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Optical Studies of Drug-Protein Complexes

III. Interaction of Flufenamic Acid and Other *N*-Arylanthranilates with Serum Albumin

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SUMMARY

The extrinsic Cotton effects generated by the binding of flufenamic acid [*N*-(α,α,α-trifluoro-*m*-tolyl)anthranilic acid], meclofenamic acid [*N*-(2,6-dichloro-*m*-tolyl)anthranilic acid], and mefenamic acid [*N*-(2,3-xylyl)anthranilic acid] to human serum albumin suggested that although these anti-inflammatory drugs are bound to the same binding site, each one takes up a unique spatial orientation to the protein. Extrinsic Cotton effects generated by the binding of flufenamic acid to different albumins indicated that the drug-binding sites on human, porcine, equine, and bovine serum albumins were similar, while those of canine, ovine, and rabbit serum albumins had somewhat different asymmetries. Spectral changes which accompanied the binding of flufenamic acid to human serum albumin strongly suggested that the aromatic portion of the drug was inserted into a hydrophobic crevice in the protein, while the carboxylate group of the drug interacted with a cationic site on the protein surface.

INTRODUCTION

The binding of a symmetric chromophoric drug molecule to serum albumin may generate an "extrinsic" Cotton effect in the normal circular dichroic spectrum of the protein (1, 2). Because these extrinsic Cotton effects result from the perturbation of electronic transitions in the drug chromophore by an asymmetric locus at or near the protein-binding site (3), they can be used to probe such sites (4).

In the preceding paper in this series (2) it was shown that the extrinsic Cotton effects generated by the binding of phenylbutazone to human serum albumin (HSA) could be abolished by introducing hydrophilic groups into the phenyl rings of the drug. These results, together with ultraviolet and fluorescence data, suggested that hydrophobic as well as electrostatic interactions were important for binding. In the present report the extrinsic Cotton effects generated by the binding of the potent anti-inflammatory drugs flufenamic acid, mefenamic acid, and meclofenamic acid to HSA suggest that although these drugs are bound to the same binding site, each one takes up a unique spatial orientation to the protein. Extrinsic Cotton effects generated by the binding of flufenamic acid to different serum albumins indicate that the drug-binding sites on human, porcine, equine, and bovine serum albumins are similar, while those of canine, ovine, and rabbit serum albumins have somewhat different asymmetries. The spectral changes which accompany the binding of flufenamic acid to HSA strongly suggest that the aromatic portion of the drug is inserted into a hydrophobic crevice in the protein, while the carboxylate group of the drug is associated with a cationic group on the protein surface.

MATERIALS AND METHODS

Materials. The serum albumins were purchased from Mann Research Laboratories. The human, rabbit, and bovine serum albumins were crystalline, the remainder being Cohn Fraction V (purity > 95%). Each albumin was dialyzed overnight at 4° against distilled water before use. The moisture content of each batch was determined by heating a small sample to 105° overnight. The concentration of each albumin was calculated with reference to the dried sample. The concentrations of the human and bovine serum albumins were checked by measuring their optical densities at 280 mμ, using $E^{1\%}_{1\,cm}$ values of 5.3 and 6.6, respectively. Flufenamic acid [*N*-α,α,α-trifluoro-*m*-tolyl)anthranilic acid], mefenamic acid [*N*-(2,3-xylyl)anthranilic acid], and meclofenamic acid [*N*-(2,6-dichloro-*m*-tolyl) anthranilic acid] were supplied by Dr. C. V. Winder (Parke, Davis and Company). The remaining *N*-arylanthranilates were generously donated by Dr. R. G. Gryglewski of the Medical Academy, Cracow, Poland, or were synthesized by the method of Ullman (5). Dansylglycine (*N*,*N*-dimethylaminonaphthalene-5-sulfonyl-*N'*-glycine) was purchased from Mann Research Laboratories. The cetrimide (hexadecyltrimethylammonium bromide) and the spectroscopically pure *n*-hexane were obtained from Fisher. All other chemicals were of reagent grade.

