MOLECULAR PHARMACOLOGY, 7, 229–237

Optical Studies of Drug-Protein Complexes

V. The Interaction of Phenylbutazone, Flufenamic Acid, and Dicoumarol with Acetylsalicylic Acid-Treated Human Serum Albumin

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(Received November 12, 1970)

SUMMARY

When acetylsalicylic acid is incubated at 37° with human serum albumin the protein is acetylated by the drug. Acetylation of human serum albumin by acetylsalicylic acid does not appear to cause any conformational changes, since the intrinsic optical activity of the protein is unaltered. However, the positive extrinsic Cotton effect generated at 287 mμ by the binding of phenylbutazone to human serum albumin increased in amplitude after the protein had been acetylated by acetylsalicylic acid. In contrast, the strong positive extrinsic circular dichroic band which appears at 296 mμ when flufenamic acid binds to human serum albumin decreased in amplitude when the protein was acetylated by acetylsalicylic acid, while the weaker negative band at 345 mμ was unchanged. Acetylsalicylic acid treatment of human serum albumin did not affect the strong negative extrinsic circular dichroic band at 305 mμ generated by the binding of dicoumarol. These results suggest that acetylation of human serum albumin by acetylsalicylic acid specifically modifies the binding sites for phenylbutazone and flufenamic acid. No change in the extrinsic optical activity of bound phenylbutazone or flufenamic acid was observed when acetylsalicylic acid was replaced by salicylic acid or by other acylating agents such as benzylpenicillin, benzylpenicillenic acid, and acetic anhydride. Equilibrium dialysis measurements showed that acetylation by acetylsalicylic acid also increased the affinity of human serum albumin for phenylbutazone but decreased its affinity for flufenamic acid. The affinity of the acetylsalicylic acid-treated human serum albumin for dicoumarol was the same as that of albumin incubated alone. Thus the plasma binding of phenylbutazone or flufenamic acid in a patient may be altered by the prior ingestion of acetylsalicylic acid.

INTRODUCTION

Hawkins and co-workers have recently reported (1) that acetylsalicylic acid can acetylate human serum albumin. Acetylation occurred both *in vivo* and *in vitro* and appeared to involve a lysine group in the protein (1, 2). Since the acetylated HSA[1] had an increased capacity to bind acetrizoate (3-acetamido-2,4,6-triidobenzoate), these workers suggested (1) that the interaction of other anionic drugs as well as biologically important anions could be altered by acetylation of the protein. We have therefore made equilibrium dialysis and circular dichroism studies of the binding of three anionic drugs—phenylbutazone, flufenamic acid, and dicoumarol—to acetylsalicylic acid-treated HSA. Our results show

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

that incubation of HSA with acetylsalicylic acid modifies the protein binding sites for phenylbutazone and flufenamic acid, but does not alter the HSA binding site for dicoumarol.

MATERIALS AND METHODS

Materials. Crystalline HSA, purchased from Mann Research Laboratories, was dialyzed overnight at 4° before use. The concentration of HSA was calculated from the optical density of solutions at 280 mμ ($E^{1\%}_{1\,cm}$ = 5.3). Flufenamic acid [*N*-(α,α,α-trifluoro-*m*-tolyl)anthranilic acid] was supplied by Dr. C. V. Winder (Parke, Davis and Company); phenylbutazone (4-butyl-1,2-diphenyl-3,5-pyrazolidinedione) was a gift from Dr. F. Clarke (Geigy Pharmaceuticals), while dicoumarol (Dicumarol; 3,3′-methylenebis[4-hydroxycoumarin]) was obtained from Abbott Laboratories. All other chemicals were of reagent grade. Sodium benzylpenicillin, purchased from Calbiochem, was converted to benzylpenicillenic acid by the method of Levine (3). Dansylglycine (1-dimethylaminonaphthalene-5-sulfonyl-*N*-glycine) was purchased from Mann Research Laboratories.

