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High Stereospecificity of the Benzodiazepine Binding Site on Human Serum Albumin

Studies with *d*- and *l*-Oxazepam Hemisuccinate

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SUMMARY

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Using *d*- and *l*-oxazepam hemisuccinate (RV 1208 and RV 1210), the stereospecificity of binding of benzodiazepines to human serum albumin was investigated. The interactions of the two enantiomers of oxazepam hemisuccinate with HSA were examined by gel filtration and circular dichroism measurements at pH 7.4 and 8.2. Both enantiomers, RV 1208 and RV 1210, were bound mainly to one binding site at pH 7.4, but there were great differences in the binding constants. Raising the pH to 8.2 decreased the affinity of RV 1208 and increased the affinity of RV 1210. In both enantiomers complex formation with HSA induced similar extrinsic Cotton effects, the signs of which were partly changed by increasing the pH to 8.2. No ligands other than tryptophan exhibit this high degree of stereospecificity of binding to HSA. A theory for the origin of the stereospecific binding is considered.

INTRODUCTION

Benzodiazepines are bound to human serum albumin principally at one binding site and in a very specific manner (1-3). Small changes of the chemical structure of the drugs can have a large influence on their binding to human serum albumin, and in some these effects are in contradiction to the commonly known influences of substituents on the binding behavior of other substances to human serum albumin (2). In this study we attempt to determine whether the specific binding site for benzodiazepines on the human serum albumin molecule exhibits stereospecificity.

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Measurement of circular dichroism was chosen for this study because the binding of benzodiazepines to human serum albumin produces typical biphasic extrinsic Cotton effects (1, 3, 4), and small changes of the ligand-protein complex can influence circular dichroism spectra (1, 3).

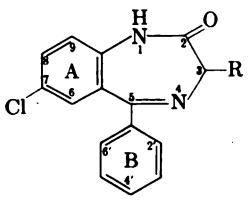
The isomers of oxazepam hemisuccinate have different biological actions; for example, their differences in antagonism of pentetrazole-induced convulsions (5, 6), anti-strychnine activity (6), plasma levels in mice after intravenous administration (5), and hydrolysis by a soluble esterase (7). Therefore they seemed useful for investigation of the stereospecificity of the benzodiazepine binding site of the human serum albumin molecule.

METHODS

Materials. Human serum albumin was obtained from Behringwerke, Marburg (*trocken, reinst;* electrophoretic purity, 100%). The benzodiazepine derivatives were obtained from the manufacturers: oxazepam, Boehringer, Ingelheim; and *d*- and *l*-oxazepam hemisuccinate sodium salts (RV 1208 and RV 1210), Ravizza, Milan. Their chemical structures are shown in Formula I. All other chemicals were of reagent grade. All solutions were prepared with deionized water.

RV 1208 and RV 1210 were almost completely hydrolyzed in 0.1 M NaOH or 0.1 M HCl within 2 hr, as detected by a total loss of their intrinsic optical activity. This hydrolysis could account for the results of Mussini *et al.* (5), who found differences in the blood levels of RV 1208 and RV 1210 given intravenously but not after oral administration. Possibly the drugs are rapidly hydrolyzed in the stomach. Under the conditions used in this work—i.e., M/15 phosphate buffer, pH 6.6–8.2—solutions of RV 1208 and RV 1210 did not show a decrease in intrinsic ellipticity during 12 hr after preparation.

Circular dichroism measurements. CD measurements were made at 27° with a Cary 61 CD spectropolarimeter calibrated with *d*-10-camphorsulfonic acid. All spectra were recorded in cylindrical cells with 10-mm path length, using a full-scale deflection of 0.02° or 0.05° θ and a spectral bandwidth of 2 nm. Results are expressed as molar ellipticities, $[\theta]$ (deg·cm^{-2} dmole^{-1}), calculated either with reference to the HSA concentration, using a molecular weight of 69,000, or with reference to the total drug concentration or the concentration of drug bound. The solutions for the CD measurements were prepared as described elsewhere (1). The final HSA concentration was always 13.1 μM (0.09%), and the drug concentrations were 20 and 50 μM. All solutions were made with M/15 phosphate buffer and adjusted to the desired pH with 1 M NaOH or 1 M HCl. Each CD spectrum reported is the average of three observations.



