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Hydrophobic Bonding of Sulfonamide Drugs with Serum Albumin†

Toshio Fujita

Department of Agricultural Chemistry, Kyoto University, Kyoto, Japan. *Received June 6, 1972*

The adsorption constants of various sulfonamide drugs with bovine serum albumin (BSA) at various pH points are analyzed with free energy related physicochemical parameters taking into account the correction for ionization of the drugs. The pH dependence of the adsorption constant is discussed in terms of the pH-dependent hydrogen ion dissociation equilibrium of BSA. It is postulated that the adsorption equilibrium is determined by the hydrophobicity of drugs and occurs through the binding of the neutral drug molecule with the hydrophobic fraction of the protein surface, the variation of which is dependent on the state of the dissociation equilibrium of basic groups on the BSA molecule.

The binding of sulfonamide drugs to serum albumin has been considered to be important for their chemotherapeutic values, such as *in vivo* antibacterial activity and the duration of action.[1] Only unbound free drug is active against bacteria,[2] susceptible to metabolism,[3] and excreted from the kidney.[4] There have been a number of efforts to elucidate the nature of the drug-protein binding force from physicochemical as well as pharmacological points of view.[2,5-8] While the view that most of the energy of binding of sulfonamide drugs is due to hydrophobic forces is receiving increasing support[9,10] some experimental evidence still seems to remain suggesting an important role of electrostatic force between the sulfonamide anion and the positive charge on the protein surface.[5,8]

We have been analyzing the bacteriostatic activity,[11] serum protein binding,[11] and the rate of elimination[12] of N^1-substituted sulfonamides in terms of their physicochemical properties with the use of free energy related substituent parameters such as $\Delta \log K_A$ for the electron-withdrawing factor and π for the hydrophobic character of the N^1 substituent. K_A is the dissociation constant for the N^1 hydrogen and π is defined as $\Delta \log P$.[13] P is the partition coefficient of drugs determined with an *i*-BuOH-H_2O system. For $\Delta \log K_A$ and $\Delta \log P$, the $\log K_A$ and $\log P$ values of unsubstituted sulfanilamide are taken as the point of reference. Taking into account the effect of dissociation at the physiological pH, our analyses show that the hydrophobic character of the N^1 substituent plays extremely important roles in the behavior of these drugs, especially in the serum protein binding[11] and the rate of elimination.[12] The purpose of the work in this paper is to obtain further insight into the role of hydrophobic force in the serum albumin binding and to further support our previous analyses, in particular, of the rate of elimination.

Experimental Section

The protein binding of sulfonamide drugs has been known to obey the Langmuir's adsorption isotherm as shown in eq 1,[5,7,8]

$$KC_F = \frac{r}{n - r} \tag{1}$$

where K is the binding constant, C_F is the equilibrium concentration of the unbound free drug, n is the maximum number of binding sites, and r is the number of sites occupied by the bound drug per single molecule of the protein.

In fact, the drugs exist as an equilibrium mixture of neutral and ionized forms and the degree of ionization varies according to dissociation constants in a series of sulfonamides at a certain pH. If the drugs are bound only as either the neutral or ionized form, eq 1 is modified to either eq 2 or 3 so that experimentally determined

$$K_n C_F(1 - \alpha) = \frac{r}{n - r} \tag{2}$$

$$K_i C_F \alpha = \frac{r}{n - r} \tag{3}$$

$$K_n = \frac{K}{1 - \alpha} \tag{4}$$

$$K_i = \frac{K}{\alpha} \tag{5}$$

apparent constant can be corrected as shown in eq 4 and 5, where K_n and K_i are constants for the neutral and ionized forms and α is the degree of ionization of a drug. We assume that the logarithmic values of binding constant, K_n and K_i, can be described by free energy related parameters as shown in eq 6 and 7, where

$$\log K_n = \log K + \log\left(\frac{K_A + [H^+]}{[H^+]}\right) = a\pi + b\Delta \log K_A + c \tag{6}$$

$$\log K_i = \log K + \log\left(\frac{K_A + [H^+]}{K_A}\right) = a'\pi + b'\Delta \log K_A + c' \tag{7}$$

$[H^+]$ is the hydrogen ion concentration of the medium and a, a', b, b', c, and c' are constants.