Methods. Circular dichroism measurements were made at 27° with a Cary 6001 attachment to the Cary model 60 spectropolarimeter. Results are expressed as molar ellipticities, $[\theta]$ (deg·cm^2 mole^{-1}), which were calculated from the formula

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

where θ_{obs} = observed ellipticity (degrees), l = pathlength (centimeters), and c = molar concentration. Molar ellipticities were calculated in terms of the concentration either of bound drug or of HSA, assuming a molecular weight of 69,000.

The binding of drugs to HSA (10 μM) (Figs. 4–7) was measured by equilibrium dialysis using multiple microdialysis cells (1-ml capacity) purchased from the Chemical Rubber Company. The cells were rocked at 25° for 24 hr, by which time equilibrium had been established. The concentrations of free phenylbutazone (ϵ_{267} = 2×10^4 M^{-1} cm^{-1}), flufenamic acid (ϵ_{288} = 1.24×10^4 M^{-1} cm^{-1}), and dicoumarol (ϵ_{308} = 1.76×10^4 M^{-1} cm^{-1}) were measured spectrophotometrically. Results have been plotted according to the method of Scatchard (4), using the relationship

$$\frac{r}{c} = Kn - Kr \qquad (1)$$

where r = number of moles of drug bound per mole of protein, c = molar concentration of free drug, K = association constant (liters per mole), and n = number of drug-binding sites per molecule of protein. The concentration of bound drug needed for the calculation of $[\theta]$ in Tables 2–4 was determined in triplicate by ultrafiltration through an Amicon cell (10 ml) equipped with a PM-10 filter. The binding values determined by this method for any given drug-protein mixture did not vary from each other by more than 5%.

Acetylation of HSA was carried out by the method of Hawkins *et al.* (1), in which the protein (0.1 mM) is incubated at 37° for 24 hr with acetylsalicylic acid (0.5 mM) and then dialyzed at 4° for 48 hr against multiple changes of 0.15 M NaCl containing 0.05 M sodium phosphate buffer, pH 7.4, and finally against distilled water. In control experiments acetylsalicylic acid was omitted or replaced by an equimolar concentration of salicylic acid, acetic anhydride, benzylpencillin, or benzylpenicillenic acid.

The quantum yield of dansylglycine bound to the treated albumins was determined by previously reported methods (5).

All experiments were carried out in the presence of 0.1 M sodium phosphate buffer, pH 7.4.

RESULTS

Circular dichroism. Legrand and Viennet (6) have reported that the circular dichroic spectrum of HSA has a positive band at 190 mμ and negative bands at 209 mμ and 222 mμ, which are characteristic of α-helical proteins. HSA also shows some optical activity in the 240–320 mμ region (6), which is probably due to the aromatic residues present in the protein. Incubation of HSA

either alone or with acetylsalicylic acid or salicylic acid did not alter the magnitude of the 222 mμ ellipticity band (Table 1), nor did it affect the circular dichroism of the protein at higher wavelengths (Fig. 1). Thus it would appear that no major conformational changes took place in HSA during the incubation period.

TABLE 1

Molar ellipticity at 222 mμ of human serum albumin

Human serum albumin (0.1 mM) was incubated with drug (0.5 mM) where indicated, then dialyzed at 4° against 0.15 M NaCl and finally distilled water.

Addition	Incubation conditions		Molar ellipticity, $[\theta] \times 10^{-4}$
	Time	Temperature	
	hr		*deg·cm² dmole⁻¹*
None	0		−1.41
None	24	37°	−1.40
Acetylsalicylic acid	24	37°	−1.43
Salicylic acid	24	37°	−1.40

