Optical Studies of Drug-Protein Complexes
II. Interaction of Phenylbutazone and Its Analogues with Human Serum Albumin

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SUMMARY

The binding of phenylbutazone to human serum albumin generated a positive ellipticity band at 287 mμ in the circular dichroic spectrum of the protein. This extrinsic Cotton effect resulted from perturbation of the carbonyl chromophore of phenylbutazone by an asymmetrical locus at the albumin binding site. Hydrophobic interactions appeared to be important for the maintenance of a rigid drug-protein complex, since the introduction of hydrophilic groups into phenylbutazone caused considerable reduction in the magnitude of induced optical activity. Phenylbutazone competitively displaced 1-dimethylamino-naphthalene-5-sulfonyl-*N*-glycine (a fluorescent probe for the hydrophobic regions of proteins) from human serum albumin. This suggested that there was a hydrophobic area at or near one of the phenylbutazone-binding sites. The red shift in the ultraviolet absorption maximum of phenylbutazone on binding to albumin provided some evidence that the phenyl groups of the drug were located in a region of the protein where the dielectric constant was less than that of water.

INTRODUCTION

When small, symmetrical, chromophoric molecules are bound to proteins, Cotton effects are often observed in the frequency region where the chromophore absorbs light (1). Although several hypotheses have been advanced to account for these "extrinsic" Cotton effects, it is now generally thought that they result from interaction of the ligand chromophore with an asymmetrical locus in the protein. Because extrinsic Cotton effects apparently reflect the characteristics of specific asymmetrical sites in proteins, they can serve as a probe for studying such sites (1).

The preceding paper in this series (2) showed that when certain anionic drugs are bound by serum albumin, extrinsic Cotton effects may be observed. In the present report, the circular dichroic spectra of phenylbutazone and some of its analogues when bound to human serum albumin have been examined. This study shows how measurements of extrinsic Cotton effects may be used to gain information about the nature of drug-protein complexes.

MATERIALS AND METHODS

Materials. The serum albumins (Mann Research Laboratories) were all crystalline, with the exception of the equine and porcine preparations, which were Cohn Fraction V (purity > 95%). The moisture content of each batch was determined by drying a small sample at 105° overnight. Albumin concentrations were calculated with reference to the dry weight. The concentrations of HSA[1] and bovine serum albumin were

[1] The abbreviation used is: HSA, human serum albumin.

checked by measuring the optical density of solutions at 280 mμ, using $E_{1cm}^{1\%}$ values of 5.3 and 6.6, respectively. All albumins were dialyzed against distilled water overnight before use. Phenylbutazone and its analogues were generously donated by Dr. Murray Weiner (Geigy Research) and Dr. Peter G. Dayton. The dansyl derivative of glycine (1-dimethylamino-naphthalene-5-sulfonyl-*N*-glycine) was purchased from Mann Research Laboratories. Spectroscopically pure *n*-hexane was obtained from Fisher. All other chemicals were of reagent grade. The 1-octanol was washed with 0.1 N NaOH, 0.1 N HCl, and water before use.

Methods. Circular dichroism measurements were made at 27° with a Cary 6001 attachment to the Cary model 60 spectropolarimeter. Absorbance was kept below 2 by using cell path lengths of 0.5–20 mm. Results are expressed as molar ellipticities, $[\theta]$ (deg cm^2 dmole^{-1}), calculated with reference either to the concentration of bound drug or to the concentration of HSA, assuming a molecular weight of 69,000.

