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BINDING OF DIAZOXIDE AND OTHER BENZOTHIADIAZINES TO HUMAN ALBUMIN*

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Abstract—The binding of seven benzothiadiazines to human albumin was studied by equilibrium dialysis. All these 1,2,4-benzothiadiazine-1,1-dioxide analogs are highly bound to human albumin. The unsubstituted benzothiadiazine nucleus is bound less than the substituted analogs. Addition of a chlorine at C-6 and C-7 markedly increases binding, but further addition of methyl or sulfamyl groups results in some reduction of binding. Binding studies on benzothiadiazines do not demonstrate independent binding sites on albumin. Binding of drugs to albumin can be evaluated by fitting a logistic function to the experimental points with a least squares method. This empirical, objective technique allows examination of the nature of the bonds between drug and protein. Diazoxide, the 7-chloro-3-methyl analog, was used for detailed investigations into the mechanism of protein binding of the benzothiadiazines. The effects of pH, temperature, ionic strength, cations and deuterium on the binding of diazoxide to human albumin indicate that the drug is bound mainly by hydrophobic interaction and to a lesser extent by hydrogen bonding. Difference spectroscopy studies show a shift in the electron distribution of diazoxide with binding.

THE EXTENT and mechanism of the binding of benzothiadiazines to human plasma proteins have not been established. Chlorothiazide, hydrochlorothiazide, benzthiazide and triflumethiazide are bound to the albumin fraction of dog plasma.[1,2] Diazoxide, a nondiuretic, hypotensive benzothiadiazine, is highly bound to human albumin.[3,4] Since the drug is eliminated mainly by glomerular filtration, this binding results in a plasma half-life of 22 hr.[3,4] It may also account for the increase with intravenous injection rate of the hypotensive response to diazoxide.[3,4] The present study was conducted to determine the extent of binding of diazoxide and of its benzothiadiazine analogs to human albumin and to define the types of bonds formed.

METHODS

Most of the analogs studied were obtained from the Schering Corp., Bloomfield, N.J. Analogs with sulfamyl substitution at C-7 were obtained from Merck & Co., Rahway, N.J. The 6-chloro-1,2,4-benzothiadiazine-1,1-dioxide analog was synthesized by refluxing 10·0 g of 4-chloro-2-aminobenzene sulfonamide in 10% excess 95% formic acid[5–7] for 2 hr followed by precipitation in ice-cold water and subsequent crystallization from butanone and then from methanol. A yield of 46·4 per cent was obtained.

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The structure of the final product was confirmed by nuclear magnetic resonance and infrared spectroscopy and the purity by melting point determination (251–253°)[6] and elemental analysis (expected C = 38·8%, H = 2·33%, N = 12·93%; obtained C = 38·63%, H = 2·34%, N = 12·86%).

The binding of diazoxide and of other analogs of 1,2,4-benzothiadiazine-1,1-dioxide to human albumin was studied by equilibrium dialysis. Dialysis bags were made by forming a continuous loop from 1/4 inch dialysis tubing. The tubing was rinsed for 3 hr with lukewarm running distilled water and stored overnight in distilled water at 6°. Prolonged storage or insufficient washing of the tubing resulted in the appearance of material in the buffer plasma which interfered with the drug assays and increased the amount of drug bound on the dialysis tubing. Immediately prior to an experiment, the tubing was rinsed with phosphate buffer and gently shaken until no excess buffer was present. The dialysis bag was filled with 2·0 ml albumin solution and immersed in 10·0 ml phosphate buffer which contained the drugs in concentrations ranging from $5{\cdot}0 \times 10^{-6}$ to $1{\cdot}0 \times 10^{-4}$ M.

Phosphate buffer was prepared from KH_2PO_4 titrated to the desired pH with a 1·0 M solution of sodium hydroxide. In the majority of experiments, 0·067 M, pH 7·4, phosphate buffer was used. Albumin solutions were prepared from electrophoretically pure crystallized human albumin (Pentex Corp., Kankakee, Ill.; Lots 27, 28, 29, 31). All albumin concentrations were corrected for water content, determined by heating a small portion of albumin for 4 hr at 80° (range of H_2O content, 4·0 to 5·5%). Albumin concentrations between $1{\cdot}45$ and $5{\cdot}80 \times 10^{-5}$ M were used. (Molecular weight of human albumin = 69,000.)[8] Within this range, changes in albumin concentration did not affect the binding isotherms. Albumin was not defatted prior to its use, since studies with defatted human albumin[9,10] showed that diazoxide binding to albumin was not significantly altered by defatting.