Methods. Circular dichroism measurements were made at 27° with a Cary 6001 attachment to the Cary model 60 spectropolarimeter. Unless otherwise stated, the concentration of *N*-arylanthranilate was 10^{-4} M, HSA was 1.45×10^{-5} M, and sodium phosphate buffer (pH 7.4), 0.1 M. Results have been expressed as molar ellipticities $[\theta]$ (deg·cm² dmole^{-1}), which were calculated from the formula

$$[\theta] = \frac{100\theta_{obs}}{lC}$$

where θ_{obs} = observed ellipticity, l = path length (centimeters), and C = molar concentration. The final result has been expressed either in terms of the concentration of bound drug or in terms of the concentration of HSA, assuming a molecular weight of 69,000. The concentration of bound drug was determined by ultrafiltration through a Diaflo model 50 ultrafiltration cell (Amicon Corporation) equipped with a PM-10 filter. Drug concentrations were measured in a Beckman DU spectrophotometer equipped with a Gilford attachment.

The optical anisotropy or dissymmetry factor ($\Delta\epsilon/\epsilon$) (6) was calculated from the expression

$$\frac{\Delta\epsilon_\lambda}{\epsilon_\lambda} = \frac{[\theta]_\lambda}{3300\epsilon_\lambda}$$

where $[\theta]_\lambda$ = molar ellipticity (calculated with respect to the concentration of bound drug), ϵ_λ = molar extinction coefficient of the drug, and λ = the wavelength of the circular dichroism maximum.

The binding of flufenamic acid to HSA was determined by the equilibrium dialysis method of Klotz *et al.* (7) and plotted according to the method of Scatchard (8), using the relationship

$$\frac{r}{C} = Kn - Kr$$

where r = number of moles of ligand bound per mole of protein, C = molar concentration of free ligand, K = association constant (liters per mole), and n = number of ligand-binding sites per molecule of protein.

Dansylglycine binds strongly to a single site on HSA, with a concomitant increase in fluorescence (2). The association constants of the *N*-phenylanthranilates for HSA were determined by measuring their ability to displace dansylglycine competitively (9). Fluorescence measurements were made at 480 mμ with an Aminco-Bowman spectrophotofluorometer, using an activating wavelength of 350 mμ (10). The initial solution (2 ml) contained the following: HSA, 1×10^{-5} M; dansylglycine, 2×10^{-4} M; and sodium phosphate buffer, 0.1 M (pH 7.4). There was no further in-

crease in fluorescence when the concentration of dansylglycine was doubled, indicating that saturation of the dye-binding sites was complete (9). The fluorescence of the mixture of dansylglycine and HSA was then measured during titration with a solution (10^{-3} M) of each of the *N*-phenylanthranilate analogues. The association constant of each analogue for HSA was then calculated from the association constant of dansylglycine (2) by the method of Flanagan and Ainsworth (9).

Partition coefficients were determined by shaking 5 ml of a 10^{-4} M solution of each *N*-phenylanthranilate (0.1 M sodium phosphate buffer, pH 7.4) with 10 ml of *n*-hexane containing 1% 1-octanol for 15 hr at room temperature and measuring the concentration of drug remaining in the aqueous phase spectrophotometrically. Difference spectra were measured by the double-cell compensation technique of Herskovits (11). Tandem double-compartment cells with 1.0-cm path lengths (Pyrocell Manufacturing Company) 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-50 L double-beam spectrophotometer.

RESULTS AND DISCUSSION

The symmetry rules which govern the sign and magnitude of a Cotton effect have been outlined recently by Schellman (3). In the previous paper in this series the application of these rules to extrinsic Cotton effects was discussed (2). Briefly, it may be stated that the space around a chromophore may be divided into regions of negative and positive contribution to a Cotton effect. A given asymmetric center may therefore give rise to either a positive or a negative Cotton effect, depending on its spatial relationship to the chromophore which it perturbs. No extrinsic Cotton effect will be observed if the complex formed between ligand and macromolecule is loose enough to allow the asymmetric center to enter regions of positive and negative contribution. The magnitude of an extrinsic Cotton effect will increase as the asymmetric center and perturbed chromophore are brought closer together (3). Some use will be made in this paper of the term "optical anisotropy" or "dissymmetry factor" (6), which enables comparisons to be made between optically active absorption bands having different extinction coefficients. In practical terms this factor may be considered an estimate of the environmental asymmetry experienced by an optically active chromophore. The theoretical significance of optical anisotropy has been discussed by Kuhn (6).