The binding of dansylglycine to HSA was estimated by measuring the resultant increase in fluorescence (3). Fluorescence measurements were made at 480 mμ with an Aminco-Bowman spectrophotofluorometer, using an activating wavelength of 350 mμ. In all experiments the HSA concentration was 1×10^{-5} M, while that of dansylglycine was varied from 2.5×10^{-6} to 5×10^{-5} M. All measurements were made in the presence of 0.1 M sodium phosphate buffer (pH 7.4) and were corrected for self-absorption. The fluorescent intensity of a fixed amount of dansylglycine was measured in the presence of a large (100-fold) excess of HSA, at which concentration it was assumed that the dansylglycine was completely bound. From this value it was possible to calculate the concentration of bound dansylglycine in mixtures of HSA and dansylglycine. The binding of drugs by HSA was determined by the equilibrium dialysis method of Klotz *et al.* (4), or by ultrafiltration using a Diaflo model 50 ultrafiltration cell (Amicon Corporation) with a PM-10 filter (which has a cut-off at a molecular weight of 10,000). All measurements were made in the presence of 0.1 M sodium phosphate buffer, pH 7.4. Drug concentrations were measured spectrophotometrically in a Beckman DU spectrophotometer. Binding results have been plotted according to the method of Scatchard (5), using the expression

$$\frac{n}{A} = KN - Kn$$

where n = number of moles of ligand bound per mole of protein, A = concentration of free ligand, K = association constant, and N = number of ligand-binding sites per protein molecule.

Partition coefficients were determined by shaking 5 ml of drug solution (0.1 M sodium phosphate buffer, pH 7.4) with 10 ml of a *n*-hexane–1-octanol mixture (60:40) for 15 hr at room temperature. The concentration of drug remaining in the aqueous phase was then determined spectrophotometrically.

Difference spectra were measured by the double-cell compensation technique of Herskovits (6). Tandem double-compartment cells with 1.0-cm path lengths (Pyrocell) were used in all experiments. All other spectra were obtained with single 1.0-cm path length cells. Spectra were recorded with a Shimadzu MPS-50L double-beam spectrophotometer.

RESULTS

Equilibrium dialysis studies showed that HSA had three binding sites for phenylbutazone (Fig. 1). Since the Scatchard plot (Fig. 1) was nonlinear, it was necessary to postulate either that the phenylbutazone binding sites were heterogeneous or that there were electrostatic interactions between sites (7, 8). By making the simplifying assumptions that electrostatic interactions were small and that there were only two kinds of site, it was possible to calculate (7, 8) that HSA had one binding site of high affinity ($K = 1 \times 10^5$ M^{-1}) and two others with a somewhat lower affinity ($K = 4 \times 10^4$ M^{-1}). Dansylglycine, a fluorescent probe for the hydrophobic regions of proteins (3), was strongly bound ($K = 4.6 \times 10^5$ M^{-1}) to HSA at a single site, from which it could be competitively displaced by phenylbutazone

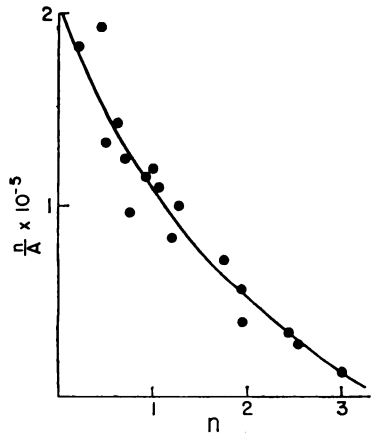


FIG. 1. *Scatchard plot of the binding of phenylbutazone to HSA*

All measurements were made in the presence of 0.1 M sodium phosphate buffer, pH 7.4. n = number of moles of phenylbutazone bound per mole of HSA. A = molar concentration of free phenylbutazone. Binding was measured by the equilibrium dialysis technique (4).

(Fig. 2). By using the method of Klotz *et al.* (9), it was possible to calculate that the affinity constant of the dansylglycine binding site for phenylbutazone was 0.9×10^5 M^{-1}. This strongly suggested that phenylbutazone and dansylglycine shared a common binding site on HSA. Solomon *et al.* have reported (10) that HSA has only one binding site for phenylbutazone, with an affinity constant of 1.17×10^5 M^{-1}. The two lower-affinity binding sites observed in the present investigation were apparently overlooked, because the concentrations of phenylbutazone used by these workers never exceeded that of HSA (10). Under these conditions, binding would be almost exclusively to the single high-affinity site.