The test tubes containing buffer, drug and protein solutions were tightly capped, placed vertically in racks, and shaken in a controlled temperature bath until equilibrium was reached. Binding results obtained from tubes placed in a horizontal rocker were identical. Equilibrium was reached in 3·5 hr at 37°, 12 hr at 27°, 15 hr at 15°, and 24 hr at 3°. Usually five replicates for each of at least five different concentrations were studied, but on occasion up to 12 replicates and 12 different concentrations were examined.

Initially, drug concentrations were determined at equilibrium in both buffer and protein phases by the extraction technique of Symchowicz *et al.*[11] In later experiments, only the equilibrium free drug concentration in the buffer phase was measured by direct reading of ultraviolet absorbance at the absorption maximum of each drug. Identical binding results were obtained with both methods of determination. Drug binding to siliconized glass test tubes and dialysis bags was determined in separate experiments for each drug and appropriate corrections were applied in the calculation of the protein-binding isotherms. Albumin, buffer, drug standards and blanks were carried through all steps of each study. The technique of Baer *et al.*[1] was used to confirm that the compounds studied underwent negligible hydrolysis[12] at the temperature, pH and duration of each experiment. Because the benzothiadiazine binding curves were shown to have no discernible maximum, an empirical curve-fitting technique was used to analyze and compare binding results under various conditions.

Binding results were analyzed by fitting a logistic function

$$E = M\frac{[D]^P}{[D]^P + K^P}$$

to the experimental points by a minimization of least squares differences of observed and expected values (E = moles of drug bound per mole of albumin, D = free drug concentration). This mathematical form indicates a curve which reaches a maximum M, has a position K along the abscissa at $M/2$, and a slope determined by the power P. The parameter M is chosen with a constant value ten times greater than the maximum experimental value for moles bound per mole of protein. This simplifies the analysis to estimating only P and K from the experimental results. The shape of the fitted curve is insensitive to 10^3 variations in choice of M, but will alter the values of the parameters K and P. While it is tempting to assign physical meaning to the parameters P, K and M or draw analogy with other analytic techniques, such generalizations are not necessarily valid, since this is an empiric curve-fitting technique. A computer program fits N curves independently to N sets of binding results, tests the parallelism among curves, and tests for differences in position between any two parallel curves. Details concerning the statistical model, weighting and mechanics of calculation for the minimization technique are given by Snedecor and Cochran[13] and Parker and Waud.[14,15]

The influence of pH, of ionic strength, and of sodium/potassium ratio on diazoxide binding was studied at four pH levels (6·0, 7·4, 8·5, 10·5), three different total ionic strengths (0·19, 0·54, 1·08 M) and three ratios of sodium to potassium ion concentration (0·83, 8·3, 23) in phosphate buffer. Sodium/potassium ratios were adjusted to exact values by adding NaCl or KCl to the buffer. With the buffer strengths employed, corrections for Donnan inequalities in ion distribution on either side of the dialysis tubing were negligible.[16] In one set of experiments the buffer was prepared in 95% deuterated water. The effect of calcium on binding was determined by comparing the binding of diazoxide in 0·1 M tris-(hydroxymethyl)-aminomethane (Tris) buffer in the absence and presence of calcium (1×10^{-4} M).

The pKa values of diazoxide, chlorothiazide, 6-Cl, 7-Cl and unsubstituted benzothiadiazine nucleus were determined by precipitation, titration and by ultraviolet spectral techniques.[17] The ultraviolet spectrum of diazoxide changes with pH and is suitable for this latter technique. Ultraviolet difference spectra of free and albumin-bound diazoxide were determined in the four sample compartments of a temperature-controlled recording Beckman DU spectrophotometer at 25° and pH 6·0, 7·4 and 8·5.[18] The partitioning of the compounds into 1-octanol from phosphate buffer, pH 7·4, was determined.[19] The partition coefficient was calculated assuming that only un-ionized drugs entered the octanol.