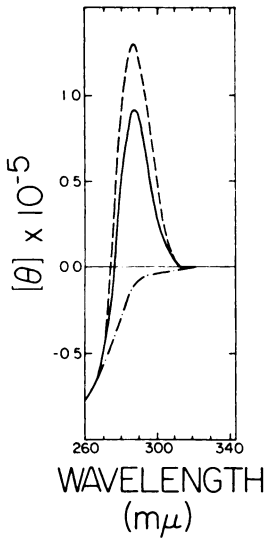

FIG. 1. *Extrinsic Cotton effects generated by binding of phenylbutazone to control (nonincubated) and acetylsalicylic acid-treated HSA*

–·–·, HSA (control and acetylsalicylic acid-treated) (13 μM); ——, phenylbutazone (50 μM) + HSA (13 μM); – – –, phenylbutazone (50 μM) acetylsalicylic acid-treated HSA (13 μM).

TABLE 2

Molar ellipticity at 287 mμ of phenylbutazone bound to human serum albumin

Human serum albumin (0.1 mM) was incubated with drug (0.5 mM) where indicated, then dialyzed at 4° against 0.15 M NaCl and finally distilled water.

Addition	Incubation conditions		Molar ellipticity, $[\theta] \times 10^{-3}$[a]
	Time	Temperature	
	hr		*deg·cm² dmole⁻¹*
None	0		+53.9
None	24	37°	+53.7
Acetylsalicylic acid	24	37°	+75.8
Salicylic acid	24	37°	+51.4
Benzylpenicillenic acid	24	37°	+53.0
Benzylpenicillin	24	37°	+52.8
Acetic anhydride	24	37°	+53.2

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

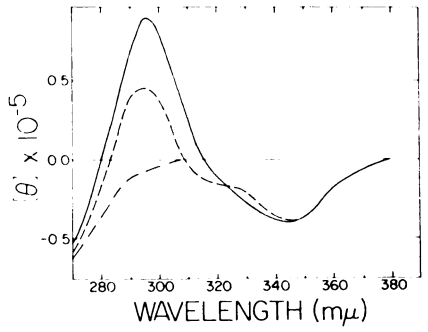

FIG. 2. *Extrinsic Cotton effects generated by binding of flufenamic acid to control (nonincubated) and acetylsalicylic acid-treated HSA*

–·–·, HSA (control and acetylsalicylic acid-treated) (14.5 μM); ——, flufenamic acid (100 μM) + HSA (14.5 μM); – – –, flufenamic acid (100 μM) + acetylsalicylic acid-treated HSA (10 μM).

In previous studies (7) it has been shown that when phenylbutazone binds to HSA a positive ellipticity band appears at 287 mμ in the circular dichroic spectrum of the protein (Fig. 1). This band is extrinsic in origin and results from perturbation of an n-π^* transition in the carbonyl group of phenylbutazone by an asymmetrical locus at or near the drug-binding site (7). When

TABLE 3

Molar ellipticity of flufenamic acid bound to human serum albumin

Human serum albumin (0.1 mM) was incubated with drug (0.5 mM) where indicated, then dialyzed at 4° against 0.15 M NaCl and finally distilled water.

Addition	Incubation conditions		Molar ellipticity, $[\theta] \times 10^{-3}$	
	Time	Temperature	296 mμ	345 mμ
	hr		*deg·cm² dmole⁻¹*	
None	0		+15.7	−6.32
None	24	37°	+16.2	−6.80
Acetylsalicylic acid	24	37°	+8.3	−6.80
Salicylic acid	24	37°	+16.2	−6.80
Benzylpenicillenic acid	24	37°	+16.4	−6.80
Benzylpenicillin	24	37°	+16.2	−6.80
Acetic anhydride	24	37°	+15.0	−6.80

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

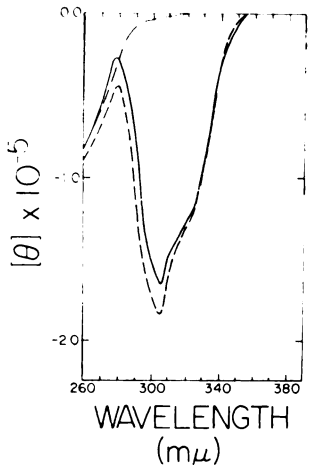

FIG. 3. *Extrinsic Cotton effects generated by binding of dicoumarol to control (nonincubated) and acetylsalicylic acid-treated HSA*

–·–·, HSA (control and acetylsalicylic acid-treated) (13 μM); ——, dicoumarol (50 μM) + HSA (13 μM); – – –, dicoumarol (50 μM) + acetylsalicylic acid-treated HSA (13 μM).