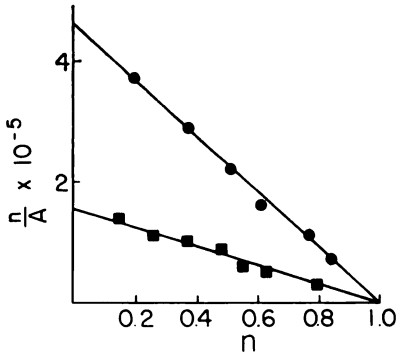


FIG. 2. *Scatchard plot of the binding of dansylglycine to HSA*

All measurements were made in the presence of 0.1 M sodium phosphate buffer. n = number of moles of dansylglycine bound per mole of HSA. A = molar concentration of free dansylglycine ●——●, Control; ■——■, 2.5×10^{-5} M phenylbutazone. Binding was measured by the fluorescence technique described under MATERIALS AND METHODS.

Below 310 mμ, HSA had a small negative circular dichroism (Fig. 3), with a definite shoulder appearing at 260 mμ. Upon the addition of phenylbutazone, a large, positive ellipticity band appeared at 287 mμ (Fig. 3). When a fixed concentration of HSA was titrated with increments of phenylbutazone, the ellipticity increased in direct proportion to the concentration of bound drug (Fig. 4). However, after each mole of HSA had taken up 2.6 moles of phenylbutazone, the observed ellipticity reached a maximum which was not changed by further addition of drug (Fig. 4). The

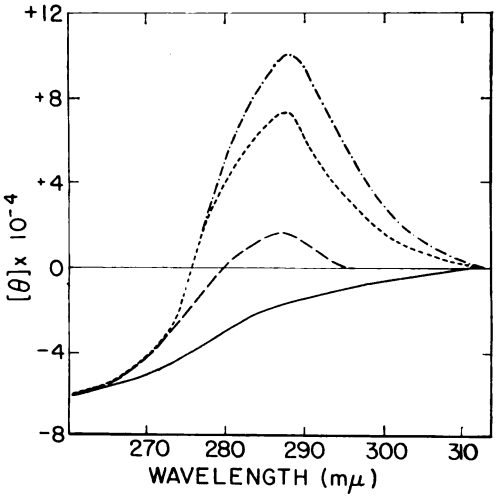


FIG. 3. *Circular dichroic spectra of phenylbutazone, oxyphenbutazone, and sulfinpyrazone when bound to HSA*

-·-·-, Phenylbutazone (5×10^{-5} M); ——, sulfinpyrazone (5×10^{-5} M); – – –, oxyphenbutazone (1×10^{-4} M); ——, HSA (1.45×10^{-5} M). All measurements were made in the presence of 0.1 M sodium phosphate buffer, pH 7.4.

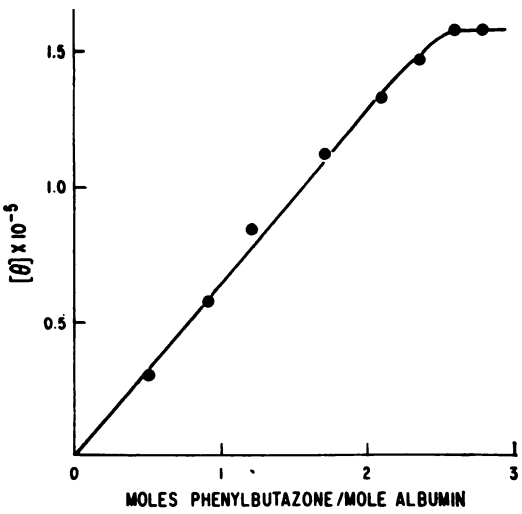


FIG. 4. *Relationship between molar ellipticity [θ] and moles of phenylbutazone bound per mole of HSA*

Molar ellipticity was calculated with reference to the concentration of HSA, which was 1.45×10^{-5} M. All measurements were made in the presence of 0.1 M sodium phosphate buffer, pH 7.4.

circular dichroic spectrum of HSA below 260 mμ did not appear to be affected by the binding of phenylbutazone. Ellipticity bands were also observed when phenylbutazone was bound to porcine, bovine, equine, and rabbit serum albumins (Table 1). The ellipticity of phenylbutazone bound to HSA was profoundly affected by substitution in the phenyl ring (Table 2). The introduction of hydroxyl (oxyphenbutazone, G-30249), methylsulfonyl (G-32568, G-34764), or nitro (G-28234) groups caused a marked reduction in optical activity, while the chloro (G-15140) and fluoro (G-32170) substituents had relatively little effect (Table 2). The changes in ellipticity which accompanied the modification of the *n*-butyl group were generally less dramatic (Table 2).

