MOLECULAR PHARMACOLOGY, 12, 279-290

A Nuclear Magnetic Resonance Study of the Kinetics of the Binding of the Renal Contrast Medium Acetrizoate to Albumin

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(Received August 6, 1975)

SUMMARY

RODRIGUES DE MIRANDA, J. F. & HILBERS, C. W. (1976) A nuclear magnetic resonance study of the kinetics of the binding of the renal contrast medium acetrizoate to albumin. *Mol. Pharmacol.*, 12, 279-290.

NMR line broadening and shift measurements in conjunction with dialysis binding experiments have been performed on the acetrizoate-bovine serum albumin (BSA) system. Two types of binding sites have been detected, for which the binding constants and the kinetic parameters have been determined. The association rate constants for the formation of both types of acetrizoate-BSA complexes appear not to be diffusion-limited. The kinetic parameters are discussed in relation to the renal tubular excretion of acetrizoate. The acetrizoate molecules bound to the second class of binding sites retain a considerable amount of freedom with respect to the albumin molecule. It is pointed out that some earlier NMR studies concerning the motional freedom of individual molecular groups of pharmacologically interesting molecules on protein surfaces should be regarded with caution.

INTRODUCTION

Protein binding of drugs, in particular by albumin, can interfere with a number of biophysical processes in the body (1-3). It may, for instance, have a profound influence on the pharmacokinetics of the drug involved. Two factors are important in this respect. First is the amount of drug bound to the protein, determined by the affinity constant and the concentrations of protein and drug, since albumin-bound drug is not susceptible to ultrafiltration or diffusion processes occurring, for instance, in kidney, liver, or brain (4, 5). Moreover, in general the fraction of bound drug is not directly involved in drug action (2). On the other hand, the drug-protein complex can serve as a drug depot; i.e., release of the drug from the complex will tend to maintain the free drug concentration (4, 6). This constitutes the second factor of importance, the dissociation rate of the drug-protein complex, which may affect the active transport processes in kidney, liver, or brain, for example.

Dynamic events occurring during protein-drug binding can be studied by magnetic resonance techniques. Exchange of the drug molecules between protein and solution may cause shifting and broadening of the NMR signals (7-9). The line broadening and shifts are determined by three independent parameters: the relaxation time of the drug molecule when bound to the protein, the exchange rate, and the difference between resonance frequencies of the protons of the drug molecule when bound to the protein or free in solution.

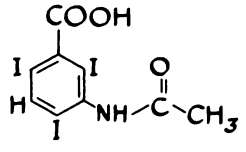
In the present paper the binding of the renal contrast medium acetrizoate (Trioignost) to bovine serum albumin has been studied by proton magnetic resonance. In

relation to its renal tubular excretion, it is of interest to know whether the dissociation rate of the acetrizoate-albumin complex is rate-limiting in the active excretion process (10, 11). Moreover, the strong binding of acetrizoate to albumin, compared to the weak binding of other contrast media of the triiodobenzoic acid family (10), raises the question of what part or group of the acetrizoate molecule may be responsible for its rather specific and strong binding to albumin.

Acetrizoate seemed to be particularly suited for a NMR study because of the simplicity of its NMR spectrum and because, according to Lang and Lasser (12), binding takes place predominantly at one site on the albumin molecule. During the course of this investigation it became evident from NMR as well as independent dialysis binding studies that there are at least two groups of binding sites on the albumin molecule. In spite of this complication, the dissociation rates of the different types of complexes could be estimated by combining NMR and equilibrium dialysis measurements. The present and other investigations also indicate that the interpretation of earlier NMR studies of pharmacologically interesting systems may be oversimplified.

MATERIALS AND METHODS

The acetrizoate (Triognost) used in the NMR as well as the dialysis experiments and the starting materal (2,4,6-triiodo-3-aminobenzoic acid) for the ^{14}C-labeled acetrizoate were kindly supplied by Dagra (Diemen, The Netherlands). Bovine serum albumin was purchased from Povite (Amsterdam). All other materials were obtained from Merck.



Acetrizoate

[^{14}C]Acetrizoate was synthesized by adding 40 mg of 2,4,6-triiodo-3-aminobenzoic acid to 4 mg of [1-^{14}C]acetic anhydride which had been dissolved in 5 ml of acetonitrile under vacuum. The acetonitrile was freshly distilled from P_2O_5; SO_3 was used as a catalyst. The radiochemical purity was checked by taking a radioautogram of a silica gel thin-layer chromatogram, developed in a solution of benzene–ethanol–acetic acid (7:1:2).

The binding of acetrizoate to albumin was determined in equilibrium dialysis experiments at 4° and 30° by measuring the radioactivity of the [^{14}C]acetrizoate, present in tracer amounts in the samples, on both sides of the membrane (13, 14). The samples were prepared by dissolving the required amounts of acetrizoate and albumin in H_2O containing 0.1 M KCl–0.05 M phosphate buffer, pH 7. The NMR samples were prepared in D_2O; the solutions were of the same composition and pH[1] as those used in the dialysis experiments.

The NMR experiments were carried out on a Varian XL-100 instrument equipped with F. T. facilities. The spectra were obtained either by accumulation in a Varian C1024 time-average computer or by accumulation using the F. T. technique. In the latter case a (180°-t-90°-T) pulse sequence was used in order to avoid dynamic range problems, connected with the limited word length of the computer memory, due to the residual HDO resonance (17, 18). Samples were freshly prepared just prior to the measurements.