I

—R = —OH
Oxazepam

—R = —O—CO—CH_2—CH_2—CO—O^-·Na^+
d-Oxazepam hemisuccinate (RV 1208)
l-Oxazepam hemisuccinate (RV 1210)

The anisotropy factors (*g* values) were calculated according to Müller and Wollert (1) and Chignell (8).

Albumin binding measurements. Binding of benzodiazepines to HSA was studied by the gel filtration technique of Krieglstein and Kuschinsky (9) on 20 × 1.2 cm columns of Sephadex G-50 (fine, Pharmacia) at room temperature (22°) (2). The suitability of this method was verified by ultracentrifugation for high, medium, and low bound benzodiazepines (2). The HSA concentration was always 145 μM (1%), and drug concentrations ranged from 40 to 200 μM. All solutions were made with M/15 phosphate buffer and adjusted to the desired pH with 1 M HCl or 1 M NaOH. The binding data were plotted by the method of Scatchard (10), using the equation.

$$\frac{\bar{r}}{c_f} = kn - k\bar{r}$$

where $\bar{r}$ = number of moles of benzodiazepine bound per mole of HSA, c_f = concentration of free drug, k = association constant or single binding constant, n = number of binding sites of benzodiazepine derivatives on the HSA molecule, and kn = K_1 = total binding constant.

From the total binding constants, K_1, the free binding energy, ΔF, was calculated according to the relationship (11)

$$\Delta F = -RT \ln K_1$$

where R = gas constant and T = absolute temperature.

Ultraviolet measurements. Spectra were recorded with a Gilford spectrophotometer, model 2400.

RESULTS

Gel filtration studies. Both enantiomers of oxazepam hemisuccinate, RV 1208 and RV 1210, are bound to HSA at pH 7.4 (Fig. 1; β values are given in Table 1). However, the affinity of the protein is about 40 times greater for RV 1208 than for RV 1210 (see the total binding constants, K_1, in Table 1). RV 1208 is bound mainly to one binding site, whereas RV 1210 seems to have two binding sites on the HSA molecule (Table 1).

The binding of both substances is influenced by the pH of the solution (Fig. 2). There is a small increase in the amount of RV 1208 bound (β value) between pH 6.6 and 8.2 (Fig. 2). The binding of RV 1210 also is increased, but for this substance a higher increase in the β values can be seen between pH and 8.2 (Fig. 2). Although the binding of both enantiomers is increased at pH 8.2, and the total and single binding constants of RV 1210 are also increased, those of RV 1208 are decreased (Table 1).

Circular dichroism measurements. RV 1208 and RV 1210 display intrinsic Cotton effects (Fig. 3 and Table 2). Such symmetrical intrinsic CD spectra (Fig. 3) are typical for the enantiomers of an optically active substance (12). The wavelengths of the CD maxima of the substances are in good accord with the ultraviolet absorbance maxima (Fig. 3). The intensities of the intrinsic CD bands of RV 1208 and RV

TABLE 1

Influence of pH on binding data

K_1, total binding constants; n, number of binding sites on the HSA molecule; k, single binding constants; $-\Delta F°$, free binding energy; β, percentage of RV 1208 and RV 1210 bound at total concentration c = 150 μM and an HSA concentration of 1% in M/15 phosphate buffer, pH 7.4 and 8.2. The equations of the regression lines in the Scatchard plot at pH 8.20 are: for RV 1208, $\bar{r}/c_f$ = 212,703 − 137,278 $\bar{r}$; r = −0.9738; for RV 1210, $\bar{r}/c_f$ = 12,843, − 7,587$\bar{r}$; r = −0.9461, where r = correlation coefficient.