If both the neutral and ionized forms of drugs are involved in binding simultaneously with their respective equilibrium constants, K_n' and K_i', the adsorption isotherms for the two forms are described as shown in eq 8 and 9 where r_n and r_i are the number of sites binding neutral and ionized forms. Taking the ratio of eq 8 to eq 9, eq 10

$$K_n' C_F(1 - \alpha) = \frac{r_n}{n - r_i - r_n} \tag{8}$$

$$K_i' C_F \alpha = \frac{r_i}{n - r_i - r_n} \tag{9}$$

$$\frac{K_n'}{K_i'}\frac{(1 - \alpha)}{(\alpha)} = \frac{r_n}{r_i} \tag{10}$$

is derived. If the bound drug molecule is also in a dissociation equilibrium on the protein surface, the ratio of r_n to r_i is $(1 - \alpha)/\alpha$

†Studies on Structure-Activity Relationships. 5.

$$[K_n'(1-\alpha)+K_i'\alpha]C_F = \frac{r_n + r_i}{n-(r_n+r_i)} = \frac{r}{n-r} \quad (11)$$

$$K = K_n'(1-\alpha)+K_i'\alpha = K_n' = K_i' \quad (12)$$

$$\log K = a''\pi + b''\Delta \log K_A + c'' \quad (13)$$

$$\log K_n + \log [H^+] - \log K_A = \log K + \log\left(\frac{K_A + [H^+]}{K_A}\right) =$$
$$a\pi + b\Delta \log K_A + c + \log [H^+] - \log K_A = a\pi + (b-1)\Delta \log K_A + c' \quad (14)$$

$$\log K_n = \log K + \log K_A - \log [H^+] \quad (15)$$

$$\log K = a\pi + b\Delta \log K_A + c - \log K_A + \log [H^+] = a\pi + (b-1)\Delta \log K_A + c'' \quad (16)$$

Table I. Binding Constants of Sulfonamides with BSA

				pH 4.9			pH 5.5			pH 6.2		
	pK_A[a]	$\Delta \log K_A$[b]	π[c]	Log K	Log K_n	Log K_i	Log K	Log K_n	Log K_i	Log K	Log K_n	Log K_i
Sulfisomidine	7.38	3.07	0.39	2.49	2.49	4.97	2.96	2.96	4.84	3.16	3.19	4.37
Sulfathiazole	7.10	3.35	0.82	2.96[d]	2.96[d]	5.16[d]	3.29[d]	3.30[d]	4.90[d]	3.65[d]	3.70[d]	4.60[d]
Sulfamonomethoxine	6.03	4.42	1.37	3.08	3.11	4.24	3.21	3.32	3.85	3.52[d]	3.91[d]	3.74[d]
Sulfaphenazole	6.50*	3.95	1.59	3.77	3.78	5.38	4.03	4.07	5.07	4.18	4.36	4.66
Sulfadimethoxine	6.05	4.40	1.86	4.22	4.25	5.40	4.52	4.63	5.18	4.76	5.14	4.99
Sulfisoxazole	4.62	5.83	2.61	4.20	4.66	4.38	4.53	5.46	4.58	4.50	6.09	4.51

	pH 7.0			pH 7.5			pH 8.0		
	Log K	Log K_n	Log K_i	Log K	Log K_n	Log K_i	Log K	Log K_n	Log K_i
Sulfisomidine	3.20	3.35	3.73	3.54	3.91	3.79	3.77	4.48	3.86
Sulfathiazole	3.78	4.03	4.13	3.86	4.41	4.01	4.04	4.99	4.09
Sulfamonomethoxine	3.46	4.47	3.50	3.47	4.95	3.48	3.47	5.44	3.47
Sulfaphenazole	4.04	4.66	4.16	4.00	5.04	4.04	3.95	5.46	3.96
Sulfadimethoxine	4.47	5.47	4.52	4.35	5.81	4.36	4.27[d]	6.22[d]	4.27[d]
Sulfisoxazole	4.56	6.94	4.56	4.12	7.00	4.12	4.18	7.56	4.18

[a] Taken from Yamazaki, *et al.*,[28] except for the one with the asterisk which is from Koizumi, *et al.*[29] [b] pK_A of sulfanilamide = 10.45.[a] [c] Calculated from the "Übergangszahlen" in ref 7 with correction for ionization in the aqueous phase. [d] Estimated by interpolation.