HSA was treated with acetylsalicylic acid, the amplitude of this ellipticity band increased while its wavelength maximum remained essentially unchanged (Fig. 1). Since the molar ellipticity $[\theta]$ in Fig. 1 was calculated from the HSA concentration, it was possible that the increased ellipticity was due merely to increased binding of phenylbutazone to the protein. However, it can be seen from Table 2 that when $[\theta]$ was expressed in terms of the concentration of bound drug the difference persisted. Moreover, this effect was specific for acetylsalicylic acid-treated HSA, since it was not observed when the protein was incubated

TABLE 4

Molar ellipticity at 305 mμ of dicoumarol bound to human serum albumin

Human serum albumin (0.1 mM) was incubated with drug (0.5 mM) where indicated, then dialyzed at 4° against 0.15 M NaCl and finally distilled water.

Addition	Incubation conditions		Molar ellipticity, $[\theta] \times 10^{-3}$
	Time	Temperature	
	hr		*deg·cm² dmole⁻¹*
None	0		−41.9
None	24	37°	−45.1
Acetylsalicylic acid	24	37°	−48.3
Salicylic acid	24	37°	−45.6

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

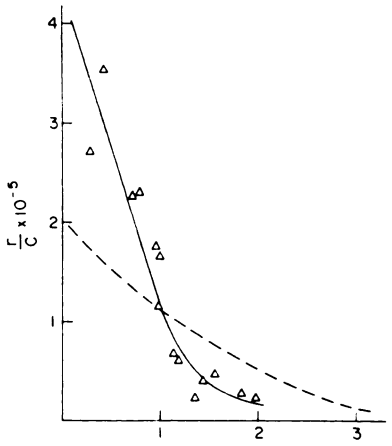

FIG. 4. *Scatchard plot of phenylbutazone binding to control (nonincubated) HSA* (– – –) *and control (incubated) HSA* (△——△)

The data for the control (nonincubated) HSA curve were taken from ref. 7.

with equimolar concentrations of salicylic acid, acetic anhydride, benzylpenicillin, or benzylpenicillenic acid (Table 2).

When flufenamic acid binds to HSA, biphasic extrinsic Cotton effects are observed (8), with a strong, positive ellipticity band appearing at 296 mμ and a weaker, negative band at 345 mμ (Fig. 2). Incubation of HSA with acetylsalicylic acid decreased the amplitude of the positive ellipticity band but left the negative band unchanged (Fig. 2). Once again it can be seen (Table 3) that this difference was still observed when the results were calculated in terms of bound drug. Treatment of HSA with salicylic acid, acetic anhydride, benzylpenicillin, and benzylpenicillenic acid did not alter the extrinsic optical activity of bound flufenamic acid (Table 3).

The binding of dicoumarol to HSA generates a strong negative extrinsic ellipticity band at 305 mμ (5) (Fig. 3). In contrast to the results obtained with phenylbutazone and flufenamic acid, the Cotton effect generated by the binding of dicoumarol to HSA was not affected by prior treatment of the protein with acetylsalicylic acid (Fig. 3 and Table 4).

Equilibrium dialysis studies. Previous studies have shown (7) that HSA has one strong binding site ($K_a = 1 \times 10^5$ M^{-1}) and two weaker binding sites ($K_a = 4 \times 10^4$ M^{-1}) for phenylbutazone (Fig. 4). Incubation of HSA alone or with salicylic acid significantly increased the affinity of the protein for phenylbutazone (Figs. 4 and 5). Unfortunately, it is not possible to determine from the Scatchard plots which sites in the HSA molecule were affected by the incubation. Nevertheless, for acetylsalicylic acid-treated HSA the affinity of the protein for phenylbutazone was so great that under the experimental conditions employed no free drug could be detected when the phenylbutazone to HSA ratio was less than unity (Fig. 5).