Substance	pH	K_1	n	k	$-\Delta F°$	β
		$M^{-1} \times 10^4$		$M^{-1} \times 10^4$	cal/mole	%
RV 1208	7.4	25.419	1.28	19.843	7295	90
RV 1210	7.4	0.729	1.80	0.405	5214	45
RV 1208	8.2	21.270	1.55	13.732	7191	92
RV 1210	8.2	1.284	1.69	0.759	5545	53

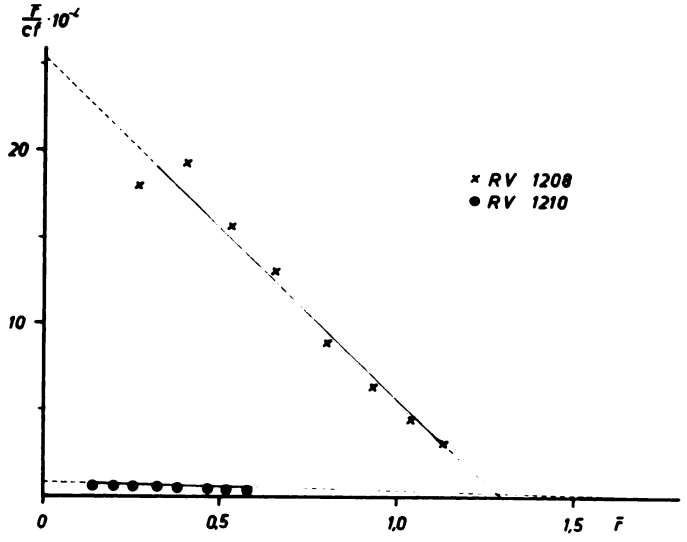


FIG. 1. *Scatchard plot of RV 1208 and RV 1210 binding to HSA*

Experiments were performed at pH 7.4 and an HSA concentration of 1%. Ordinate: $\bar{r}/c_f$ (liters per mole); c_f = molar concentration of the substances; $\bar{r}$ = moles of benzodiazepines bound per mole of HSA. Abscissa: $\bar{r}$. Each point represents the mean value of two experiments. The equations of the regression lines are: for RV 1208, $\bar{r}/c_f$ = 254,193 − 198,356$\bar{r}$; r = −0.9827; for RV 1210, $\bar{r}/c_f$ = 7290 − 4052$\bar{r}$; r = −0.9491, where r = correlation coefficient.

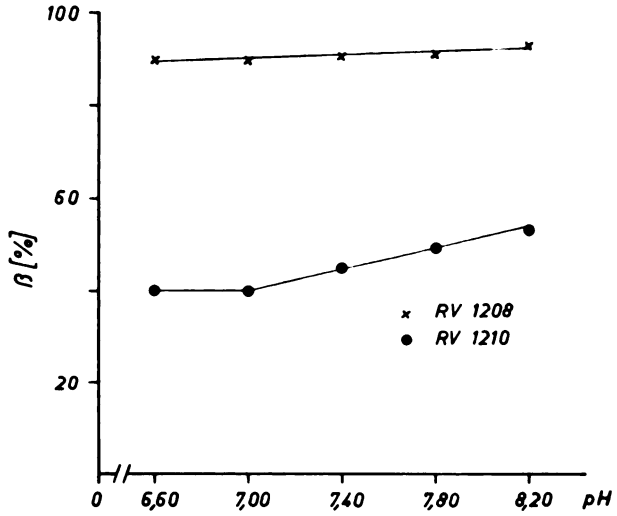


FIG. 2. *Influence of pH on percentage of RV 1208 and RV 1210 bound to HSA*

The pH dependence of RV 1208 and RV 1210 (150 μM binding to HSA (145 μM) is expressed as β. Each point represents the mean value of two experiments. Ordinate, percentage of drug bound, β; abscissa, pH.

1210 near 260 nm (Table 2) are smaller than the intensities of the extrinsic CD band near 260 nm of oxazepam bound to HSA (Table 2).

Extrinsic Cotton effects resulting from the binding of the ligands to human serum albumin are found to be superimposed upon the intrinsic Cotton effects of RV 1208 and RV 1210 (Fig. 4). The signs of the extrinsic Cotton effects are identical for RV 1208, RV 1210, and oxazepam; the only exception is the negative band of RV 1208 near 275 nm (Fig. 4).

The intensities of the extrinsic Cotton effects of RV 1210 are smaller than those of RV 1208; this can be seen mainly with small drug concentrations (Table 3). The extrinsic Cotton effects of RV 1208 and RV 1210 are smaller than those of oxazepam (Fig. 4). The wavelengths of the extrinsic CD maxima of all three substances are nearly identical (Fig. 4).