RESULTS

Influence of chemical substitution on binding. The best fit lines to the experimental points for the binding of each of the seven 1,2,4-benzothiadiazine analogs to human albumin at 3° are shown in Fig. 1. It is evident that over the range of free drug concentrations studied there is no saturation of binding. The least bound analog is the unsubstituted benzothiadiazine nucleus. Addition of a chlorine at C-6 or C-7 markedly increases binding. Further addition of a methyl group, a sulfamyl group, or both to the 6- and 7-chloro compounds significantly decreases binding. Large substi-

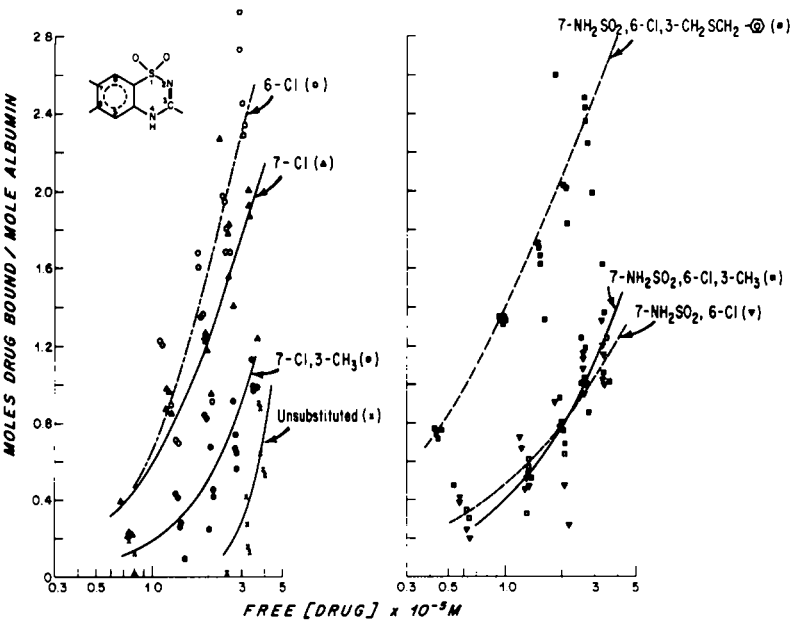


FIG. 1. Binding of benzothiadiazine analogs to human albumin. Studies conducted in 0·067 M phosphate buffer, pH 7·4, at 3°.

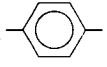
tutions on C-3 such as 3-[(Benzylthio)methyl] are associated with a marked increase in binding. The binding of all compounds studied was fully reversible.

The data shown in Fig. 1 were also plotted by the more commonly used Scatchard plot. Curves fitting the points were distinctly curved for all drugs without evidence of different classes of binding sites. The lack of evidence of discrete binding sites was the primary reason an objective and empiric curve-fitting procedure was used. Table 1 shows the calculated values for M, P and K, and the results of the statistical comparison of the curves shown in Fig. 1. All curves except that of the 3-[(Benzylthio)-methyl]-substituted compound can be fitted equally well with the same common value for the slope P as with different values for P for each curve. All curves differ significantly in position ($P < 0{\cdot}01$) from each other except those for the 7-chloro,3-methyl (diazoxide), the 7-sulfamyl,6-chloro (chlorothiazide) and the 7-sulfamyl,6-chloro,3-methyl substituted analogs.

Relation of physical characteristics and biologic activity of benzothiadiazine analogs. Table 2 lists the pKa values and octanol partition coefficients of the non-sulfamyl-substituted analogs and the values of M, P and K which characterize their binding isotherms. Results for the 7-sulfamyl-substituted compounds could not be obtained because each compound has two titratable protons.

The pKa of the diazoxide N-4 proton as determined spectrophotometrically in aqueous buffers is $8{\cdot}74 \pm 0{\cdot}04$. By precipitation and titration, pKa values of 8·5 and 8·58 were obtained. Diazoxide and the other three analogs are predominantly unionized at the physiologic pH of 7·4. The binding parameter K does not correlate as determined by rank correlation with 1-octanol partition coefficient (C') or potency of the drugs as antagonists of barium-induced contraction of rat aortic segments (pA_2) but increases as pKa increases.

TABLE 1. BINDING OF 1,2,4-BENZOTHIADIAZINE-1,1-DIOXIDES TO HUMAN ALBUMIN*

Substitution	Maximum *M*	Slope *P*	Position *K* $\times 10^{-5}$ M	Variance ratio			
				Slope	d.f.	Position	d.f.
7-NH_2SO, 6-Cl, 3-CH_2SCH_2- 	5	0·88	2·9				
6-Cl	5	1·6	3·3	4·8†	1·40	12·4†	1·41
7-Cl	5	0·98	5·9	2·8(NS)	1,34	11·1‡	1,35
7-NH_2SO, 6-Cl, 3-CH_3	5	1·1	9·3	0·11(NS)	1,34	36·1‡	1,35
7-NH_2SO_2, 6-Cl	5	1·0	10·9	0·06(NS)	1,35	0·57(NS)	1,36
7-Cl, 3-CH_3	5	1·6	7·7	1·4(NS)	1,35	4·33(NS)	1,36
Unsubstituted	5	3·8	6·3	1·9(NS)	1,30	25·9‡	1,31

* See text for description of binding parameters. All experiments done in 0·067 M phosphate buffer, pH 7·4, at 3°, with human albumin solutions, 2 g/l., prepared from crystallized albumin. Abbreviations: d.f. = degrees of freedom; NS = not significant.