Several workers have appreciated the importance of hydrophobic interactions in the binding of drugs and other small molecules to serum albumin (11, 12). Efforts to quantitate the hydrophilic or hydrophobic nature of drug molecules have involved the determination of partition coefficients between aqueous buffer and 1-octanol or isobutyl alcohol (11, 12). In this investigation, the partition of phenylbutazone between aqueous sodium phosphate buffer (pH 7.4) and

TABLE 1

Molar ellipticities of complexes between phenylbutazone and different serum albumins

Serum albumin	Wavelength of circular dichroism maximum	[θ][a]
	mμ	(deg cm² dmole⁻¹) × 10⁻³
Human	287	+52.2
Bovine	287	+88.0
Porcine	287	+79.5
Equine	287	+45.0
Rabbit	275–280	+22.0

[a] Calculated with reference to the concentration of bound phenylbutazone. The following concentrations were employed: serum albumin, 0.1%; phenylbutazone, 5×10^{-6} M; sodium phosphate buffer (pH 7.4), 0.1 M.

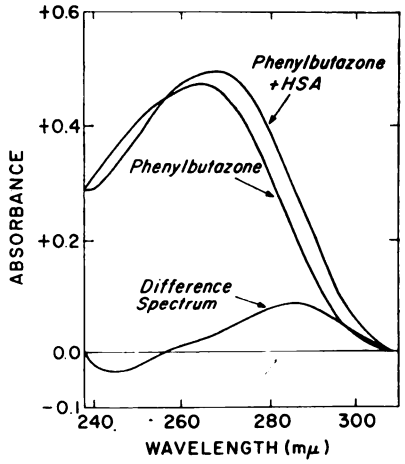
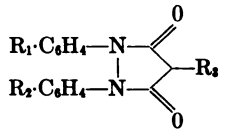


FIG. 5. *The ultraviolet absorption spectum of phenylbutazone bound to human serum albumin*

The spectrum of phenylbutazone alone was obtained by using a buffer blank. For the phenylbutazone + HSA spectrum, the blank was HSA. All path lengths were 1.0 cm. The difference spectrum was obtained (see MATERIALS AND METHODS) by combining drug and protein in the sample beam and separating them in the reference beam. The following concentrations were employed: phenylbutazone, 2.5×10^{-6} M; HSA, 0.76×10^{-6} M; sodium phosphate buffer, pH 7.4, 0.1 M.

TABLE 2
Molar ellipticities of complexes between phenylbutazones and human serum albumin



Drug	R_1	R_2	R_3	Wavelength maximum: Ultraviolet absorption	Wavelength maximum: Circular dichroism	$[\theta]^a$
				$m\mu$		*deg cm² dmole⁻¹*) × 10^{-3}
Phenylbutazone	H	H	$CH_3(CH_2)_3$	265	287	52.2
Oxyphenbutazone	*p*-OH	H	$CH_3(CH_2)_3$	264	287	6.6
Sulfinpyrazone	H	H	$C_6H_5SO(CH_2)_2$	254	288	49.4
G-13838	H	H	$(CH_3)_2CH$	263	288	48.0
G-25671	H	H	$C_6H_5S(CH_2)_2$	256	286	63.0
G-30249	*p*-OH	H	$C_6H_5CH_2CO$	268	293	9.5
G-28234	*p*-NO_2	H	$CH_3(CH_2)_3$	260		0
G-15140	*p*-Cl	*p*-Cl	$CH_3(CH_2)_3$	265	296	28.8
G-32170	*p*-F	*p*-F	$CH_3(CH_2)_3$	262	290	29.8
G-34764	*p*-CH_3SO_2	H	$CH_3(CH_2)_3$	263		0
G-32568	*m*-CH_3SO_2	*m*-CH_3SO_2	$CH_3(CH_2)_3$	271		0
Ketazone	H	H	$CH_3CO(CH_2)_2$	261	287	29.0
Benzopyrazone	H	H	$C_6H_5CO(CH_2)_2$	251	287	74.0