The circular dichroic spectrum of HSA exhibited a small negative ellipticity band at wavelengths immediately below 320 mμ (Fig. 1) (12). Upon the addition of flufenamic acid, a large, positive ellipticity band appeared at 296 mμ while a smaller, negative band was generated at 345 mμ. Intrinsic Cotton effects generated by proteins at wavelengths above 240 mμ have generally been attributed to aromatic side

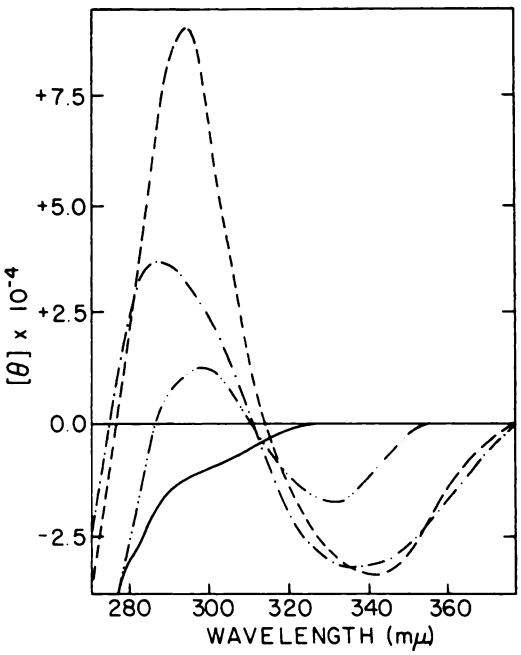


FIG. 1. *Circular dichroic spectra of flufenamic, mefenamic, and meclofenamic acids in the presence of HSA* (1.45×10^{-5} M)

– – –, Flufenamic acid (1×10^{-4} M); – · – ·, mefenamic acid (1×10^{-4} M); – · · –, meclofenamic acid (1×10^{-4} M); ——, HSA (1.45×10^{-5} M) alone.

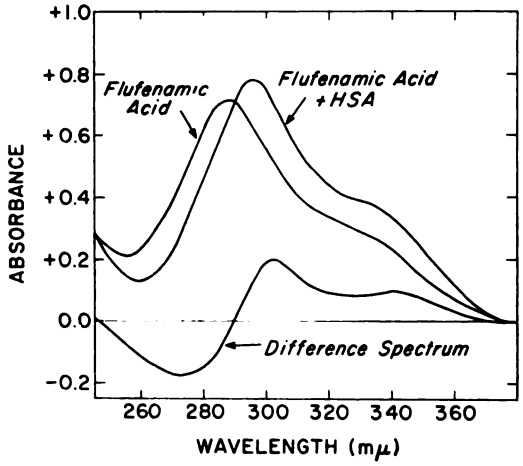


FIG. 2. *Ultraviolet absorption spectra of flufenamic acid alone and bound to human serum albumin*

For the flufenamic acid + HSA spectrum, the blank was HSA. All path lengths were 1.0 cm. The difference spectrum was obtained by combining drug and protein in the sample beam and separating them in the reference beam (see MATERIALS AND METHODS). The following concentrations were employed: flufenamic acid, 1×10^{-4} M; HSA, 1.45×10^{-5} M; sodium phosphate buffer (pH 7.4), 0.1 M.

chain interactions (13). Because the difference spectrum generated by the binding of flufenamic acid to HSA (Fig. 2) did not show any evidence of perturbation of protein tyrosine or tryptophan residues (11, 14), there seemed little doubt that the observed Cotton effects were extrinsic. In addition, the negative ellipticity band at 345 mμ occurred at a wavelength where HSA does not absorb light, and hence could only have resulted from perturbation of the drug chromophore. The binding of mefenamic acid and meclofenamic acid to HSA was also accompanied by the appearance of biphasic extrinsic Cotton effects similar to those observed for flufenamic acid (Fig. 1). No Cotton effects were generated by any of these drugs in the absence of HSA.