THEORETICAL BACKGROUND

As will be shown in the next section, to a good approximation the binding of acetrizoate to BSA[2] can be described by two classes of independent identical sites on the albumin molecule. Exchange of the acetrizoate molecules bound to the protein and free in solution results in a transfer of magnetization between three different environments. The transition probabilities between these states can be defined as follows:

[1] It has been shown (15, 16) that at equal pH meter readings in H_2O and D_2O solutions, the degree of ionization of the protein is approximately equal. It is to be expected, therefore, that the protein will bind the same number of acetrizoate molecules in the dialysis and NMR experiments. Possible intrinsic isotope effects do not affect the conclusions below.

[2] The abbreviation used is: BSA, bovine serum albumin.

$$\tau_{IS}^{-1} = k_I^-; \qquad \tau_{SI}^{-1} = k_I^+ \sum_{j=0}^{n_I} (n_I - j)(PS_j)_I \quad \text{all configurations} \tag{1}$$

$$\tau_{IIS}^{-1} = k_{II}^-; \qquad \tau_{SII}^{-1} = k_{II}^+ \sum_{j=0}^{n_{II}} (n_{II} - j)(PS_j)_{II} \quad \text{all configurations}$$

where k_I^- and k_{II}^- are the dissociation rate constants for the complexes of types I and II, and k_I^+ and k_{II}^+ are the corresponding association rate constants, defined by the following reaction equation:

$$PS_j + S \underset{k^+}{\overset{k^-}{\rightleftharpoons}} PS_{j+1} \tag{2}$$

PS_j is a particular configuration of j molecules S bound to a certain class of binding sites on the protein molecule. The $\sum$ signs indicate a summation over all particles PS_j and over all their configurations present in solution. The definitions in Eqs. 1 implicitly assume that binding to the sites takes place independently and that the sites within a class are identical. Moreover, it is assumed that no direct exchange takes place between the binding sites of classes I and II.

The dissociation and association transition probabilities are related by the following equation:

$$\tau_{Si}^{-1} = \frac{P_0}{[S]} \bar{r}_i \, \tau_{iS}^{-1} \tag{3}$$

where $\bar{r}_i$ represents the average number of acetrizoate molecules bound per albumin molecule for a particular class i of binding sites, P_0 is the concentration of protein weighed into the sample, and $[S]$ is the concentration of free substrate molecules. Within the approximations mentioned above, i.e., independent and identical binding sites, the average number of molecules bound per albumin molecule for particular class of binding sites is given by (13, 19)

$$\bar{r}_i = \frac{n_i K_i[S]}{1 + K_i[S]} \tag{4}$$

where K_i is the equilibrium constant characterizing reaction Eq. 2 and n_i is the total number of sites of class i available for binding. The total average number of acetrizoate molecules bound to BSA is

$$\bar{r}_T = \bar{r}_I + \bar{r}_{II} = \frac{n_I K_I[S]}{1 + K_I[S]} + \frac{n_{II} K_{II}[S]}{1 + K_{II}[S]} \tag{5}$$

The NMR experiments were carried out with a large excess of substrate molecules, so that using Eq. 3, the dissociation rates can be directly related to the observed line broadening (20):

$$T_2^{-1} - T_{2S}^{-1} = \frac{P_0}{S_0} \{\bar{r}_I X_I + \bar{r}_{II} X_{II}\} \tag{6}$$

where T_2^{-1} equals $\pi \Delta\nu_{1/2}$, with $\Delta\nu_{1/2}$ being the observed linewidth at half-height of the substrate resonances in the presence of protein, T_{2S}^{-1} is the reciprocal transverse relaxation time of the substrate resonance in the absence of protein, and S_0 is the concentration of substrate weighed into the sample. The line broadening parameters X_I and X_{II} are concentration-independent. They are a function of the dissociation rate constant, τ_{iS}^{-1}, the relaxation rate of the nucleus in the substrate molecule while bound to the protein molecule, T_{2i}^{-1}, and the chemical shift difference $\Delta\omega_i$ between the resonance position free in solution and bound to one of the sites of class i; i.e. (20, 21),

$$X_i = \frac{\tau_{iS}^{-1}\{\tau_{iS} T_{2i}^{-1}(1 + \tau_{iS} T_{2i}^{-1}) + (\Delta\omega_i \tau_{iS})^2\}}{(1 + \tau_{iS} T_{2i}^{-1})^2 + (\Delta\omega_i \tau_{iS})^2} \tag{7}$$

In an analogous fashion, the observed changes in the resonance positions are related to the binding parameters by (20)

$$\delta = \frac{P_0}{S_0} \{\bar{r}_I \, \Delta_I + \bar{r}_{II} \, \Delta_{II}\} \tag{8}$$

with

$$\Delta_i = \frac{\Delta\omega_i}{(1 + \tau_{iS} T_{2i}^{-1})^2 + (\Delta\omega_i \tau_{iS})^2} \tag{9}$$

In practice expressions 7 and 9 can often be simplified. Recently Granot and Fiat (22) have systematically reviewed the possible simplifications of Eqs. 7 and 9. These results will not be reproduced here, but for the reader's convenience those limiting cases employed in the following sections are given.