Increasing the pH from 7.4 to 8.2 influences the extrinsic CD maxima of RV 1208 at 325 and 275 nm and of RV 1210 at 323 nm (Table 3). In contrast, the CD bands of both substances at 260 nm are not changed (Table 3). The intrinsic CD spectra of RV 1208 and RV 1210 are not influenced by raising the pH to 8.2.

Ultraviolet difference spectroscopy measurements showed that the absorbance of RV 1208 and RV 1210 is not influenced between 350 and 250 nm by binding of the substances to HSA.

TABLE 2

Comparison of intrinsic Cotton effects of RV 1208 and RV 1210 and extrinsic Cotton effects of oxazepam

Molar ellipticity and anisotropy factors (intrinsic effects) of RV 1208 and RV 1210 were measured at a total concentration of 50 μM in M/15 phosphate buffer at pH 7.4. Molar ellipticity and anisotropy factors (extrinsic effects) of oxazepam (50 μM) in the presence of HSA (13.1 μM) in M/15 phosphate buffer at pH 7.4 were calculated with reference to the concentration of substance bound (3).

Substance	λ_{max} CD	$[\theta]$ $[\bar{x} \pm s_{\bar{x}} (n = 6)]$	g
	nm	$\times 10^{-4}$	$\times 10^{4}$
RV1208	237	−2.882 ± 0.035	−3.410
	253	+4.925 ± 0.063	+10.917
	273	+4.638 ± 0.067	+27.422
	313	−2.098 ± 0.020	−30.943
RV 1210	237	+2.925 ± 0.024	+3.521
	253	−3.870 ± 0.071	−8.699
	273	−3.807 ± 0.079	−22.799
	313	+1.823 ± 0.028	+27.143
Oxazepam	258	+7.31 ± 0.052[a]	+15.27
	317	−2.33 ± 0.015[a]	−25.69

[a] $n = 3$.

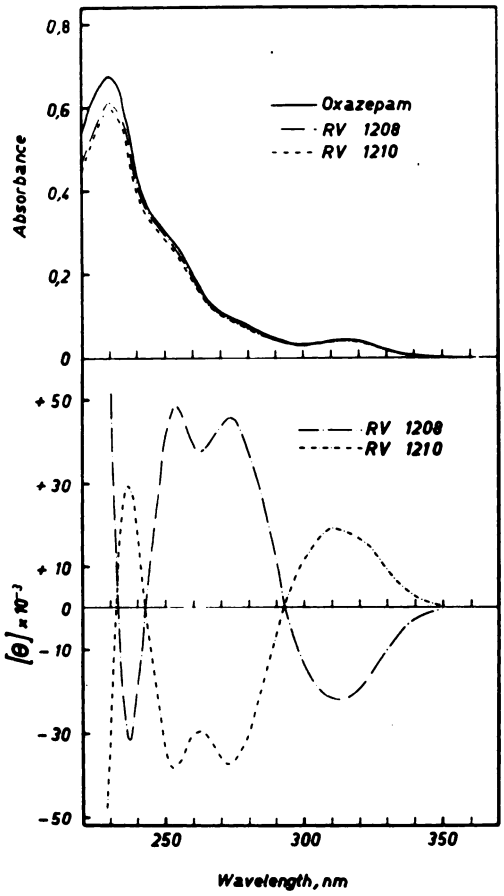
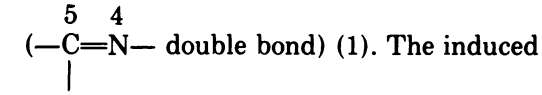


FIG. 3. *Comparison of ultraviolet and CD spectra*
Upper: ultraviolet spectra of oxazepam, RV 1208, and RV 1210 measured at a concentration of 20 μM in M/15 phosphate buffer, pH 7.4. Ordinate, absorbance; abscissa, wavelength.

Lower: intrinsic CD spectra of RV 1208 and RV 1210 measured at a concentration of 50 μM in M/15 phosphate buffer, pH 7.4. Ordinate; molar ellipticity calculated with respect to the concentrations of the two substances; abscissa, wavelength.