† $P < 0{\cdot}05$.

‡ $P < 0{\cdot}01$.

TABLE 2. RELATION OF PHYSICAL CHARACTERISTICS AND BIOLOGIC ACTIVITY OF BENZOTHIADIAZINE ANALOGS

1,2,4-Benzothiadiazine substitution	pKa	Octanol partition coefficient (C)	C'*	pA_2†	M	Slope P	K‡ $\times 10^{-5}$
6-Cl	7·80	10·4	36·5	4·92	5	1·6	3·3
7-Cl	7·98	13·0	62·4	4·12	5	0·98	5·9
7-Cl, 3-CH_3	8·74	12·0	273·0	5·11	5	1·6	7·7
Unsubstituted	8·55	1·5	22·7	3·62	5	3·8	6·3

* $C' = C(1 + 10^{pKa - pH})$.
† Reference 20.
‡ Experiments done in 0·067 M phosphate buffer, pH. 7·4, at 3°.

Mechanism of binding of diazoxide to albumin. In order to define the mechanism of benzothiadiazine binding to albumin, the binding of diazoxide was examined under a variety of conditions. Diazoxide exists as a 4-H resonance hybrid in acidic and in electrophilic solvents.[6] The two tautomeric forms of the drug are shown in Fig. 2.

pH. The effect of pH on the binding of diazoxide to albumin was examined at two temperatures. At 3°, binding of diazoxide to albumin increases from pH 6·0 to 8·5, but then decreases at pH 10·5 (Fig. 3). At 37°, changes in pH have no significant effect on diazoxide binding.

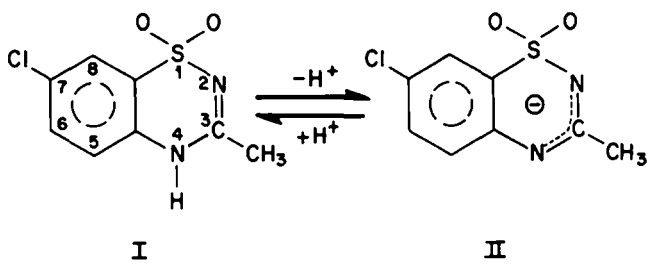


FIG. 2. Dissociation of 4—H diazoxide tautomer into anionic form.

Deuteration of phosphate buffer. When the phosphate buffer was prepared in 95% deuterated water, diazoxide binding to solutions of crystalline albumin was depressed at free diazoxide concentrations $> 8 \times 10^{-6}$ M (Fig. 4).

Temperature. Figure 5 shows the effects on diazoxide–albumin binding of increasing temperature at three pH levels. At pH 8·5, binding decreases significantly as temperature is raised stepwise from 3° to 15° to 25° to 37°. At pH 7·4, the temperature-induced decrease is still significant but is not as marked as at pH 8·5. There is no significant influence of temperature on binding at pH 6·0.

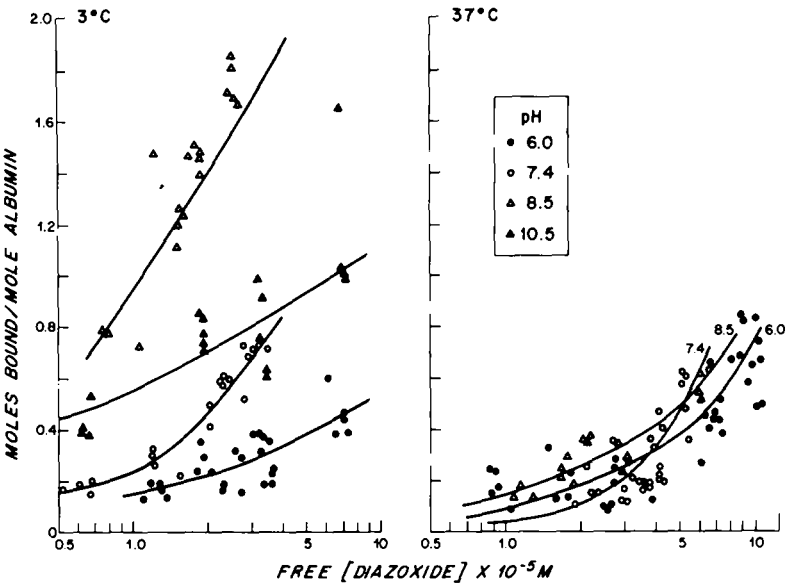


FIG. 3. Influence of pH on diazoxide binding to albumin. Studies conducted in 0·067 M phosphate buffer.