Human serum albumin has three very strong binding sites ($K > 1.5 \times 10^6$ M^{-1}) and about eight weaker binding sites ($K < 7 \times 10^4$ M^{-1}) for flufenamic acid (Fig. 6) (8). Incubation of HSA with acetylsalicylic acid reduced the affinity of flufenamic acid

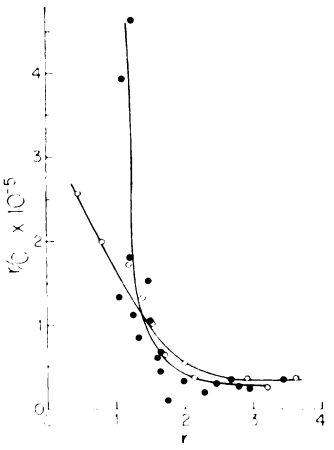

FIG. 5. *Scatchard plot of phenylbutazone binding to acetylsalicylic acid-treated HSA* (●——●) *and salicylic acid-treated HSA* (○ ——○)

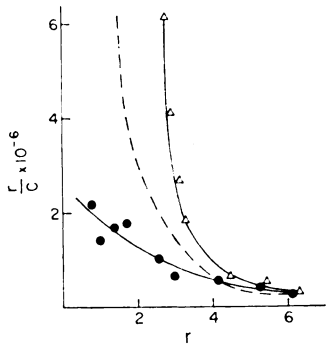

FIG. 6. *Scatchard plot of flufenamic acid binding to control (nonincubated) HSA* (- - -), *acetylsalicylic acid-treated HSA* (● ——●), *and control (incubated) HSA* (△— △)

The data for the control (nonincubated) HSA curve were taken from ref. 8.

for the strong binding sites. In contrast, the affinity of flufenamic acid for the albumin incubated alone (Fig. 6) was greater than its affinity for the control, nonincubated albumin. The Scatchard plot for the binding of flufenamic acid to the salicylic acid-treated albumin (not shown) resembled that for the incubated albumin (Fig. 6).

Incubation of HSA either alone or with acetylsalicylic acid dramatically increased the affinity of the protein for dicoumarol (Fig. 7). The Scatchard plot for the binding of dicoumarol to the salicylic acid-treated albumin (not shown) was the same as that for the incubated albumin (Fig. 7).

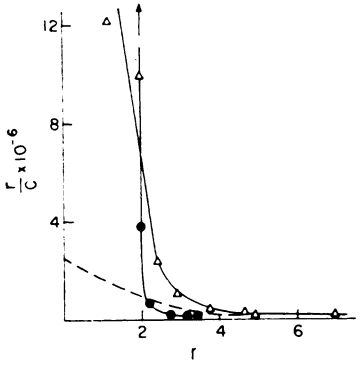

FIG. 7. *Scatchard plot of dicoumarol binding to control (nonincubated) HSA* (- - -), *acetylsalicylic acid-treated HSA* (●——●), *and control (incubated) HSA* (△ · · △)

The data for the control (nonincubated) HSA curve were taken from ref. 9.

TABLE 5

Quantum yield of dansylglycine bound to human serum albumin

Quantum yields were determined using solutions containing 10 μM dansylglycine and 8.5 μM HSA. The activation wavelength was 350 mμ, with a bandwidth of 12 mμ. Quantum yields were calculated by previously reported methods, using a quinine sulfate standard. HSA was incubated and dialyzed as described in Table 2.