DISCUSSION

It is surprising that the *g* value of the extrinsic CD band of oxazepam at 258 nm is much higher than the *g* values of the intrinsic CD bands of RV 1208 and RV 1210 at 253 nm (Table 2). These observations could confirm our conclusion that the A benzene ring of the benzodiazepines is the primary binding moiety of the molecule (2). Schellman (13) noted that the intensity of intrinsic or extrinsic Cotton effects decreases as the distance between the asymmetrical center and the perturbed chromophore is lengthened. The ultraviolet band of the benzodiazepines at 260 nm is mainly due to electronic transitions in the A benzene ring of the substances (1). The intrinsic CD bands of RV 1208 and RV 1210 at 253 nm indicate that the two atoms are located between the asymmetrical center (C_3) and the perturbed chromophore (A benzene ring). The extrinsic CD band at 258 nm of the oxazepam-HSA complex could indicate that the distance between the chromophore (A-benzene ring) and the asymmetrical center of the protein is smaller than the bond distance of two atoms. This would be possible if the A benzene ring were the binding group of the benzodiazepine molecule and therefore were located very close to the asymmetrical center of the protein at or near the binding site. In case of the longer wavelength intrinsic CD bands of RV 1208 and RV 1210 at 313 nm the asymmetrical center (C_3) is located near the perturbed chromophore



(1). The induced optical activity of the oxazepam-HSA complex at this wavelength is smaller than the intrinsic activity of the two enantiomers (Table 2), as one would expect if the A benzene ring is the binding moiety of the molecules.

Previous experiments have shown that the binding of benzodiazepines to HSA is highly dependent on their structure (2). The very high stereospecificity of the binding of both enantiomers to HSA shown in this paper is surprising (Fig. 1 and Table 1). Only a few drugs are known to be bound stereospecifically to HSA, e.g., anionic azo dyes (14), warfarin (15), barbiturates (16), and an optically active analogue of clofibrate (17, 18). In each case the difference in binding between the isomeric forms is relatively small (15, 17, 18) and has been observed mostly at high drug to protein ratios (14, 16). Oxazepam hemisuccinate is the first drug known to exhibit such stereospecific binding mainly to one binding site of the HSA molecule.

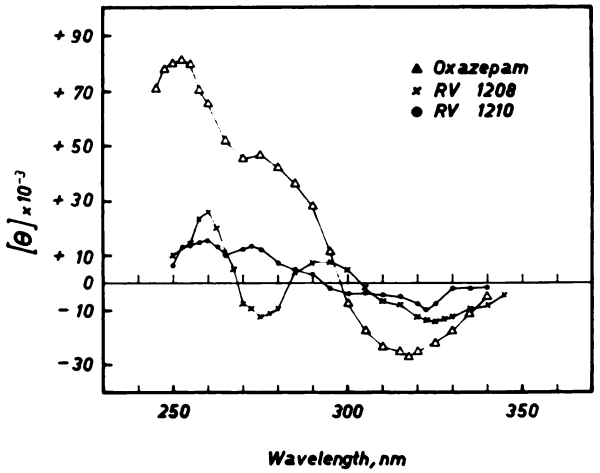


FIG. 4. *Induced optical activity of oxazepam and its two isomeric hemisuccinates*

Extrinsic CD spectra of oxazepam, RV 1208, and RV 1210 bound to HSA (13.1 μM) were measured at a concentration of 50 μM at pH 7.4. Each point represents the mean value of three experiments. In the case of oxazepam the contributions of HSA, and for RV 1208 and RV 1210 the contributions of HSA and the drugs themselves at the same wavelength, have been subtracted.

TABLE 3

Extrinsic Cotton effects of RV 1208 and RV 1210

Molar ellipticities ([θ]) of RV 1208 and RV 1210 in the presence of HSA (13.1 μM) in M/15 phosphate buffer, pH 7.4 and 8.2, were calculated with reference to the HSA concentration at the wavelength of the CD maxima (λ_{max} CD).