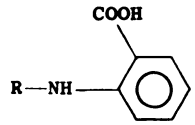
The generation of extrinsic Cotton effects by the binding of these fenamates to HSA must mean that the resultant drug-protein complexes are fairly rigid (2). Within these complexes an asymmetric locus at or near the protein binding site perturbs two electronic transitions in the drug molecules (Fig. 1). Spectral studies have shown[1] that the strong fenamate absorption band at 290–300 mμ results from resonance between the two phenyl groups across the nitrogen, while the weaker band at about 340 mμ (seen only as a shoulder, Fig. 2 and Table 1) is due to electronic transitions occurring in the anthranilic acid portion of the molecule. The biphasic Cotton effects generated by the three fenamate drugs must mean that the symmetry rules (3) governing the interaction of these two electronic transitions with the HSA binding site are such that the protein asymmetric center is located in a region of positive contribution to a Cotton effect for the short wavelength transition (290–300 mμ), but in a region of negative contribution for the long wavelength transition (330–345 mμ). Furthermore, each drug molecule must have a preferred side for binding to the protein-binding site; otherwise Cotton effects of both signs would be generated at each wavelength and no optical activity would be observed.

In an attempt to determine what parameters were important for the generation of extrinsic Cotton effects by these drugs, the interaction of other *N*-phenylanthranilates with HSA was studied (Table 1). With the exception of *N*-phenylanthranilic acid and *N*-(3-methoxyphenyl)anthranilic acid, all the analogues studied generated biphasic extrinsic Cotton effects similar to those observed for the three drug molecules (Table 1). It would appear, therefore, that the majority of the *N*-phenylanthranilates had similar spatial relationships to the drug-binding sites. Nevertheless, each analogue experienced a slightly different asymmetric environment, which in turn gave rise to a unique dissymmetry factor (Table 1). Differences were observed among compounds which were positional isomers (cf. 2-, 3-, and 4-chloro derivatives, Table 1). The successive replacement of chlorine by bromine and then iodine resulted in a decrease of the dissymmetry factor at the lower wavelength but left the higher wavelength factor relatively unchanged (Table 1). The increase in the bulk of the halogens

[1] C. V. Winder, personal communication.

TABLE 1

Molar ellipticities of N-arylanthranilates bound to human serum albumin



R	Ultraviolet absorption maxima		Circular dichroism maxima		
	Wavelength	ϵ	Wavelength	$[\theta]^a$	$\Delta\epsilon/\epsilon^b$
		(M^{-1} cm^{-1})		(deg · cm^2 $dmole^{-1}$)	
	mμ	× 10^{-4}	mμ	× 10^{-4}	× 10^4
C_6H_5	287	1.34	307	+1.00	+4.46
	325[c]	0.49			
4-Cl·C_6H_4	291	1.55	293	+1.97	+3.87
	330[c]	0.60	344	−0.91	−6.00
3-Cl·C_6H_4	288	1.40	306	+1.97	+6.49
	330[c]	0.54	342	−1.17	−8.06
2-Cl·C_6H_4	286	1.37	315	+0.80	+3.78
	330[c]	0.51	343	−0.48	−3.83
4-Br·C_6H_4	292	1.62	297	+1.79	+3.52
	340[c]	0.54	344	−0.93	−5.87
4-I·C_6H_4	294	1.70	300	+1.56	+2.88
	340[c]	0.67	347	−1.02	−5.74
4-CH_3O·C_6H_4	283	1.19	288	+1.77	+4.71
	330[c]	0.68	327	−1.02	−6.45
3-CH_3O·C_6H_4	304	0.94			
	340[c]	0.46	324	+0.92	+4.65
4-C_2H_5O·C_6H_4	284	1.24	284	+0.93	+2.27
	325[c]	0.49	325	−0.40	−1.66
3-C_2H_5O·C_6H_4	290	1.15	300	+2.06	+6.01
	330[c]	0.49	341	−0.51	−3.68
1-Naphthyl	337	0.95	337	−0.72	−2.32
2-Naphthyl	307	1.52	304	+3.48	+7.13
	340	0.84	355	−0.93	−4.03
3-CF_3·C_6H_4	288	1.24	296	+2.01	+5.70
(flufenamic acid)	322[c]	0.54	345	−0.69	−7.04
2,6-Dichloro-*m*-tolyl	277	0.88	302	+0.44	+2.40
(meclofenamic acid)	315[c]	0.56	332	−0.36	−2.80
2,3-Xylyl	284	1.40	292	+2.24	+4.70
(mefenamic acid)	332[c]	0.64	340	−1.44	−6.54