Case I. Complexation of acetrizoate to

the specific binding site on albumin resulted in zero values for Δ_i (see RESULTS). Under these circumstances the following conditions apply:

$$\Delta\omega_i \ll T_{2i}^{-1} \quad \text{or} \quad \Delta\omega_i \approx 0$$

That is, Eqs. 7 and 9 can be simplified to

$$X_i = (\tau_{iS} + T_{2i})^{-1} \tag{10}$$

and

$$\Delta_i = \Delta\omega_i \tau_{iS}^2 (\tau_{iS}^{-1} + T_{2i}^{-1})^{-2} \tag{11}$$

Δ_i approaches zero when $\Delta\omega_i$ approaches zero and/or τ_{iS} is much larger than T_{2i}.

Case II. For the binding of azetrizoate to the second class of binding sites, we had no clue as to the relative values of T_{2i}^{-1} and and $\Delta\omega_i$. We therefore start by assuming that $\Delta\omega_i \approx T_{2i}^{-1}$. Since the temperature dependence of the line broadening indicates the existence of a fast exchange situation, i.e., $(\Delta\omega_i\tau_{iS})^2 \ll 1$, Eqs. 7 and 9 can be written as

$$X_i = (\tau_{iS} + T_{2i})^{-1} + \Delta\omega_i^2 \tau_{iS} \cdot(1 + \tau_{iS}T_{2i}^{-1})^{-2} \tag{12}$$

and

$$\Delta_i = \Delta\omega_i(1 + \tau_{iS}T_{2i}^{-1})^{-2} \tag{13}$$

Since in the present circumstances

$$(\tau_{iS}T_{2i}^{-1})^2 \ll 1,$$

these equations can be simplified to

$$X_i = (\Delta\omega_i + \Delta)(2T_2\Delta\omega_i)^{-1} + \tau_{iS}\,\Delta_i\,\Delta\omega_i \tag{14}$$

and

$$\Delta_i = \Delta\omega_i(1 - 2\tau_{iS}T_{2i}^{-1}) \tag{15}$$

Fast exchange conditions applied to the limiting cases in which $T_{2i}^{-1} \ll \Delta\omega_i$ or $T_{2i}^{-1} \gg \Delta\omega_i$ lead to formulae which are not compatible with the observed line broadenings and shifts.

RESULTS

Concentration dependence. An example of the line broadening observed for the acetrizoate resonances as a result of binding to albumin is shown in Fig. 1. The lower half of the figure displays the spectrum of a 6 mM acetrizoate solution without albumin, and the upper half was recorded with 24 μM albumin present in the sample. The intense signal at about 430 Hz is due to residual HDO. The resonances of interest, at 58, 160, 260, and 780 Hz, were recorded on an expanded scale and at a higher amplification factor and were inserted in the figure at their corresponding frequencies. Proceeding from left to right, they represent the ϕ-H of acetrizoate, the methyl groups of tetramethylammonium chloride, the methyl group of acetrizoate, and the methyl groups of *tert*-butyl alcohol. The methyl resonances of tetramethylammonium chloride and *tert*-butyl alcohol served as a check on the field homogeneity and as internal references. Addition of albumin clearly shows line broadening of the acetrizoate resonances, while the reference signals remain virtually unchanged. For a series of solutions with constant acetrizoate concentration S_0, but increasing BSA concentration P_0, the linewidths were measured. Chemical shift changes δ were determined with respect to tetramethylammonium chloride. In all samples acetrizoate was present in large excess. The data thus obtained were plotted as a function of P_0/S_0. Figure 2 presents such a plot of T_2^{-1} as function of P_0/S_0 for the CH_3 and ϕ-H resonances of acetrizoate, while Fig. 3 shows analogous plots of δ. As expected on the basis of Eqs. 6 and 8, T_2^{-1} and δ increase linearly with increasing BSA concentration.

Before the slopes of the lines in Fig. 2 can be interpreted in terms of exchange and relaxation phenomena, it is necessary to show whether the broadening is due to specific or multiple binding. To this end a series of experiments, like those given in Fig. 2, were conducted with varying acetrizoate concentrations. The slopes of the lines, tan α, obtained in the individual experiments, turned out to be a function of the acetrizoate concentration. This points to the existence of binding sites on the albumin molecule which are not yet occupied in the acetrizoate concentration range studied in the NMR experiments. In view of the data of Lang and Lasser (12), this result was somewhat unexpected, and therefore a series of equilibrium dialysis experiments were carried out.