Ionic strength. Table 3 summarizes the diazoxide–albumin binding parameters at three ionic strengths of the buffer. The fitted curves for binding at a total ionic strength of 0·19 and 0·54 M are parallel but differ in position ($P < 0·01$). The increase in K indicates that the binding curve is shifted to the right and that binding is decreased by increasing total ionic strength from 0·19 to 0·54 M. In contrast, a further increase of ionic strength to 1·08 M increases binding, as reflected in a significant

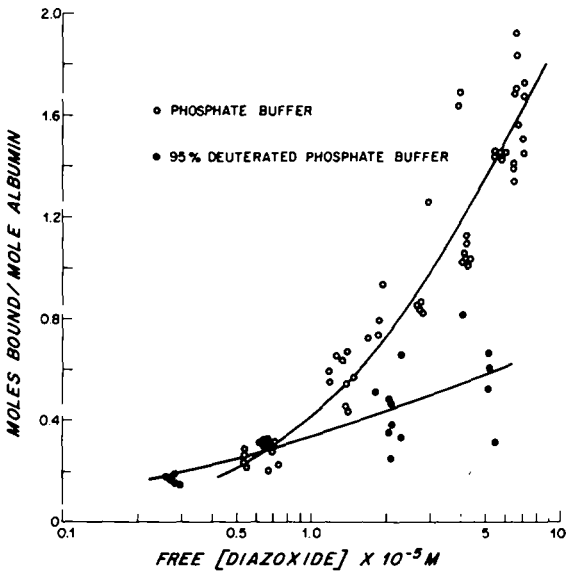


FIG. 4. Influence of deuteration of phosphate buffer on diazoxide binding to albumin. Studies conducted at 3° in 0·067 M phosphate buffer, pH 7·4.

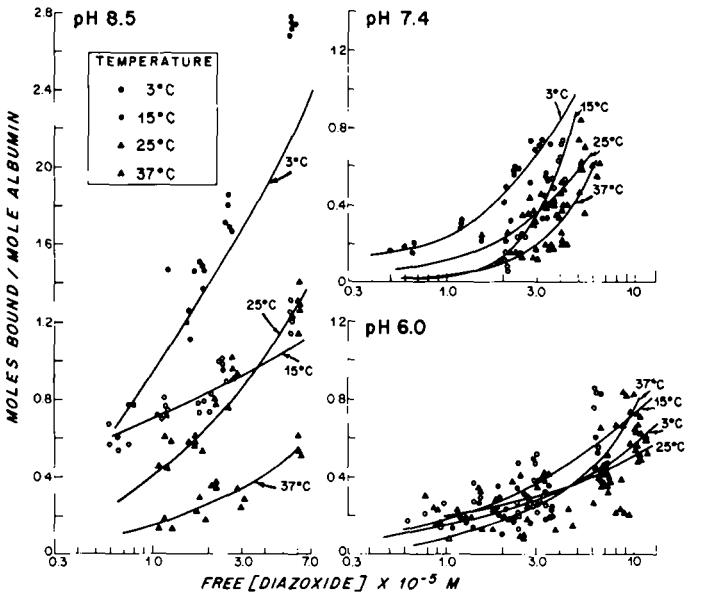


FIG. 5. Influence of temperature on diazoxide albumin binding in 0·067 M phosphate buffer.

change of the slope (P) of the curve ($P < 0{\cdot}01$). Although these changes are significant, they represent only small changes (*ca.* 10 per cent) in amount of drug bound.

Sodium, potassium and calcium. The effects of monovalent and divalent cations on diazoxide binding to albumin were studied at 3° and pH 7·4. Diazoxide binding in 0·067 M phosphate buffer under the above conditions was not significantly different with three ratios of Na^+/K^+ (0·83, 8·3, 23).

Parameters of the binding of diazoxide to albumin in 0·1 M Tris buffer at pH 7·4 and 3° were: $P = 1{\cdot}0$, $K = 1{\cdot}6 \times 10^{-4}$ M, $M = 5$ in the absence of calcium; and $P = 1{\cdot}1$, $K = 3{\cdot}3 \times 10^{-4}$ M, $M = 5$ in the presence of 10^{-4} M calcium. Since the slopes of the two curves (given by P) are the same but the curves differ in position (given by K) along the abscissa, calcium significantly depresses diazoxide binding to albumin.