[a] Calculated with reference to the concentration of bound drug. The number of moles of drug bound per mole of HSA was kept below 2 for all measurements.

a mixed solvent system consisting of 60% *n*-hexane and 40% 1-octanol was measured. This became necessary when it was found that all the phenylbutazone analogues were completely extracted into either 1-octanol or isobutyl alcohol. Those phenylbutazone analogues which showed little solubility in the organic phase (Table 3) exhibited weak extrinsic Cotton effects when bound to HSA (Table 2).

TABLE 3
Partition coefficients and association constants of phenylbutazone and some of its analogues

Drug	Partition coefficient[a]	Association constant: K_1	Association constant: K_2
		$M^{-1} \times 10^{-5}$	
Phenylbutazone	1.96	1.0	0.4
G-15140	20.00	4.15	4.15
G-32170	1.33	0.92	0.18
G-28234	0.42	3.18	0.67
Oxyphenbutazone	0.40	2.28	0.37
G-34764	0.02	3.36	0.60
G-32568	0.01	1.40	0.39

[a] Partition between *n*-hexane–1-octanol (60:40) and 0.1 M sodium phosphate buffer (pH 7.4). For details, see MATERIALS AND METHODS.

[b] The assumption was made that there were no electrostatic interactions and that there was only one strong binding site (K_1). The remaining sites were assumed to be homogeneous, with an association constant K_2.

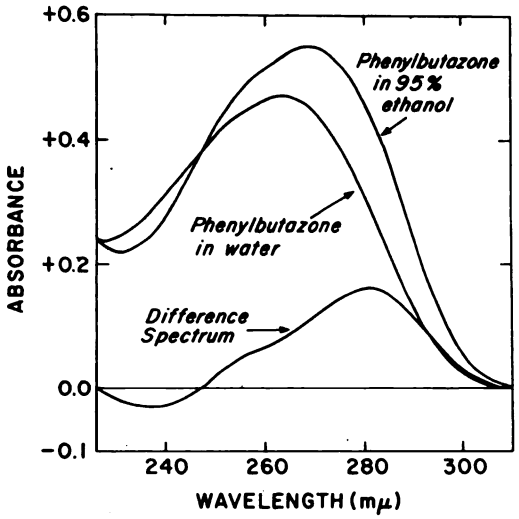


FIG. 6. *The ultraviolet absorption spectrum of phenylbutazone in ethanol*

Blanks contained the corresponding solvent without the drug. All path lengths were 1.0 cm. The difference spectrum was obtained (see MATERIALS AND METHODS) by placing the ethanol solution of phenylbutazone in the sample beam and the aqueous solution of the drug in the reference beam. No buffers were employed. Instead, sodium hydroxide was added, so that the pH was approximately 10, to keep the drug in an ionized form.

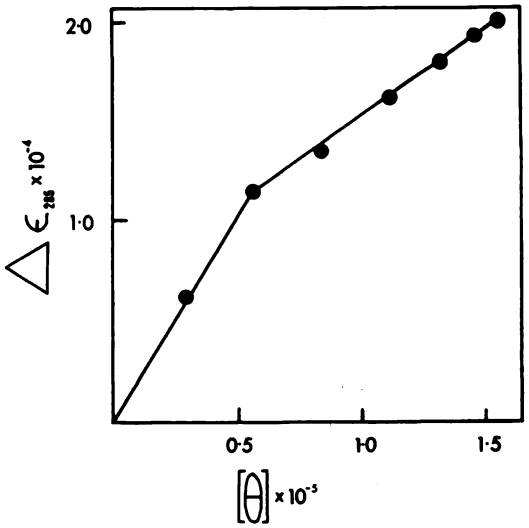


FIG. 7. *The relationship between molar ellipticity and the difference spectrum generated when phenylbutazone is bound to HSA*

The concentration of HSA was 1.45×10^{-6} M. The concentrations of phenylbutazone added were exactly the same as those in Fig. 4.