The results of these experiments, con-

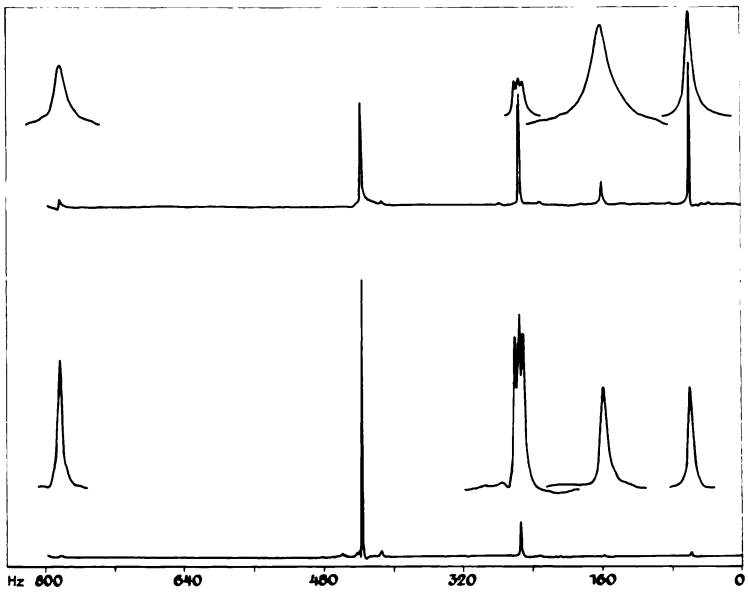


FIG. 1. *NMR spectra of acetrizoate (6 mM) in D_2O-phosphate buffer, pH 7.4*
Lower spectrum, without; upper spectrum, with albumin (24 μM). The resonances at 58, 160, 260, and 780 Hz were recorded on an expanded scale and at a higher amplification factor and inserted at their corresponding frequencies.

ducted at 4°, are given in Fig. 4, where the average total number of acetrizoate molecules $\bar{r}_T$ bound per albumin molecule is plotted against the logarithm of acetrizoate concentration free in solution. The points show that in a concentration region around 1 μM a first binding site is titrated. A second set of sites is titrated at much higher concentrations, from 1 to 10 mM, and does not show any saturation up to the highest concentration studied. On the assumption that two different classes of binding sites are present on the albumin molecule, a curve was fitted to the experimental points (solid line in Fig. 4), using Eq. 5 to describe the binding. To this end a computer fitting procedure was employed, based on a gradient method for the nonlinear parameter K and a linear regression method for the linear parameter n. The resulting binding parameters, together with their standard errors, are given in Table 1 for two series of experiments, at 4° and 30°. From Fig. 4 it can be concluded that in the concentration range in which the NMR experiments were conducted (1–20 mM) there are at least two types of complexes present in solution. Moreover, in this concentration range, the first class of binding sites is saturated; i.e., $\bar{r}_I = 0.9$ throughout the concentration range of acetrizoate explored in the NMR experiments. The values of tan α obtained at 4° and 30° are summarized in Table 2, together with the experimentally determined values of $\bar{r}_T$. The chemical shift data were evaluated in an analogous fashion; i.e., the slopes of the lines, tan β, obtained by plotting δ vs. P_0/S_0 (see Fig. 3) were determined for different concentrations of acetrizoate and are listed in Table 2.

Evaluation of line broadening parameters. According to Eq. 6,

$$\tan \alpha = \bar{r}_I X_I + \bar{r}_{II} X_{II} \qquad (16)$$

The binding experiments (see Fig. 4) have shown that in all NMR experiments $\bar{r}_I = 0.9$, so that

$$\tan \alpha = 0.9 X_I + (\bar{r}_T - 0.9) X_{II} \qquad (17)$$

Plotting tan α vs. $(\bar{r}_T - 0.9)$ should yield a straight line with a slope given by X_{II}, and an intercept given by $0.9\, X_I$. The experimental results obtained at 4° and 30° are given in Fig. 5 for both the CH_3 and the ϕ-H protons. A least-squares fit to the points

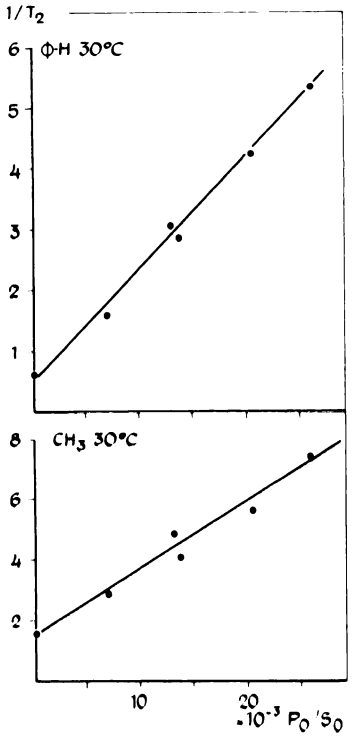


FIG. 2. *Plot of T_2^{-1} of ϕH and CH_3 protons of acetrizoate as a function of ratio of amounts of protein (P_0) and substrate (S_0) weighed into sample*

The acetrizoate concentration was kept constant at 3.5 mM. Each point is an average of at least six independent linewidth measurements. Temperature, 30°. The solid lines represent least-squares fits to the experimental points, based on Eq. 6.

results in the values for X_I and X_{II} given in Table 3.

It should be noted in passing that, independently of the interpretation of the binding curve (Fig. 4), the NMR data are inconsistent with the assumption of one class of binding sites for acetrizoate on the albumin molecule. If albumin were to possess only one class of independent and identical binding sites, the line broadening parameter $\tan \alpha/\bar{r}_T$ would be concentration-independent. Examination of Fig. 6, in which $\tan \alpha/\bar{r}_T$ of the phenyl protons, obtained at 30°, is plotted as a function of S_0, clearly reveals a concentration dependence, which is compatible only with the existence of more than one class of binding sites. The line drawn through the points has been calculated using the expression

$$\frac{\tan \alpha}{\bar{r}_T} = \frac{0.9X_I + (\bar{r}_T - 0.9)X_{II}}{\bar{r}_T} \quad (18)$$

The $\bar{r}_T$ values were taken from Fig. 4, and X_I and X_{II}, from Table 3.