Table II. Equations Correlating Binding Data Shown in Table I.[a] log K, log K_n, log $K_i = a\pi + b\Delta \log K_A + c$

	a	b	c	s	r	eq	a	b	c	s	r	eq
			pH 4.9						pH 7.0			
Log K	0.845		2.236	0.304	0.925	(17)	0.612		3.037	0.287	0.881	(44)
		0.566	1.094	0.505	0.776	(18)		0.407	2.221	0.413	0.734	(45)
	1.842	−0.835	4.282	0.142	0.988	(19)	1.368	−0.634	4.590	0.225	0.947	(46)
Log K_n	1.025		2.065	0.237	0.967	(20)	1.565		2.567	0.300	0.977	(47)
	(±0.376)		(±0.604)				(±0.476)		(±0.766)			
		0.727	0.508	0.474	0.859	(21)		1.225	−0.287	0.402	0.958	(48)
	1.701	−0.566	3.453	0.165	0.988	(22)	1.114	0.378	1.641	0.314	0.981	(49)
Log K_i	−0.168		5.164	0.541	0.263	(23)	0.371		3.566	0.342	0.688	(50)
		−0.273	6.058	0.474	0.533	(24)		0.225	3.163	0.402	0.522	(51)
	1.701	−1.566	9.003	0.165	0.967	(25)	1.114	−0.622	5.091	0.314	0.817	(52)
			pH 5.5						pH 7.5			
Log K	0.794		2.613	0.345	0.896	(26)	0.292		3.469	0.281	0.673	(53)
		0.525	1.567	0.520	0.742	(27)		0.160	3.223	0.337	0.462	(54)
	1.816	−0.856	4.710	0.225	0.968	(28)	1.098	−0.675	5.124	0.194	0.897	(55)
Log K_n	1.163		2.283	0.325	0.953	(29)	1.373		3.210	0.236	0.981	(56)
	(±0.515)		(±0.828)				(±0.374)		(±0.601)			
		0.854	0.395	0.513	0.877	(30)		1.076	0.699	0.326	0.964	(57)
	1.551	−0.325	3.080	0.353	0.958	(31)	0.956	0.350	2.353	0.236	0.986	(58)
Log K_i	−0.031		4.781	0.537	0.053	(32)	0.179		3.709	0.298	0.466	(59)
		−0.146	5.345	0.513	0.298	(33)		0.076	3.649	0.326	0.248	(60)
	1.551	−1.325	8.030	0.353	0.822	(34)	0.956	−0.650	5.303	0.236	0.794	(61)
			pH 6.2						pH 8.0			
Log K	0.675		2.990	0.360	0.854	(35)	0.186		3.678	0.283	0.500	(62)
		0.428	2.176	0.507	0.679	(36)		0.096	3.548	0.309	0.321	(63)
	1.770	−0.918	5.240	0.219	0.962	(37)	0.781	−0.499	4.900	0.264	0.713	(64)
Log K_n	1.314		2.506	0.281	0.971	(38)	1.339		3.763	0.299	0.969	(65)
	(±0.446)		(±0.717)				(±0.476)		(±0.764)			
		0.993	0.259	0.466	0.919	(39)		1.066	1.246	0.312	0.966	(66)
	1.401	−0.073	2.684	0.323	0.972	(40)	0.725	0.515	2.502	0.284	0.979	(67)
Log K_i	0.121		4.305	0.454	0.227	(41)	0.146		3.762	0.294	0.398	(68)
		−0.007	4.509	0.466	0.017	(42)		0.066	3.696	0.312	0.226	(69)
	1.401	−1.073	6.934	0.323	0.800	(43)	0.725	−0.486	4.952	0.284	0.643	(70)

[a] s is the standard deviation and r is the correlation coefficient. The figures in parentheses are the 95% confidence intervals.

so that K_n' becomes equal to K_i'. By adding eq 8 and 9 together, eq 11 is obtained. Thus, the experimentally obtained binding constant, K, can be expressed by eq 12. In this situation, the effect of ionization on the apparent binding constant may be neglected and its logarithmic value can be directly used for the analysis with physicochemical parameters as shown in eq 13, where a'', b'', and c'' are constants. The fitting of the experimentally obtained binding data into eq 6, 7, and 13 is performed with the method of least squares.