In sodium phosphate buffer at pH 7.4, the ultraviolet absorption spectrum of phenylbutazone had a maximum at 265 mμ ($\epsilon = 2.0 \times 10^4$ M^{-1} cm^{-1}) (Fig. 5). The binding of phenylbutazone to HSA resulted in a shift of the absorption maximum to 270 mμ, with a concomitant increase in the intensity of absorption (Fig. 5). The difference spectrum generated by this shift showed a positive maximum at 285 mμ (Fig. 5). The absorption spectrum of phenylbutazone was also shifted to longer wavelengths when the drug was dissolved in ethanol (Fig. 6); the resultant difference spectrum was very similar to that generated when phenylbutazone was bound to HSA. When the intensity of the difference spectrum generated by the binding of phenylbutazone to HSA was plotted as a function of the observed ellipticity, a biphasic relationship was obtained (Fig. 7). The sharp break in the line occurred when 1 mole of drug had been bound per mole of HSA (cf. Fig. 4).

DISCUSSION

Induced Cotton effects are often observed when the electrons of a chromophore are perturbed by electrostatic forces associated with a nearby asymmetrical locus (13). The sign of such an induced Cotton effect, whether extrinsic or intrinsic, is governed by the configuration of the asymmetrical center and its spatial relationship to the perturbed chromophore. Schellman has shown (13) that the space around a chromophore may be divided into regions of positive and negative contribution to a Cotton effect, according to well-defined symmetry rules. The latter are determined by the symmetry of the perturbed transition. An example of the simplest type of interaction is the purines and pyrimidines, which obey a planar rule, with the plane of the π-electron system as the nodal plane. Placing an asymmetrical center on one side of the plane will give a Cotton effect. Moving it to the other side will reverse the effect (13). The extrinsic Cotton effect induced in ATP on binding to creatine-ATP transphosphorylase is thus explainable if

there is a preferred side for binding to the protein.[2] Because induced Cotton effects result from an electrostatic interaction, their intensity is inversely proportional to the distance between the asymmetrical locus and the perturbed chromophore (13). For extrinsic Cotton effects, where the asymmetrical center and the perturbed chromophore are not part of the same molecule, the rigidity of the ligand-macromolecule complex is of paramount importance. A loose complex may allow the ligand sufficient freedom of movement so that the protein asymmetrical center moves into regions of positive and negative contribution to a Cotton effect. Under these conditions no optical activity would be observed.

Although phenylbutazone had an ultraviolet absorption maximum at 265 mμ (Fig. 5), the ellipticity band generated by binding to HSA was located at 287 mμ (Fig. 3). This observation suggested that it was not the $\pi \rightarrow \pi^*$ transition of the phenyl groups which was being perturbed, but the $n \rightarrow \pi^*$ transition of the ring carbonyl (14). For the $n \rightarrow \pi^*$ transition of amides and peptides, a quadrant rule has been proposed (15), and it appeared likely that either the quadrant or planar rules could apply to phenylbutazone (13). Since a positive ellipticity band was observed when phenylbutazone was bound to HSA, it was obvious that the protein asymmetrical center was located in a region of the drug chromophore which made a positive contribution to a Cotton effect, and that the protein-drug complex was rigid enough to prevent the asymmetrical center from entering regions of negative contribution. The spatial relationship between phenylbutazone and at least two of the HSA binding sites appeared to be the same (Fig. 4). It is still not clear, however, why there was no further increase in the ellipticity after 2.6 moles of drug had been bound to each mole of protein. Although not all phenylbutazone analogues gave rise to circular dichroic spectra on being bound to HSA, those that did had positive ellipticity bands (Table 2). If it was the carbonyl chromophore which was being perturbed, then phenylbutazone and its analogues must have similar spatial relationships to the protein-binding site and its associated asymmetrical center. Phenylbutazone also generated positive ellipticity bands when bound to serum albumin from sources other than man (Table 1). This would suggest that the binding sites are similar in all the albumins studied. The considerable variation in the size of the observed ellipticities probably reflected the differences in the rigidity of the drug-protein complexes as well as the relative distances between the asymmetrical center and the chromophore.