Treatment	Incubation conditions		Quantum yield
	Time	Temperature	
	hr		
Control			0.443
Control	24	37°	0.460
Acetylsalicylic acid	24	37°	0.446
Salicylic acid	24	37°	0.470

Fluorescence. Since the binding of certain dansylamino acids to serum albumin results in an increase in the fluorescence yield of the ligand and a blue shift in its fluorescence emission maximum, Chen (9) has suggested that they can be used as fluorescent probes for the hydrophobic region of proteins (10). Chignell (7) has shown by fluorescence titration that dansylglycine can competitively displace phenylbutazone from its high-affinity site on HSA. Since the circular dichroism and equilibrium dialysis studies suggested that acetylsalicylic acid modified the high-affinity phenylbutazone-binding site on HSA, it was of interest to see whether such a change would alter the polarity of the site. It can be seen from Table 5 that there was little difference in the quantum yield of dansylglycine bound to the different albumins. The fluorescence emission maximum of dansylglycine bound to HSA was also unaffected by treatment with acetylsalicylic acid (Table 5).

DISCUSSION

Circular dichroism. Extrinsic Cotton effects generated by the binding of drugs such as phenylbutazone, flufenamic acid, and dicoumarol to serum albumin result from the perturbation of the electronic transitions occurring in a drug chromophore by electrostatic forces associated with an asymmetrical locus at the albumin binding site (11). An extrinsic Cotton effect may be characterized by three parameters: sign, magnitude, and wavelength location. The sign of an extrinsic Cotton effect is governed by the spatial relationship between the asymmetrical center and the perturbed chromophore. Schellman (11) has shown 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. For example, the purines and pyrimidines 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 (11). The n-π^* transition in the carbonyl group of phenylbutazone, which becomes optically active when the drug binds to HSA, probably obeys either the quadrant rule or the octant rule (11) (Fig. 8). For the remainder of this discussion it will be assumed that the simpler quadrant rule is in operation. The magnitude of an extrinsic Cotton effect is dependent upon the distance between the perturbed chromophore and the asymmetrical center as well as on the rigidity of the ligand-macromolecule complex. While the precise relationship depends to

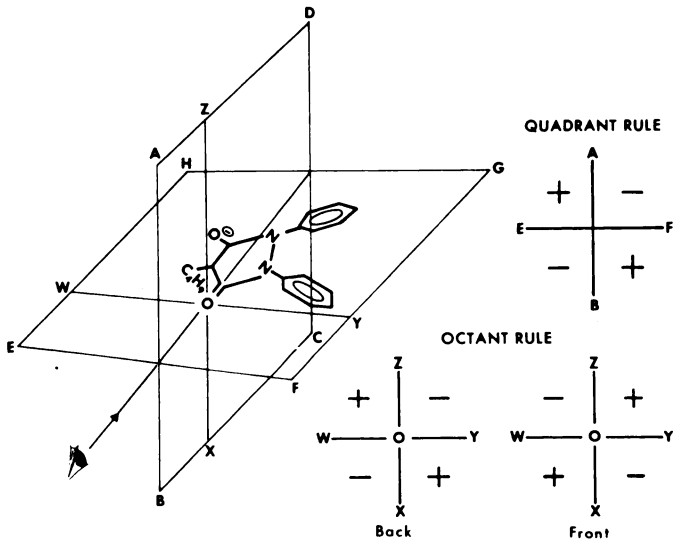

FIG. 8. *Symmetry rules for the carbonyl chromophore of phenylbutazone*
Planes *ABCD* and *EFGH* are at right angles.

some extent on the system involved, the magnitude of a Cotton effect generally is inversely proportional to the fifth or sixth power of the distance between the asymmetrical locus and the perturbed chromophore. The maximal ellipticity of an extrinsic Cotton effect occurs in the same wavelength region where the perturbed chromophore absorbs anisotropic (i.e., nonpolarized) light (12, 13). Extrinsic Cotton effects appear to mirror the characteristics of specific asymmetrical sites in proteins and thereby offer an experimental means of exploring the detailed features of such sites (12, 13).