[a] Calculated with reference to the concentration of bound drug.

[b] Optical anisotropy or dissymmetry factor.

[c] Shoulder.

in going from chlorine to iodine may have resulted in an increase in the distance between the asymmetric locus and the perturbed chromophore, thereby decreasing the strength of the interaction. Meclofenamic acid was much less influenced by the asymmetric environment than were the other *N*-phenylanthranilates (Table 1). Once again it is possible that the bulky chlorine and methyl substituents carried by this drug molecule may have prevented a closer interaction with the binding site. Replacement of the *N*-phenyl group by *N*-naphthyl produced a new series of optically active bands (Table 1).

While extrinsic Cotton effects can be

TABLE 2
Molar ellipticities of flufenamic acid bound to different serum albumins

Serum albumin	Wavelength maximum	$[\theta]^a$	$\Delta\epsilon/\epsilon$
		(deg · cm² dmole⁻¹)	
	mμ	*× 10⁻⁴*	*× 10⁴*
Human	296	+2.01	+5.70
	345	−0.61	−7.04
Porcine	292	+1.90	+4.43
	352	−0.31	−3.92
Equine	292	+0.49	+1.14
	348	−0.36	−3.63
Bovine	305	+0.77	+2.65
	348	−0.26	−2.63
Canine	295	+1.08	+2.64
Ovine	302	+0.90	+2.84
Rabbit	297	+1.23	+3.22
	325[b]	+0.40	+1.95

[a] Calculated with reference to the concentration of bound drug.
[b] Shoulder.

used to study the orientation of ligands at the same binding site, they may also reflect differences between binding sites (2). The biphasic Cotton effects generated when flufenamic acid bound to porcine, equine, and bovine serum albumins indicated that the binding sites on these proteins were similar to those of HSA (Table 2). In contrast, canine and ovine serum albumins only perturbed the short wavelength transition of flufenamic acid, suggesting that the asymmetric center did not interact with the anthranilic acid portion of the drug. The binding of flufenamic acid to rabbit serum albumin (Table 2) was unique in that it gave rise to an extrinsic Cotton effect with two positive maxima. It is noteworthy that the binding site for phenylbutazone on rabbit serum albumin also differs from that of other albumins (2).

The binding of flufenamic acid to HSA was measured by equilibrium dialysis, and the results were plotted in the form of a Scatchard curve (Fig. 3). When n was less than 2, n/C became infinitely large because all the flufenamic acid appeared to be bound. For this reason, calculation of individual association constants for the binding sites was not attempted. Nevertheless, it did appear that HSA had at least three binding sites with a very high affinity for flufenamic acid. It was not surprising to find, therefore, that when a fixed concentration of HSA was titrated with increments of flufenamic acid, the rise in ellipticity at 295 mμ was rapid until each mole of protein had bound 3 moles of drug (Fig. 4). Further addition of flufenamic acid produced only a small increase in ellipticity (Fig. 4). Thus the binding of flufenamic acid to the three high-affinity sites on HSA generated strong extrinsic Cotton effects, while binding to the low-affinity sites gave rise to much weaker ellipticities. This supports the hypothesis that strong extrinsic Cotton effects only occur when the complex between ligand and macromolecule is rigid. The titration curve (Fig. 4) showed some evidence of sigmoid character, suggesting either that a cooperative effect occurred between sites, or that one of the strong binding sites induced a larger extrinsic Cotton effect than did the others.