Substance	λ_{max} CD	[θ] $[\bar{x} \pm s_{\bar{x}} (n = 6)]$		p
		pH 7.4	pH 8.2	
	nm	$\times 10^{-3}$	$\times 10^{-3}$	
RV 1208, 50 μM	325	−10.774 ± 1.142	−2.510 ± 1.473	0.005
	275	−10.023 ± 2.900	+0.872 ± 2.615	0.02
	260	+28.927 ± 3.111	+35.569 ± 3.802	NS[a]
RV 1210, 50 μM	323	−7.634 ± 0.706	+5.004 ± 1.247	0.001
	273	+7.769 ± 2.750	+8.520 ± 1.818	NS[a]
	260	+11.760 ± 2.134	+12.517 ± 1.833	NS[a]
RV 1208, 20 μM	325	−9.412 ± 1.803[b]		
	275	−7.769 ± 1.803[b]		
	260	+23.547 ± 1.758[b]		
RV 1210, 20 μM	323	−2.750 ± 0.661[b]		
	273	+1.999 ± 1.638[b]		
	260	+4.508 ± 1.984[b]		

[a] Not significant ($p > 0.05$).

[b] $n = 3$.

The only substance known to bind stereospecifically to HSA in similar fashion is L-tryptophan, which has many times the affinity of the D isomer for primarily one binding site of the albumin molecule (19).

In addition to this stereospecific binding site a second one seems to exist for RV 1210. The present data (Fig. 1, Table 1) do not exclude the possibility that RV 1208 can also bind to this second site. However,

its affinity might be so low that binding to the second binding site would be obscured by its high binding affinity at the first site and thus would not be detectable on the Scatchard plot (Fig. 1) in the concentration range employed.

Binding to HSA induces similar extrinsic Cotton effects in both enantiomers (Fig. 4 and Table 3). The signs of the two main bands near 260 nm and 310 nm are identical with those of other benzodiazepines bound to HSA (1, 3) (see oxazepam in Fig. 4). That the observed Cotton effects are extrinsic in origin is clearly established by the following points: (a) the similarity of the CD spectra of *d*- and *l*-oxazepam hemisuccinate to those of other symmetrical benzodiazepines (1), which excludes perturbation by the asymmetrical carbon atom of the enantiomers itself, (b) the correspondence of the wavelengths of the CD bands with the ultraviolet maxima at 315 nm and the shoulder at 255 nm (Fig. 3), and (c) the similarity of the extrinsic and intrinsic CD spectra of the two enantiomers, which excludes a drug-induced alteration of the protein conformation. Because it has been shown that these typical biphasic CD spectra of the benzodiazepines in the presence of HSA are produced at only one binding site (3), it seems clear that RV 1208 and RV 1210 also are primarily bound to this binding site. Linearity in Scatchard plots (Fig. 1) shows that a single binding constant will describe binding to the high-affinity site (Table 1).

The intensities of the extrinsic Cotton effects of RV 1208 at two ligand concentrations do not differ (Table 3), which indicates that the binding site is saturated at both concentrations. This is consistent with the high affinity we observed for the RV 1208–albumin complex (Table 1). However, the binding site does not seem to be saturated at the lower concentration of RV 1210. By increasing the total concentration, complete saturation will be approached, and therefore the intensities of the extrinsic Cotton effects will increase.

It has been shown for some substances that binding to macromolecules induces similar Cotton effects in both enantiomers, e.g., *d*- and *l*-fenoprofen bound to HSA (20) and *d*- and *l*-*sec*-butylaminoacridines bound to DNA (21). It was concluded from these observations that these substances are not bound stereospecifically (20, 21) and that the enantiomers are bound in an identical manner (21). Our results differ from the above observations, because we found high stereospecific binding which induces similar Cotton effects in both enantiomers. The only explanation for this phenomenon can be that binding of both enantiomers to the protein must be sterically similar and therefore induces equal Cotton effects in two ultraviolet transitions of the benzodiazepine molecule. A precise explanation for the trough of RV 1208 at 275 nm cannot be given by the existing data. The part of the molecule to which this band corresponds is unknown. A different orientation of the two enantiomers at the HSA binding site would in any case cause largely differing spectra.

Although no doubt is left that both enantiomers are bound to the binding site for the benzodiazepines at the HSA molecule in the same orientation, an explanation is needed for the nearly 40-fold lower affinity of RV 1210, especially since the optically active atom of the molecule (C_3) seems to be not involved in the binding of the substances to HSA (2).