Although acetylation of HSA by acetylsalicylic acid increased the amplitude of the extrinsic Cotton effect generated by the binding of phenylbutazone (Table 2), the wavelength of maximal circular dichroism was unchanged (Fig. 1). There is little doubt, therefore, that in the complex between phenylbutazone and acetylsalicylic acid-treated albumin it is still the n-π^* transition of the drug carbonyl group which becomes optically active (7). It is also important to note that the Cotton effect remains positive when phenylbutazone binds to the acetylated HSA (Fig. 1). From Fig. 8 it may be seen that this will occur only if the asymmetrical locus remains in the same quadrant or if it moves diagonally into the opposite quadrant having the same sign. If it is assumed that the asymmetrical center remains in the same quadrant, the increase in the extrinsic optical activity of phenylbutazone bound to acetylsalicylic acid-treated HSA suggests that the drug interacts to a greater extent with its binding site in the acetylated albumin. In simplest terms, this may mean that treatment with acetylsalicylic acid has modified the phenylbutazone site so that the carbonyl group of the drug is much closer to the asymmetrical center in the acetylated protein.

Incubation of HSA with acetylsalicylic acid did not alter either the wavelength location or the sign of the extrinsic Cotton effects generated by the binding of flufenamic acid. However, the magnitude of the strong positive Cotton effect at 296 mμ was smaller in the acetylsalicylic acid-treated protein (Table 3 and Fig. 2). Spectral studies have shown[2] that the transition at 296 mμ involves resonance between the two phenyl groups across the nitrogen, while the transition at 345 mμ originates in the anthranilic acid portion of the molecule. Since acetylation affected only the optical

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

activity of the transition at 296 mμ, it appears that the changes brought about by acetylation altered only the spatial relationship between the *N*-phenyl portion of the molecule and the asymmetrical locus at the binding site. Possibly acetylation of HSA may result in distortion of flufenamic acid at its binding site.

At the present time it is not possible to interpret changes in the extrinsic optical activity of HSA-bound drugs in terms of specific alterations at their protein-binding sites. Nevertheless it can be stated unequivocally that treatment of HSA with acetylsalicylic acid somehow alters the molecular architecture of the binding sites for phenylbutazone and flufenamic acid. These alterations must be highly specific, since they are not observed with dicoumarol, nor are they seen with phenylbutazone or flufenamic acid when acetylsalicylic acid is replaced by other acylating agents, such as acetic anhydride, benzylpenicillenic acid, or benzylpenicillin, which are known to react with the amino groups of proteins (3, 14, 15). The inability of acetic anhydride to modify the binding sites of phenylbutazone and flufenamic acid is of interest, since Pinckard and co-workers (16) have shown that acetylation of HSA with this reagent increases the binding of acetrizoate to the protein. At first glance this would suggest that acetrizoate occupies a different binding site on HSA from phenylbutazone or flufenamic acid. However, since Pinckard and co-workers (16) did not indicate the conditions under which they acetylated HSA with acetic anhydride, it is not possible to compare their findings with those reported in this paper. In our experiments, when acetic anhydride and acetylsalicylic acid were used in equimolar concentrations, only the acetylsalicylic acid-treated HSA exhibited alterations in the binding sites for phenylbutazone and flufenamic acid. This suggests that the specificity of acetylsalicylic acid toward the binding sites for phenylbutazone and flufenamic acid probably resides in the salicylic acid portion of the molecule.

The syndrome of "aspirin intolerance" characterized by asthma, rhinitis, and nasal polyps is induced by ingestion of acetylsalicylic acid but not salicylic acid (17). While the pathogenesis of this syndrome is unknown, one possible explanation may be that acetylation of proteins such as albumin may render them antigenic (1). Circular dichroism studies of drug-treated HSA, such as those described here, may therefore provide a means for detecting small structural changes in HSA which could possibly intensify its antigenicity.