There seems to be little doubt that electrostatic forces play an important role in the binding of anionic drugs to serum albumin (15). In accord with the suggestion of Skidmore and Whitehouse (16) that the

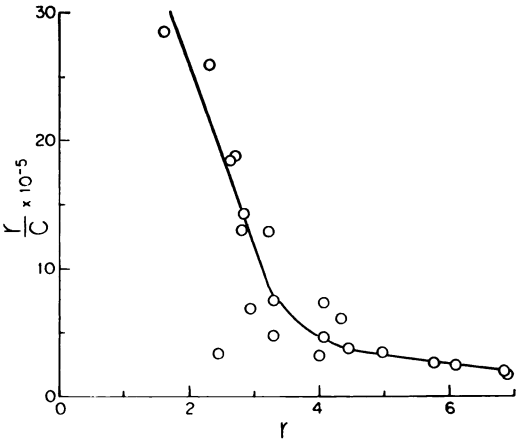


FIG. 3. *Scatchard plot of the binding of flufenamic acid to HSA*

All measurements were made in the presence of 0.1 M sodium phosphate buffer, pH 7.4. r = number of moles of flufenamic acid bound per mole of HSA; C = molar concentration of free flufenamic acid.

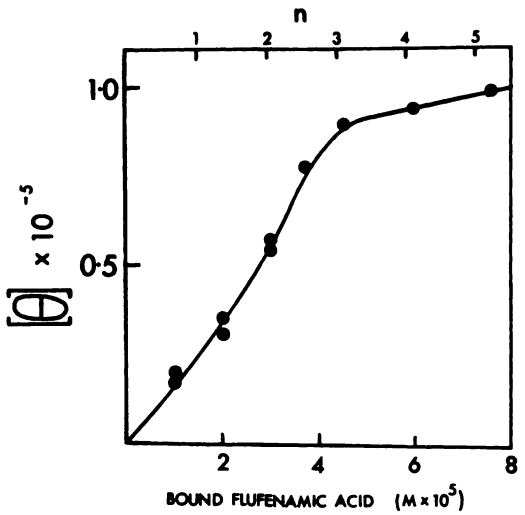


FIG. 4. *Relationship between molar ellipticity [θ] measured at 295 mμ and the concentration of bound flufenamic acid*

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.

ε-amino group of lysine may be the point of attachment for anionic drugs, unpublished work in our own laboratory has shown that prior treatment of HSA with *O*-methylisourea or succinic anhydride drastically reduces the binding of flufenamic acid. However, a one-point electrostatic attachment of flufenamic acid to HSA would allow the drug molecule to rotate freely, thus precluding the generation of extrinsic Cotton effects. It has been shown (2) for phenylbutazone that the extrinsic Cotton effects generated by binding to HSA depend not only on electrostatic interactions but also on van der Waals attraction between the phenyl groups of the drug and a hydrophobic area on the protein. In order to determine what role, if any, hydrophobic interactions played in the binding of *N*-phenylanthranilates to HSA, an attempt was made to correlate the association constant of a given analogue for HSA with its hexane/water partition coefficient (Fig. 5). Association constants for the *N*-phenylanthranilates were determined by measuring their ability to displace dansylglycine competitively from

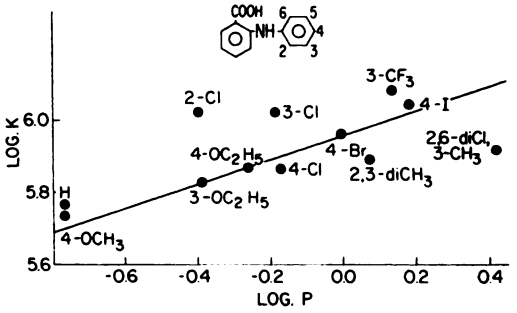


FIG. 5. *Relationship between the association constants (K) of the N-phenylanthranilates for HSA and their partition coefficients (P) between n-hexane (containing 1% 1-octanol) and 0.1 M sodium phosphate buffer, pH 7.4.*

