F. Rodrigues de Miranda, and G. A. J. Vanos, *Biochemistry*, **10**, 3217 (1971).
(10) L. H. M. Janssen and T. H. A. Nelen, *J. Biol. Chem.*, **254**, 4300 (1979).
(11) K. Diem and C. Lentzer, "Wissenschaftliche Tabellen," 7th ed., 556, Ciba-Geigy AB, Basel, Switzerland, 1976.
(12) J. H. Perrin and J. J. Vallner, *J. Pharm. Sci.*, **64**, 1508 (1975).
(13) A. S. Brill and H. E. Sandberg, *Biophys. J.*, **8**, 669 (1968).
(14) Scatchard, *Ann. N.Y. Acad. Sci.*, **51**, 660 (1949).
(15) J. G. Perrin, J. J. Vallner, and S. Wold, *Biochim. Biophys. Acta*, **371**, 482 (1974).
(16) H. H. Solmon, J. J. Schrage, and D. Williams, *Biochem. Pharmacol.*, **17**, 143 (1968).
(17) C. F. Chignell, *Mol. Pharmacol.*, **5**, 244 (1969).
(18) A. Rosen, *Biochem. Pharmacol.*, **19**, 2075 (1970).
(19) A. B. Gutman, P. G. Dayton, T. F. Yii, L. Berger, W. Chen, L. E. Sican, and J. J. Burns, *Am. J. Med.*, **29**, 1017 (1960).
(20) F. Helmer, K. Kiehs, and C. Hansch, *Biochemistry*, **7**, 2858 (1968).
(21) W. Scholtan, *Arzneim.-Forsch.*, **18**, 505 (1968).