There are certain aspects which must be considered in discussing the data-fitting results. Equation 6 can be modified to eq 14. Thus, eq 6 and 7 are interrelated *a priori* by relations such as $a = a'$, $b - 1 = b'$, $c + \log [H^+] - \log K_A^{standard} = c'$. Similar relations also exist between eq 6 and 13 when the medium pH value is much higher than each of the pK_A values of drugs. In this region, eq 6 tends to eq 15, from which eq 16 is derived. Thus, $a = a''$, $b - 1 = b''$, and $c + \log [H^+] - \log K_A^{standard} = c''$. Therefore, if a good correlation is derived for $\log K_n$ values obtained at a certain pH and both of the π and $\Delta \log K_A$ terms are justified statistically, a correlation of the same quality is expected for $\log K_i$ values and sometimes for $\log K$ values so that it does not necessarily mean that the neutral form is responsible for the binding. However, the parameters on the right side of eq 6, 7, and 13 might not be always of significance statistically. Thus, the quality of correlations and physicochemical significance obtained for single- and two-parameter equations should be examined carefully. The calculations were carried out by a FACOM 230/60 computer of the Data Processing Center of this University.

Results

The binding constants of six sulfonamide drugs with bovine serum albumin (BSA) were determined by Nakagaki, *et al.*, at various pH points between 4.5 and 11.[8] From an apparent relationship between the electrostatic charge of BSA and the binding capacity of six sulfonamides, they concluded that the electrostatic force is the most important factor for binding without taking into account the hydrophobic character of the drugs. Their data obtained at the six points are shown in Table I. Each set of $\log K$, $\log K_n$, and $\log K_i$ values at each pH is analyzed with $\Delta \log K_A$ and π singly and together to yield sets of three equations as shown in Table II.

The equations in Table II are underlined when their correlation is justified at least at better than the 0.90 level of significance. When more than one of each set of the three are justified, the most significant equation is chosen by comparing their F values. If the addition of a second parameter to a justified single-parameter equation is not justified at better than the 0.90 level by examining the $F_{1,3}$ value, the two-parameter equation is not taken as significant, even though it is justified in terms of the $F_{2,3}$ value.

Although the standard deviation is smallest in the correlation of $\log K$ values with two parameters at each pH, eq 46 at pH 7 and eq 64 at pH 8 are not significant. The two-parameter equations for $\log K_i$ values are not justified except for eq 25 at pH 4.9. The $\log K_n$ values, however, are correlated legitimately only with the π term throughout the pH range studied. At four out of six pH points, the best correlation coefficient and standard deviation for single-parameter "π equations" are observed in that of $\log K_n$ values. Even though the two-parameter equations for $\log K_n$ and $\log K_i$ values at each pH point and those for $\log K_n$ and $\log K$ values at pH 7.5 and 8.0 are interrelated with each other by relations observed in eq 14 and 16, it is unnecessary for $\log K_n$ values to be accounted for with both of the parameters. The coefficients of the π term of underlined equations are very similar to each other, while the value of the constant term increases gradually with increasing pH indicating that the binding force increases with increasing pH. If we assume that the constant term is expressed by a linear function of pH, the $\log K_n$ values of six sulfonamides obtained

$$\log K_n = a\pi + b\Delta \log K_A + d\text{pH} + c \quad (71)$$

at 6 pH points could be expressed by eq 71, where a, b, d, and c are constants. Fitting the 36 data points to eq 71, eq 72 is obtained. The addition of a $\Delta \log K_A$ term does not improve the correlation. Similar correlations for $\log K$ and $\log K_i$ values, eq 73 and 74, show that the $\Delta \log K_A$ term is required to account for the binding data. Although eq 72, 73, and 74 are almost of equivalent quality in terms of the standard deviation, the F ratio is largest for eq 72.

The above results strongly suggest that the neutral form is responsible for the binding process and that the binding force is due to the hydrophobicity of drugs and the pH-dependent binding capacity of the protein.