HSA (see MATERIALS AND METHODS). This method was employed because multiple binding sites (Fig. 3) made the comparison with results obtained from equilibrium dialysis difficult. In addition, the dansylglycine-binding site on HSA has been shown to be located in a hydrophobic region of the protein (2), where short-range dispersive interactions become important for binding. While there appeared to be some correlation between the associ-

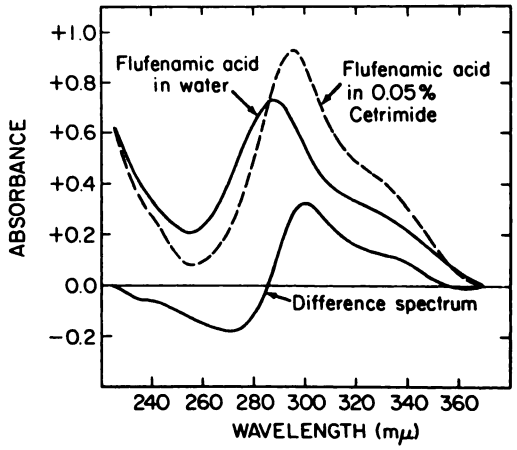


FIG. 6. *Ultraviolet absorption spectra of flufenamic acid measured in the presence of 0.05% cetrimide*

Blanks contained the corresponding solvent, without drug. All path lengths were 1.0 cm. The difference spectrum was obtained by placing the cetrimide solution of flufenamic acid in the sample beam and the aqueous solution in the reference beam. All solutions contained 0.05 M sodium phosphate, pH 7.4.

ation constant and the hexane/water partition coefficient of a given analogue, some of the *N*-phenylanthranilates gave anomalous results (Fig. 5). This suggested that factors other than short-range dispersive interactions may have to be taken into consideration. It was particularly noteworthy that the association constant of meclofenamic acid for HSA was much lower than would have been predicted from its partition coefficient (Fig. 5).

The ultraviolet absorption spectrum of flufenamic acid was also shifted to longer wavelengths on binding to HSA, with a concomitant increase in extinction coefficient (Fig. 2). The shape and location of the resultant difference spectrum (Fig. 2) would not have resulted from a perturbation of the aromatic residues of HSA (11, 14). It was found, however, that the spectral shift observed when flufenamic acid was bound to HSA was qualitatively very similar to that seen when the drug was dissolved in a solution containing the cationic detergent cetrimide (Fig. 6). No effect on the absorption spectrum of flufenamic acid was seen at cetrimide concentrations below the critical micellar concentration (0.02%, w/v) or in the presence of the anionic detergent sodium lauryl sulfate. Thus the spectral changes generated by flufenamic acid on binding to HSA could be duplicated by dissolving the aromatic portion of the drug in a micelle while allowing the carboxylate group to remain in the aqueous medium in association with a quaternary ammonium moiety. This would suggest that the HSA binding site for flufenamic acid consists of a cationic site on the surface of the protein with a hydrophobic crevice to accommodate the phenyl rings. It is the asymmetry of this crevice which is reflected by the extrinsic Cotton effects generated by the binding of the fenamates to HSA. An examination of molecular models showed that the chlorine substituents of meclofenamic acid forced the two phenyl groups into planes which were mutually at right angles. If the hydrophobic crevice available for the binding of the fenamates is narrow, it is easy to see how meclofenamic acid might have difficulty in entering. This would explain both the low dissymmetry factor (Table 1) and the relatively small association constant (Fig. 5).

This study has shown how an examination of the optical properties of drug-albumin complexes, particularly their extrinsic Cotton effects, can yield information on the nature of the binding sites involved. It is to be hoped that some of these techniques may be useful in the study of drug interaction with other biological macromolecules.

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