NMR studies have shown that the benzodiazepine molecule is present in a boat conformation (22, 23), the inversion of which can produce two different forms (22). Substitution at C_3 gives only a conformation in which the larger substituent at C_3 is in the quasiequatorial position (24). In case of RV 1208 and RV 1210 this means that both enantiomers have not only different configurations but also different conformations of the benzodiazepine ring, in which the hemisuccinate residue is in the equatorial position (see Fig. 5, Ie and IIe). The existence of two different conformations for RV 1208 and RV 1210 could explain the large differences in their affinities for HSA. The similar extrinsic Cotton effects of both enantiomers suggest that only one conformation will be bound. Therefore, it is assumed that an inversion

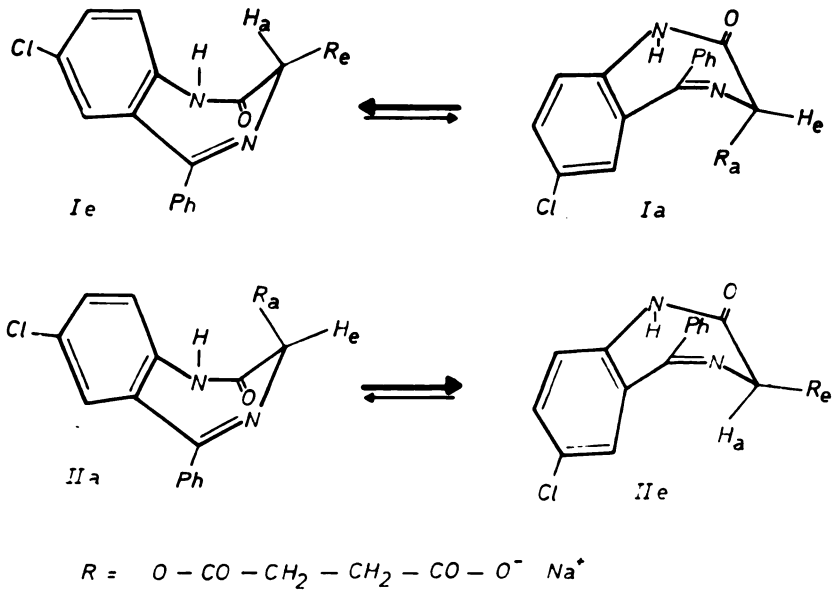


FIG. 5. *Boat conformation of the benzodiazepine molecule*

The influence of a large substituent at C_3 (24) on the boat conformation of the benzodiazepine molecule according to Bley *et al.* (22) is shown.

of the boat conformation is associated with the binding of RV 1210 to HSA.

In a previous paper (3) it was suggested that the low alkaline structure transition of the HSA molecule N → B transition (25), will influence the specificity of the benzodiazepine binding site for the two different boat conformations. If this were true, the affinity of RV 1208 for this binding site must decrease and the affinity of RV 1210 must increase from raising the pH to 8.2. The results of our binding studies at pH 8.2 seem to confirm this suggestion, because the affinity for the first binding site increases in the case of RV 1210 and decreases in the case of RV 1208 (see the single binding constants, k, Table 1). This possibly indicates, that the specificity of the HSA binding site for both enantiomers is changed.

Increasing the pH to 8.2 alters the extrinsic Cotton effects of primarily the longer-wavelength CD bands (Table 3). This indicates that the symmetry rules (13) are affected for the $-\overset{5}{\underset{|}{C}}=\overset{4}{N}-$ chromophore. The fact that the short-wavelength CD bands at 260 nm remain unchanged (Table 3) suggests that the symmetry rules of the A benzene chromophore remain constant. The possibility that the chromophores of a molecule are effected in a different manner has been shown by Chignell and Starkweather (26), who found that acetylation of HSA with acetylsalicylic acid alters only one of the two extrinsic CD bands of flufenamic acid bound to HSA.

Sadée (23) found that for derivatives with large substituents at N_1 the inversion of the boat conformation is severely hindered. For benzodiazepine derivatives bound to HSA we have found (3) that the N → B transition produces an inversion of the signs of both CD bands. At present we cannot explain why for RV 1208 and RV 1210 the longer-wavelength CD bands and for the other benzodiazepines both CD bands are influenced by the N → B transition of the protein. Possibly the asymmetrical configuration of both enantiomers itself influences the extrinsic Cotton effects.

The findings reported in this paper substantiate that the benzodiazepines show a

high site selectivity and a specific orientation when bound to human serum albumin. This can explain why their binding behavior differs in some cases (2) from many other substances bound to albumins.

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