Equilibrium dialysis. Incubation of HSA alone at 37° for 24 hr increased the affinity of the protein for phenylbutazone, flufenamic acid, and dicoumarol. The most dramatic increase was observed with dicoumarol, which was bound so strongly that when the dicoumarol to HSA ratio was less than 2 no free drug could be detected (Fig. 7). These results strongly suggest that equilibrium dialysis measurements made after prolonged incubation at 37° may give anomalously high association constants for certain drugs.

When HSA was incubated at 37° with salicylic acid, the affinity of the protein for phenylbutazone, flufenamic acid, and dicoumarol was not very different from that of HSA incubated in the absence of salicylic acid. However, when salicylic acid was replaced by acetylsalicylic acid, the acetylated HSA bound phenylbutazone to a greater extent (Figs. 4 and 5) but bound flufenamic to a lesser extent (Fig. 6) than control incubated HSA. These results suggest that the plasma binding of phenylbutazone and flufenamic acid may be altered by the ingestion of acetylsalicylic acid. Recently Van Arman and Nuss (18) have shown that the prior administration of acetylsalicylic acid to rats decreases the ability of both phenylbutazone and flufenamic acid to antagonize adjuvant-induced arthritis. While we have not studied the effect of acetylsalicylic acid on rat serum albumin, the possibility exists that acetylation of this protein may increase its affinity for phenylbutazone and flufenamic acid and thereby prevent these drugs from reaching the site of inflammation.

Fluorescence. No difference was detected in either the fluorescence emission maximum or the fluorescence quantum yield of dansylglycine bound to HSA when the protein was acetylated by acetylsalicylic acid. It has been shown previously (6) that dansylglycine

competes for phenylbutazone at its strong binding site on HSA. Since circular dichroism and equilibrium dialysis studies have shown that this site is modified by acetylsalicylic acid, it seems probable that acetylation does not greatly alter the hydrophobicity of the site which interacts with dansylglycine.

REFERENCES

1. D. Hawkins, R. N. Pinckard and R. S. Farr, *Science* **160,** 780 (1968).
2. D. Hawkins, R. N. Pinckard, I. P. Crawford and R. S. Farr, *J. Clin. Invest.* **48,** 536 (1969).
3. B. B. Levine, *Arch. Biochem. Biophys.* **93,** 50 (1961).
4. G. Scatchard, *Ann. N. Y. Acad. Sci.* **51,** 660 (1949).
5. C. F. Chignell, *Mol. Pharmacol.* **6,** 1 (1970).
6. M. Legrand and R. Viennet, *C. R. Hebd. Seances Acad. Sci. Paris* **259,** 8 (1964).
7. C. F. Chignell, *Mol. Pharmacol.* **5,** 244 (1969).
8. C. F. Chignell, *Mol. Pharmacol.* **5,** 455 (1969).
9. R. F. Chen, *Arch. Biochem. Biophys.* **120,** 609 (1967).
10. G. M. Edelman and W. O. McClure, *Accts. Chem. Res.* **1,** 65 (1968).
11. J. A. Schellman, *J. Chem. Phys.* **44,** 55 (1968).
12. D. D. Ulmer and B. L. Vallee, *Advan. Enzymol.* **27,** 37 (1965).
13. C. F. Chignell, *in* "Methods in Pharmacology" (C. F. Chignell, ed.), Vol. 2. Appleton-Century-Crofts, New York. In press.
14. C. H. Schneider and A. L. DeWeck, *Biochim. Biophys. Acta* **168,** 27 (1968).
15. J. F. Riordan and B. L. Vallee, *Methods Enzymol.* **11,** 565 (1967).
16. R. N. Pinckard, D. Hawkins and R. S. Farr, *Fed. Proc.* **27,** 543 (1968).
17. M. Samter and R. F. Beers, *J. Allergy* **40,** 281 (1967).
18. C. G. Van Arman and G. W. Nuss, *Pharmacologist* **12,** 202 (1970).