TABLE 3. INFLUENCE OF IONIC STRENGTH ON DIAZOXIDE BINDING TO ALBUMIN

Ionic strength (M)	Slope M	Slope P	Position $K \times 10^{-4}$ M	Variance ratio* Slope	Variance ratio* Position
0·19	3	1·1	0·9	0·05(NS) (1,27)	13·3† (1,28)
0·54	3	1·2	1·1		
1·08	3	1·6	1·9	9·1† (1,26)	

* See text description of binding parameters. NS = not significant. Degrees of freedom are in brackets below each variance ratio.

† $P < 0{\cdot}01$.

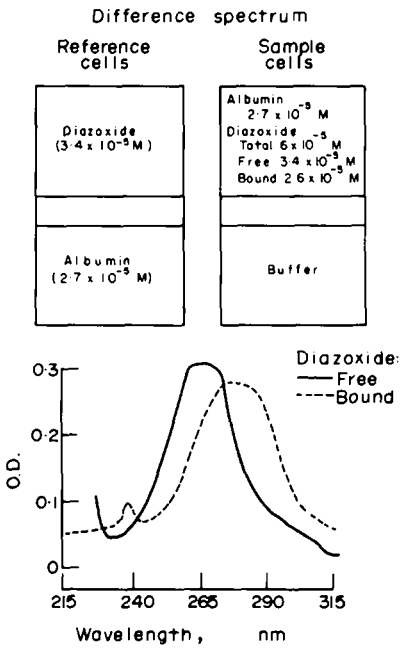


FIG. 6. Ultraviolet spectra of free and bound diazoxide. Studies conducted in 0·067 M phosphate buffer, pH 7·4, at 25° by difference spectroscopy.

Viscosity. The effect of diazoxide (1·0 to 10·0 × 10^{-5} M) on the viscosity of 7·0 × 10^{-5} M human albumin solutions was studied at 30·0° in a Cannon–Ubbelohde semi-micro dilution viscometer. No changes in viscosity were found.

Difference spectroscopy. The spectra of equal concentrations of diazoxide in free and bound forms were compared at pH 7·4 by ultraviolet difference spectroscopy (Fig. 6). Binding to albumin shifts the diazoxide absorption maximum from 268 to 278 nm and decreases the drug extinction coefficient. The maximum difference in absorption occurs at 278 nm.

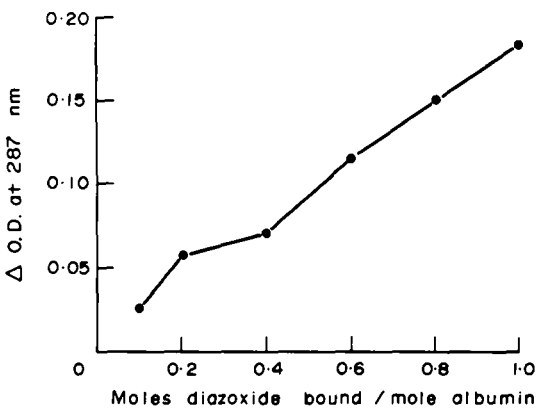


FIG. 7. Diazoxide–albumin difference spectral changes. Studies conducted in 0·067 M phosphate buffer, pH 7·4, at 25°.

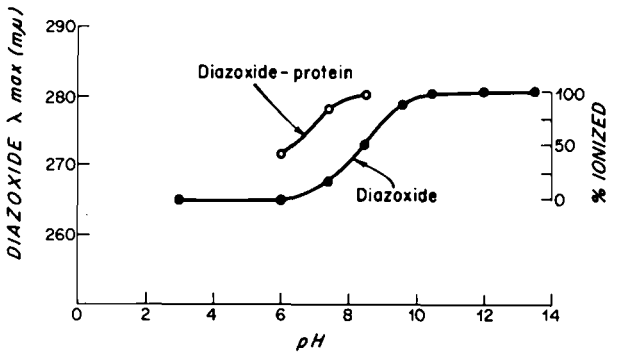


FIG. 8. Influence of pH and albumin binding on the position of the diazoxide ultraviolet absorption peak.

At pH 7·4, absorbance at 287 nm increases progressively with free drug concentration (Fig. 7). This increased absorbance reflects increased binding. Over the range of free drug concentrations that can be studied there is no evidence of a plateau of absorbance or saturation of binding. Qualitatively similar results were obtained at pH 6·0 and 8·5.