It has been shown that many anionic drugs are bound to serum albumin either at the same sites or at closely located sites (10, 16, 17). Furthermore, Skidmore and Whitehouse have proposed (17) that the ε-amino group of lysine is the point of attachment for anionic drugs. Treatment of HSA with *O*-methylisourea or succinic anhydride caused a considerable reduction in the binding of phenylbutazone.[3] However, it would seem probable that 1-point electrostatic attachment to HSA would leave a bound phenylbutazone molecule fairly free to rotate. Under such conditions, the generation of an extrinsic Cotton effect would appear unlikely. On the other hand, if one or both phenyl groups could form van der Waals bonds with a hydrophobic region of the protein, a fairly rigid complex might result. Dansylglycine underwent a considerable increase in fluorescent quantum yield (see MATERIALS AND METHODS) on binding to one of the phenylbutazone binding sites. Chen (3) has shown that dansylglycine binds preferentially to the hydrophobic regions of proteins. Evidence that the phenyl groups of phenylbutazone associate with a hydrophobic region in HSA was provided by the spectral shift generated on binding (Fig. 5). It is well known that when the secondary and tertiary structure of proteins is destroyed, their ultraviolet absorption

[2] J. H. R. Kägi and T. K. Li, unpublished observations cited by Ulmer and Vallee (1).

[3] C. F Chignell, unpublished observations.

maxima shift to shorter wavelengths, with a slight decrease in intensity (18). This shift has been attributed by Yanari and Bovey to the movement of aromatic residues from a relatively hydrophobic environment within the protein to an aqueous milieu (19). Conversely, it might be expected that if a ligand with a phenyl chromophore moved from an aqueous environment into a hydrophobic binding region, a red spectral shift, accompanied by an increase in intensity, might occur. This is precisely what was observed when phenylbutazone bound to HSA (Fig. 5). It is interesting that hydrophobic interactions were stronger at the first binding site (Fig. 7). It should also be emphasized that the spectral shift observed when phenylbutazone bound to HSA (Fig. 5) was qualitatively very similar to that seen when the drug was dissolved in ethanol (Fig. 6), a solvent which has a lower dielectric constant than water. Finally, when certain groups, such as OH, CH_3SO_2, or NO_2, were introduced into the phenyl ring of phenylbutazone, there was a marked reduction in the observed extrinsic optical activity (Table 2). However, these same modifications also rendered the phenylbutazone molecule more hydrophilic, as may be seen from the low hexane (octanol)/water partition coefficients (Table 3). It is easy to see that a phenyl ring bearing a hydrophilic group would have less tendency to form van der Waals bonds with hydrophobic areas at or near the drug binding site. Under these conditions, the drug-protein complex would be less rigid and a decrease in optical activity might be expected. It was felt, however, that electrostatic forces were probably the most important in the binding of phenylbutazone to HSA, with short-range dispersive interactions playing only a minor role. This would explain why there was a relatively poor correlation between the binding constant of HSA for a given phenylbutazone analogue and the magnitude of the resultant ellipticity (Table 3). Thus, although oxyphenbutazone had a relatively strong affinity for the cationic binding site in HSA (Table 3), van der Waals forces were insufficient to produce the rigid complex necessary for high optical activity.

Mizushima and Kobayashi have shown (20) that phenylbutazone and other anti-inflammatory drugs stabilize serum proteins against heat denaturation. They have also suggested that there may be some connection between this phenomenon and the mode of action of these drugs *in vivo*. If the ability of such compounds to interact with proteins is important to their pharmacological effect, the measurement of extrinsic Cotton effects may be an important tool for studying this effect.

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