Except for class I at 4°, where the standard errors in the line broadening parameters are too large, it can be concluded that the values of X_i for the aromatic and methyl protons are significantly different from each other for both classes of binding sites (see Table 3).

Evaluation of shift parameters. According to Eq. 8, $\tan \beta$ is related to the chemical shift parameter Δ_i as

$$\tan \beta = 0.9\,\Delta_I + (\bar{r}_T - 0.9)\,\Delta_{II} \quad (19)$$

Plotting $\tan \beta$ vs. $(\bar{r}_T - 0.9)$ should yield a straight line with a slope given by Δ_{II} and an intercept by $0.9\,\Delta_I$. The experimental results obtained at 4° and 30° are given in

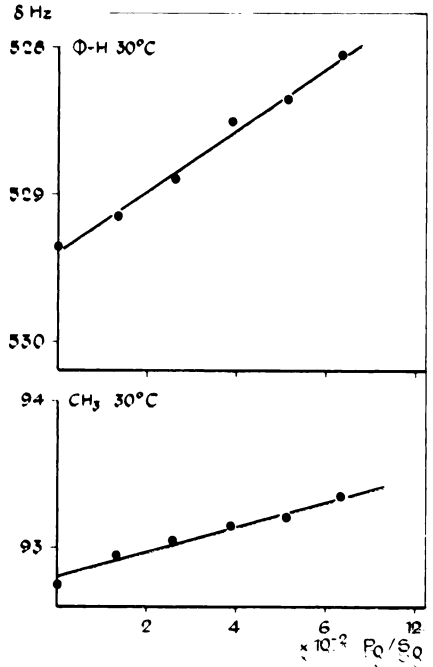


FIG. 3. *Plot of chemical shift values δ of Φ-H and CH_3 protons of acetrizoate as a function of ratio of amounts of protein (P_0) and substrate (S_0) weighed into sample*

Each point is an average of at least six independent shift measurements. The acetrizoate concentration was 3.5 mM; temperature, 30°. The solid lines represent least-squares fits to the experimental points, based on Eq. 8.

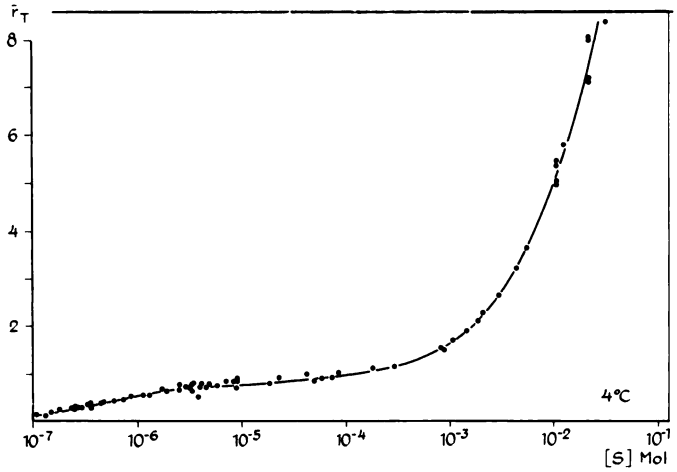


FIG. 4. *Average total number of acetrizoate molecules $\bar{r}_T$ bound per albumin molecule, plotted as a function of acetrizoate concentration free in solution*

The points were obtained from equilibrium dialysis experiments conducted at 4°. The solid line is a least-squares fit to the experimental values, based on Eq. 5.

TABLE 1

Parameters for binding of acetrizoate to BSA

The values in parentheses are standard errors.

Temperature	Class I		Class II	
	n_I	$K_I \times 10^{-5}$	n_{II}	K_{II}
		M^{-1}		M^{-1}
4°	0.85 (0.02)	16.0 (0.8)	12 (1)	60 (10)
30°	0.98 (0.02)	4.6 (0.2)	9 (1)	120 (20)

TABLE 2

Linewidth and chemical shift parameters of acetrizoate-BSA complex

The values in parentheses are standard errors.

Temperature	S_0[a]	$\bar{r}_T$[b]	Linewidth		Chemical shift	
			tan α (ϕ-H)	tan α (CH_3)	tan β (ϕ-H)	tan β (CH_3)
	mM					
30°	1.0	2.0	93 (3)	102 (8)	30 (3)	16 (5)
	2.0	2.8	157 (16)	146 (10)		
	3.5	3.7	207 (18)	219 (15)	103 (10)	43 (5)
	6.0	4.7	282 (20)	265 (12)	114 (13)	62 (15)
	10.0	6.1	376 (28)	318 (11)	148 (7)	59 (17)
	15.0	7.5			208 (11)	87 (8)
	20.0	8.7	609 (22)	516 (30)	253 (22)	101 (38)
4°	1.0	1.7	85 (4)	114 (9)	22 (3)	10 (3)
	2.0	2.2	179 (10)	134 (18)		
	4.0	3.1	234 (15)	216 (42)	31 (8)	15 (4)
	6.5	4.0	485 (22)	393 (25)	60 (7)	12 (2)
	10.0	5.1	565 (11)	457 (41)	95 (8)	32 (7)
	18.4	7.0	896 (13)	651 (39)	126 (22)	28 (23)

[a] S_0 is the total concentration of acetrizoate.