Figure 8 shows the wavelengths of maximum absorption by the bound form of diazoxide at pH 6·0, 7·4 and 8·5, and the position of the wavelength of maximum absorption as a function of pH in aqueous solution for free diazoxide. The pH associated with one-half the maximal wavelength shift for drug–protein is 6·4, which is 2·3 pH units less than the p*K*a of diazoxide in aqueous media.

DISCUSSION

Analysis of binding results. The interaction of small molecules with proteins has been the subject of extensive theoretical and experimental research.[16,21–25] The usual model of the drug–protein binding system[21,22] is based on the assumption that a protein has *n* independent non-interacting sites for ligand binding. If all the binding sites are identical in their dissociation constants, the binding isotherm can be characterized by the equation:

$$\bar{v} = n\frac{[D]}{[D] + K_d} \tag{1}$$

$\bar{v}$ = average number of moles of drug bound/mole of protein; $[D]$ = free drug concentration; K_d = dissociation constant in moles/liter. In this form, the two parameters that characterize drug–protein binding are the dissociation constant and number of binding sites.[22] Usually these parameters are determined from the slopes and intercepts of one of three linear transformations of the equation.[21–23,26] The most commonly used is the Scatchard plot.[26]

Binding results for benzothiadiazine and for many other drugs [24,27–30] do not fit the model of *n* independent binding sites. This is indicated by a curved line on the Scatchard plot. Such curved lines often appear to break near the abscissa, suggesting the existence of two classes of binding sites. One frequently used approach to curved lines on the Scatchard plot is to assume two classes of binding sites and to obtain

a best fit line to the experimental results, which yields average values for two association constants and two classes of binding sites.[31] Solutions to such two-term expressions are not unique and it is not certain that the number of binding sites and association constants derived in this fashion have physical significance. Thermodynamic quantities are commonly calculated from the apparent association constants,[25,32,33] but their meaning is not clear. Recent evidence suggests that these problems in analysis of binding results arise in part from the covalent structural "microheterogeneity" of albumin.[9,34,35] Defatted, deionized, depolymerized bovine albumin is a population of molecules that differ in acid-titration spectra and solubility.[10,34,35]

Since one cannot deduce the nature of the drug–protein binding process from experimental observations and since the heterogenous nature of albumin prevents devising a satisfactory *a priori* model for drug binding to albumin, an empirical but objective method of evaluating protein-binding results is the most useful approach at present. Desirable features for such a method of analysis are that it be mathematically simple, satisfy at least the majority of existing data, and have a form consistent with our expectations of a binding system. One function would be preferred over another if inspection of the variable values indicated the relative position, slope and height of the curve and if it could be easily adapted to include the special case of only one class of binding sites. These considerations argue against the use of polynomials and other more complex mathematical functions.[14,15] The conditions are met, however, by the logistic function[36], which is a generalization of the hyperbola (see Methods) and is similar to equations used at present to represent protein binding. The use of an empirical curve-fitting procedure for binding results precludes quantitative determination of the thermodynamic changes associated with binding. However, it is possible to reach valid and useful qualitative conclusions about the thermodynamic changes that occur and to infer the types of bonding between drug and protein from these changes.

Mechanism of diazoxide binding to albumin. At pH 6·0, diazoxide binding is not significantly altered by increasing temperature, suggesting that the free energy of binding is primarily derived from a change in entropy. In aqueous solvents, such an entropic change seems due to increased ordering of water structure that accompanies the binding of hydrophobic compounds to protein ("hydrophobic bonding").[37] Hydrophobic bonding is characteristic of the binding of many drugs to albumin.[25,29,32,38,39] The octanol partition coefficients of the benzothiadiazines and their low solubility in water indicate that the drugs are relatively hydrophobic (Table 2). At pH 7·4 and 8·5 binding is increased compared to that at pH 6·0, but at the higher pH levels binding is decreased by increasing temperature, and at 37°, is identical at all three pH levels. The exact contribution of entropic change at the higher pH levels cannot be stated. One interpretation of the increase in temperature-sensitive binding at pH 7·4 and 8·5 compared to that at pH 6·0 may be that the drug is participating in ionic or hydrogen bonding, which is decreased by increasing temperature. The residual binding at 37° at all pH levels would then be due to hydrophobic interaction.

Evidence for ionic bonding. Drugs which are bound to albumin by ionic bonding must be appreciably ionized, and their binding decreases with increasing ionic strength. Diazoxide is completely un-ionized at pH 6·0 and only 50 per cent ionized

at pH 8·5. Changes in phosphate buffer ionic strength and in sodium and potassium content have only a small effect on binding compared to temperature-induced changes. Thus, ionic binding is likely unimportant in the binding of diazoxide to albumin.