Discussion

On the surface of the BSA molecule, there are various types of pH-sensitive groups such as carboxyl, imidazolyl, amino, and phenolic hydroxyl. They are in dissociation equilibria to various extents according to their dissociation constants, hydrogen ion concentration of the medium, and the net electrostatic charge of the protein molecule. According to the work of Tanford and his coworkers on the hydrogen ion dissociation equilibria of BSA, all of the dissociable groups of any one type have the same intrinsic dissociation constant, K_A^{int}.[14] The pK_A^{int} values are 4.02 for β- and γ-carboxyl, 6.90 for imidazolyl, 9.80 for ϵ-amino, and 10.35 for phenolic hydroxyl. No remarkable conformational change of the protein occurs between pH 4.3 and 10.5.

In the pH range studied, 4.9–8, the number of maximum binding sites, n, was found to be nearly constant being around 2 regardless of variation in the N^1 substituent,[8,15] suggesting that major conformation changes do not occur even for the sulfonamide-binding BSA. In this pH range, almost all of the carboxyl groups are ionized and the phenolic hydroxyl groups are essentially un-ionized, while the fractions of neutral imidazole and ϵ-amino groups increase gradually with increasing pH. The pH dependence of the binding force in this pH region may be attributable to the state of dissociation equilibria of imidazole and/or ϵ-amino groups. We assume that it is due to the state of dissociation of one of these two types of basic groups. Then, the total protein surface would be divided into two fractions which are covered by cationic and neutral forms of the basic groups. These surface fractions are proportional to $(1 - \alpha')$ and α', respectively, where α' is the degree of dissociation of the cationic base.

When the neutral drug molecules are able to bind only with the "neutral zone" of the protein surface, the adsorp-

	n	s	r		
$\log K_n = -1.581 + 1.297\pi + 0.662\text{pH}$ (±0.606) (±0.133) (±0.087)	36	0.280	0.975	$F_{2,33} = 316.49$	(72)
$\log K_i = 8.755 - 0.954\Delta \log K_A + 1.241\pi - 0.338\text{pH}$	36	0.284	0.863	$F_{3,32} = 31.05$	(73)
$\log K = 3.972 - 0.736\Delta \log K_A + 1.446\pi + 0.128\text{pH}$	36	0.275	0.877	$F_{3,32} = 35.41$	(74)

$$K_n^n C_F(1-\alpha)\alpha' = \frac{r}{n-r} \quad (75)$$

tion isotherm can be written as shown in eq 75, where K_n^n is an intrinsic 1-to-1 binding constant between the neutral drug molecule and "neutral site" of the protein surface. Thus, the experimentally obtained binding constant, K, is expressed as eq 76 or its logarithmic form, eq 77.

$$K = K_n^n(1-\alpha)\alpha' \quad (76)$$

$$\log K + \log\left(\frac{K_A + [H^+]}{[H^+]}\right) = \log K_n^n + \log\alpha' + \text{constant} \quad (77)$$

According to Tanford and his coworkers,[14] the degree of dissociation or the dissociated fraction, α', of a certain type of titrable groups is related to the value of K_A^{int} by eq 78

$$\log [H^+] + \log \frac{\alpha'}{1-\alpha'} = \log K_A^{int} + 0.022Z \quad (78)$$

between pH 4.3 and 10.5, where the term, $+0.022Z$, takes into account the electrostatic interaction between the dissociating hydrogen ion and the net charge Z of the protein at any pH. From their hydrogen ion titration curve of the protein which shows that the value of Z decreases almost linearly with increasing pH in this pH region, eq 79 can be

$$Z = 6.5 \log [H^+] + 35 \quad (79)$$

derived. Substitution of eq 79 into eq 78 and collecting of terms yields eq 80, from which eq 81 and 82 can be given

$$\log\left(\frac{\alpha'}{1-\alpha'}\right) = \log K_A^{int} - 0.86 \log [H^+] + 0.77 = \log\left(\frac{5.9K_A^{int}}{[H^+]^{0.86}}\right) \quad (80)$$

$$\alpha' = \frac{5.9K_A^{int}}{5.9K_A^{int} + [H^+]^{0.86}} \quad (81)$$

$$1-\alpha' = \frac{[H^+]^{0.86}}{5.9K_A^{int} + [H^+]^{0.86}} \quad (82)$$

$$\log K + \log\left(\frac{K_A + [H^+]}{[H^+]}\right) = \log K_n^n - \log(5.9K_A^{int} + [H^+]^{0.86}) + \text{constant} \quad (83)$$

for the fractions dissociated and undissociated, respectively. After substitution of eq 81 into eq 77, collecting of terms yields eq 83.