[b] $\bar{r}_T$ is the total average number of acetrizoate molecules bound per albumin molecule.

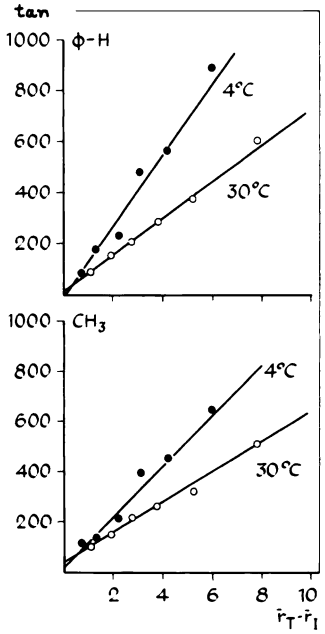


FIG. 5. *Plot of tan α vs. average number of acetrizoate molecules bound to second class of binding sites,* $\bar{r}_T - \bar{r}_I$

Each point is the slope of a line representing the T_2^{-1} dependence on P_0/S_0, of which examples are given in Fig. 2. The solid lines are least-squares fits based Eq. 17.

TABLE 3

Line broadening (X) and chemical shift (Δ) parameters of acetrizoate-BSA complex

The values in parentheses are standard errors.

Class of binding sites	Group	X		Δ	
		4°	30°	4°	30°
		sec^{-1}		sec^{-1}	
I	CH_3	27 (20)	43 (10)	0 (10)	0 (10)
	ϕ-H	0 (20)	15 (10)	0 (5)	0 (5)
II	CH_3	100 (12)	59 (3)	4 (2)	12 (2)
	ϕ-H	141 (14)	72 (4)	20 (3)	32 (2)

Fig. 7 for both the CH_3 and the ϕ-H protons. A least-squares fit to the points results in values for Δ_I and Δ_{II} given in Table 3. Examination of these data shows that the shift parameters Δ_I of the first class of binding sites do not differ from zero within experimental error for both the CH_3 and the ϕ-H protons. On the other hand, the Δ_{II} values of the second class of binding sites do differ appreciably from zero. Moreover, these values decrease significantly with decreasing temperature.

Determination of dissociation and relaxation rates. As indicated above, no chemical shift changes occur upon binding of acetrizoate to the specific binding site (class I) of BSA. Consequently Eq. 10 will be used to interpret the line broadening parameters. At 30° the X_I values of the phenyl and methyl protons are signifi-

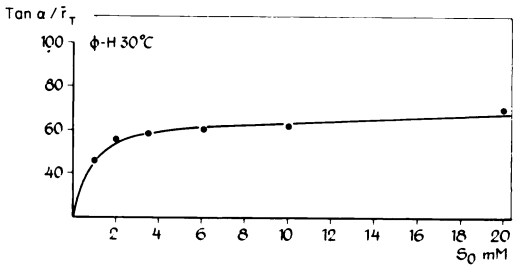


FIG. 6. *Plot of tan* $\alpha/\bar{r}_T$ *vs.* S_0 *for ϕ-H proton*

Temperature, 30°. The solid line was calculated according to Eq. 18 (see the text).

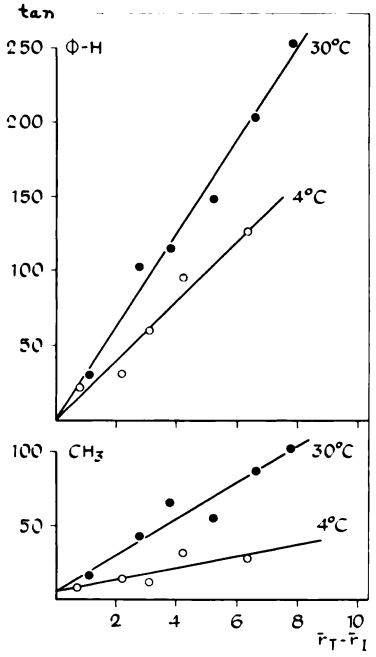


FIG. 7. *Plot of tan β as a function of average number of acetrizoate molecules bound to second class of binding sites,* $\bar{r}_T - \bar{r}_I$

Each point is the slope of a line representing the δ dependence on P_0/S_0, of which examples are given in Fig. 3. The solid lines are least-squares fits based on Eq. 19.

cantly different (see Table 3). In view of Eq. 10, this inequivalence is indicative of a situation in which only the relaxation rate T_{2I}^{-1}, or both the exchange rate τ_{IS}^{-1} and the relaxation rate T_{2I}^{-1}, contribute to the value of X_I. If the lifetime of the albumin-acetrizoate complex were much longer than the transverse relaxation times T_{2I} of the acetrizoate protons when bound to the albumin, the line broadening parameters X_I for the ϕ-H and CH_3 protons should be equal, contrary to what is observed.