Evidence for hydrogen bonding. Substitution of deuterium for exchangeable protons on a drug should increase hydrogen bond strength.[40] Diazoxide binding is decreased by deuterium substitution, suggesting that hydrogen bonding may not play a role in diazoxide binding to albumin. However, deuterium substitution occurs not only on the drug but also in the protein. The altered intra-protein hydrogen bonds may cause conformational changes which may be responsible for the decrease in binding.

Strong evidence for the presence of hydrogen bond formation comes from the results of the difference spectroscopy studies. The binding of many anionic dyes to albumin is accompanied by changes in their absorbance spectra.[22] Usually these changes are easily followed because there is not significant absorption in the near-visible spectrum by pure albumin preparations. In contrast, the causes of spectral changes recorded by ultraviolet difference spectroscopy are more difficult to determine, since both drug and protein may absorb in the same spectral region. In the studies presented here, the size of the changes in optical density, the position of absorption peaks between 270 and 280 nm, the red shift in peak in maxima, and the absence of drug-induced viscosity changes in the protein solutions indicate that the difference spectral changes are due to perturbation of the drug spectra.[16,41–43] The wavelength of maximum absorption by diazoxide is pH dependent and can serve as an indicator of the drug form.[6] In neutral and acidic solutions, the 4-H resonance hybrid is the form primarily present.[6,20,44] In alkali and nucleophilic solvents, the drug exists in form II (Fig. 2).[6] In the presence of albumin, the pH associated with one-half of the maximal wavelength shift is 2·3 units less than in aqueous solvent alone. This shift indicates that the environment of the bound form of drug is more favorable for the formation of the form II tautomer.[6]

Three mechanisms could account for this shift. First, in the aqueous phase at pH 7·4 diazoxide exists predominantly in form I. The shift in double bond could be initiated by loss of the N-4 proton. The two electrons on N-4 would then be delocalized into the ring resonance system. This in turn would create a relative excess of electrons on N-2 conducive to H-bonding at N-2 with a proton donated from an amino acid on albumin (e.g. tyrosine, histidine, lysine). Second, the reaction could be initiated by a proton donated from the protein to N-2 in form I. Fixed substitutions on N-2 convert the drug to the 3-4 double-bond form.[6] As a primary mechanism this is less likely, since N-2 (form I) will be a poor proton acceptor. Electrons associated with a nitrogen in a heterocyclic ring are not readily available for H-bonding.[40,45] Third, it is possible that a concerted reaction occurs at both N-2 and N-4 or that some other perturbation of the drug produces these spectral changes. A shift in the acidity constant for the $(CH_3)_2N$ group of 5-dimethyl, amino-1-naphthalene sulfonyl conjugated to bovine serum albumin has been reported.[45,46,47] In the protein environment, the "pK" shifted from 3·99 to 1·67.[45] These authors interpreted the shift they observed as being due to hydrophobic bonding rather than to proton donation to the ring.

Evidence for other charge effects. Despite the fact that diazoxide is predominantly un-ionized and that there is little evidence for ionic bonding, the finding that binding

increases with increasing pH at 3° suggests that other charge factors on the protein play a role. As shown by optical rotatory dispersion, human albumin undergoes slight reversible conformational changes in the pH range 6 to 8·5.[48] These changes are not detectable as changes in viscosity. Between pH 6 and 8·5, accumulation of negative charge on the protein induces minor changes in conformation.[22,29,49] Above pH 9·5, as protons on tyrosines and 6-amino groups of lysine are lost, further accumulation of negative charge induces larger conformational changes and decreases drug binding.[25,29,30] Decreased diazoxide binding at pH 10·5 (3°) may reflect the charge-induced protein conformation change. However, the absence of pH effect on binding at 37° suggests that such minor pH-induced conformational changes in albumin are important only at lower temperatures and are not of major importance at lower pH levels.

Benzothiadiazines bind extensively to human albumin. This binding has fewer specific structural requirements than the diuretic, hyperglycemic or vascular smooth muscle-relaxing actions of this class of agents. The existence of discrete binding sites on albumin for benzothiadiazines cannot be demonstrated. An empirical but objective approach to the evaluation of binding results is to fit a logistic function to the experimental points with a least squares method. In this way the nature of the bonds between drug and protein and the factors that alter this binding can be examined. The binding of diazoxide to human albumin is due to hydrophobic interaction and hydrogen bonding.

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