The lifetime of the acetrizoate-BSA complex of type I is estimated as follows. First, from Eq. 10 and the X_I values at 30° (Table 3), it immediately follows that $\tau < 0.025$ sec. Second, by making a reasonable estimate of the upper limit of T_{2I} (CH_3), a lower limit of the lifetime of the specific acetrizoate-albumin complex can be computed. The T_{2I} (CH_3) value can be derived from Fig. 1 of a paper of Werbelow and Marshall (23). To this end we assume that the acetrizoate molecule is immobilized on the protein surface, while the methyl group retains the motional freedom around its axis. Taking the rotational correlation time τ_c of BSA equal to 10^{-8} sec (24) and the rotational correlation time τ_{int} for the methyl group rotation around its axis equal to 0.5×10^{-11} sec (25), we find T_{2I} (CH_3) = 10^{-2} sec. This estimate of T_{2I} (CH_3) is not very dependent on the choice of τ_{int}, but it is sensitive to changes in τ_c. The value of 10^{-8} sec chosen for the rotational correlation time τ_c of the protein can be considered a lower limit, since the rotational correlation times of BSA have been found to be up to one order of magnitude higher (24). Higher values of τ_c lead to smaller values of T_{2I} (CH_3), so that $\tau_{IS} > 0.015$ and consequently, at 30°,

$$30 < k_I^- < 80 \text{ sec}^{-1}$$

where the experimental errors (Table 3) have been taken into account. It should be noted that within these limits τ_{IS} is of the same order of magnitude as T_{2I} (ϕ-H) and smaller than T_{2I} (CH_3). This is in accordance with the temperature dependence of the line broadening parameters X_I (CH_3) and X_I (ϕ-H). It is well known that the temperature coefficients of τ^{-1} and T_2^{-1} have opposite signs; i.e., τ^{-1} has a positive and T_2^{-1} a negative temperature coefficient (7). Since X_I is found to increase with increasing temperature, this indicates the importance of τ_{IS} in the right-hand side of Eq. 10, in agreement with the findings above.

The nonspecific acetrizoate-albumin complex (class II) presents a different picture. The X_{II} values have negative temperature coefficients. Moreover, definite changes of the resonance positions δ are observed upon binding of acetrizoate to albumin. The line broadening parameters X_{II} and the chemical shift parameters Δ_{II} of both the methyl and the phenyl protons and their temperature dependence can be described by Eqs. 14 and 15 and their temperature derivatives. These form a set of eight equations with eight unknowns which can be solved using the results in Table 3. It turned out that these parameters could be varied only slightly within their error limits to give physically acceptable solutions for τ_{IIS}, T_{2II}, and $\Delta\omega_{II}$ for both the methyl and the phenyl protons. Subsequently the values obtained for the latter parameters were substituted in the more general Swift and Connick Eqs. 7 and 9, and varied in such a way that the differences between the calculated and observed values of X_{II} and Δ_{II} became minimal. This resulted in the following lifetimes of the acetrizoate-albumin complexes (class II):

At 4° τ_{IIS} = 2–2.5 msec
At 30° τ_{IIS} = 0.4–0.6 msec

These results imply that there is a relatively large exchange contribution to the line broadening parameter X_{II}. However, as would be expected from the temperature dependence of the line broadening parameters, the T_2 parameters do dominate; i.e., at 30°,

T_{2II} (ϕ-H) = 17–23 msec
T_{2II} (CH_3) = 16–18 msec

At 4° these values are about halved. The $\Delta\omega$ values are indeed found to be of the same order of the T_{2II}^{-1} values, in accordance with the assumptions leading to Eqs. 14 and 15; i.e.,

$\Delta\omega$ (ϕ-H) = 220 rad/sec
$\Delta\omega$ (CH_3) = 80 rad/sec

DISCUSSION

Mode of binding of acetrizoate at type II binding sites. From the results above we find for the spin-spin relaxation rates of the methyl and the phenyl protons at 30°

$$T_{2II}^{-1}\ (\phi\text{-H}) = 50 \pm 10\ \text{sec}^{-1}$$
$$T_{2II}^{-1}\ (CH_3) = 59 \pm 4\ \text{sec}^{-1}$$

The spin-spin relaxation time is determined by the correlation time τ_c, which is characteristic of the tumbling time of the molecule or of an individual molecular group, and by the distances between the nucleus considered and magnetic moment-bearing nuclei in the vicinity. Thus the T_2 values of different groups of acetrizoate may yield information about their mobility with respect to the BSA molecule.

In the bound state the spin-spin relaxation time of the aromatic proton will be determined predominantly by protein nuclei at an unknown distance. Thus little can be said about the mobility of the phenyl group from T_2 values alone. However, the ratio of the spin lattice relaxation time T_1 to the spin-spin relaxation time T_2 is only a function of τ_c. In a temperature- and frequency-dependent study of the binding of sulfonamide to carbonic anhydrase, Navon and Lanir (26) have shown that these ratios yield reliable τ_c values. For this reason spin lattice relaxation rates T_1^{-1} have been determined under the same conditions used for the T_2 experiments. For T_1^{-1} a formula analogous to Eq. 6 can be derived. T_1 measurements at various BSA and acetrizoate concentrations yielded a value of T_{1II}^{-1} (ϕ-H) = 10 sec^{-1} at 30°. From the ratio $T_{1II}/T_{2II} = 5$ we obtain $\tau_c = 3 \times 10^{-9}$ sec at 30°. T_1 measurements of the methyl group exhibited only slight changes with respect to the nonbonded situation, except for the disappearance of the nonexponential behavior (25). As a result the T_{1II} (CH_3) could not be established with sufficient accuracy and the correlation time was directly calculated from the T_{2II} (CH_3), yielding a value of 3×10^{-9} sec. Again Fig. 1 of the paper of Werbelow and Marshall (23) was used, while assuming that the rotation of the methyl group around its axis is not restricted. The results are listed in Table 4 together with the values for nonbonded acetrizoate and albumin. Examination of Table 4 shows that although the mobility of the phenyl group of acetrizoate is slowed down as a result of binding to the type II binding sites, it still has a considerable amount of motional freedom with respect to the BSA molecule. The τ_c value obtained for the methyl group is equal to the value obtained for the phenyl group, which indicates that the phenyl group and the methyl group have about equal mobility on the surface of the protein, except for the rotation of the methyl group around its axis.

TABLE 4

Correlation times at 30°

Substance	Correlation time
	sec^{-1}
Albumin[a]	$(2\text{-}20) \times 10^{-8}$
Acetrizoate bound to type II binding sites	
CH_3	3×10^{-9}
ϕ-H	3×10^{-9}
Acetrizoate free in solution[b]	
CH_3	8×10^{-12}
ϕ-H	2.4×10^{-10}

[a] From Wallach (24).

[b] From Rodrigues de Miranda and Hilbers (25).

Influence of binding of acetrizoate to albumin on renal excretion. At clinical doses of 200 mg of acetrizoate per kilogram of body weight, plasma levels up to 1 mM occur (10). At this concentration about 70% is bound to bovine albumin [and probably somewhat less to human albumin (12)], of which about three-quarters is bound to the first specific binding site. Above, the dissociation rate of type I complexes was found to be in the region $30 < k_I^- < 80\ \text{sec}^{-1}$, which corresponds to a lifetime of the albumin-acetrizoate complex between 0.03 and 0.012 sec. This is at least an order of magnitude less than the cortical transit time, which may vary from 0.3 to 3 sec (27, 28). Thus, in a first approximation, little influence of the dissociation rate on the active tubular excretion of acetrizoate is to be expected. However, such a conclusion should be regarded with some care. Although the dissociation rate is high with

respect to the reciprocal transit time, the high affinity constant of the acetrizoate-albumin complex results in a very high association rate constant which might compete with the excretion rate. Model calculations (29) show that in the region where the albumin complex lifetime equals the transit time the system is most sensitive to changes in dissociation rate and that, irrespective of the dissociation rate, protein binding decreases the amount of actively excreted drugs as long as the maximum transport capacity has not been reached.

CONCLUSIONS

It has been shown, by a combination of NMR and equilibrium dialysis experiments, that the rather complicated binding of acetrizoate to albumin can be examined in detail. Binding constants for two different classes of binding sites have been obtained.

The lifetime of the specific albumin-acetrizoate complex (class I) is in the region of 0.012–0.03 sec. This value is much less than the cortical transit time; further decrease of the lifetime of the complex will have little influence on the active renal excretion rate. Comparison of the kinetic parameters obtained for the two classes of binding sites shows that the difference in the corresponding binding constants is caused mainly by the difference in the association rate constants; i.e., the decrease in the binding constant of 4.6×10^5 M^{-1} for type I binding sites to 120 M^{-1} for type II binding sites comes mainly from the decrease of $k_1^+ \approx 2.5 \times 10^7$ sec^{-1} M^{-1} to $K_{II}^+ \approx 2 \times 10^3$ sec^{-1} M^{-1} rather than from an increase in the dissociation rate constants. Such a phenomenon has been observed earlier for sulfonamide binding to carbonic anhydrase (30). Hence also in the present study the association between protein and substrate cannot be described by diffusion-limited complex formation.

The mobility of the acetrizoate molecule when bound to the second class of binding sites could be estimated. In this case the correlation time of the acetrizoate molecule was an order of magnitude less than the correlation time of the protein, indicating an appreciable amount of motional freedom with respect to the protein.

The potential possibilities which NMR offers for studying the molecular dynamics of a substrate on protein surfaces have led to a number of line broadening studies of pharmacologically highly interesting systems. In these investigations line broadenings have often been interpreted to yield values of T_2^{-1} (bound) of the order of 10^4 sec^{-1}, which correspond to linewidths of the substrate resonances of the order of 10^3 Hz (31, 32). These values are improbably large, especially when one realizes that these linewidths exceed the total spectral width of most proteins hitherto observed at 100 MHz. Moreover, the individual resonances of protein residues, for instance, of hemoglobin, are of the order of 100 Hz (7), again indicating the unlikely high value of T_2^{-1} (bound) obtained in some of these binding studies. It should therefore be stressed that before interpreting linewidth alterations, the exchange limit has to be established. Moreover, it should be checked whether line broadening is a result of binding to only one, or one class of, binding site(s) in the concentration region studied in the NMR experiments.

ACKNOWLEDGMENTS

The technical assistance of A. J. G. M. Henderix and J. W. M. van Kessel is gratefully acknowledged.

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