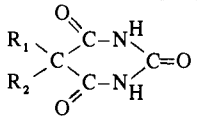
Comparison of eq 83 with eq 72 would indicate that the intrinsic binding energy, $\log K_n^n$, is determined eventually by the hydrophobicity of the drug molecule. For the value of K_A^{int} in eq 83, we could adopt that of the ϵ-amino groups. Their K_A^{int} value is $10^{-9.8}$ which is far smaller than the value of $[H^+]^{0.86}$ in the present pH range so that the pH-dependent term in eq 83 turns into 0.86pH which is fairly close to that in eq 72. If we take the imidazolyl groups, the K_A^{int} value is $10^{-6.90}$ so that the term, $-\log(5.9K_A^{int} + [H^+]^{0.86})$, varies from 4.20 at pH 4.9 to 6.06 at pH 8.0. The increment per unit pH is, on an average, 0.68, which is very close to the slope in eq 72. Although the state of dissociation of imidazolium ions seems, in effect, more likely to participate in the pH-dependent equilibrium, it would be unfair to draw a definite conclusion from the above considerations alone.

At any rate, the basic groups, which exist as the conjugate cation, can be hydrated with water molecules, while the degree of hydration is much lower for the neutral form. The increase in the degree of dissociation of the immonium (or ammonium) ions with increasing pH results in the decrease in the surface fraction which is strongly hydrated. Thus, the pH dependence of the binding constant could be due to the pH-dependent variation of the strongly hydrated fraction of the protein surface. The less hydrated the protein surface, the more chance the neutral drug molecule has to impact the hydrophobic surface.

We have examined similarly each of the other possibilities where the adsorption equilibrium may be due to the binding between the neutral drug and the "ionized" surface fraction and that between the ionized drug and each of the surface fractions or the simultaneous binding of neutral and ionized drug molecule with each or both of the two fractions. However, with these possibilities, the pH dependence appearing in eq 72-74 is not explained satisfactorily. Thus, it would be reasonable to conclude that the adsorption equilibrium of sulfonamide drugs with BSA is determined by the hydrophobicity of drugs and occurs through the binding of the neutral drug molecule with the hydrophobic fraction of the protein surface, the hydration shell over which is uncovered by the dissociation of cationic bases.

Similar analyses are also performed for the binding constants of barbiturate drugs of various structures at the 2 pH points shown in Table III. Equations 84 and 85 are derived

Table III. Binding Constants of Barbiturates



					pH 5.8		pH 7.4		
					Log K			Log K_n	
R_1	R_2	pK_A[a]	$\Delta \log K_A$	Log P[b]	Obsd	Calcd[d]	Log K	Obsd	Calcd[e]
Et	Et	7.91	0	0.65	−2.00	−1.97	−1.28	−1.16	−1.03
Et	*i*-Pr	8.01	−0.10	0.97	−1.79	−1.80	−0.83	−0.73	−0.86
Et	Ph	7.41	0.50	1.42	−1.19	−1.31	−0.60	−0.30	−0.28
Et	1-Me-Bu	8.11	−0.20	2.03	−1.14	−1.16	−0.23	−0.15	−0.08
Et	*i*-Am	7.94	−0.03	2.07	−1.10	−1.07	−0.27	−0.15	−0.19
All	1-Me-Bu	8.08	−0.17	2.30[c]	−0.97	−0.97	−0.10	−0.02	0.01
Et	Cyclohexenyl	7.50	0.41	1.45[c]	−1.47	−1.32	−0.48	−0.23	−0.30
All	Et	7.68	0.23	0.95	−1.71	−1.70			
All	All	7.79	0.12	1.19	−1.51	−1.58			

[a] Taken from Kakemi, *et al.*[30] [b] Taken from Kakemi, *et al.*,[31a] and Hansch, *et al.*[31b] [c] Estimated values. [d] Calculated by eq 85. [e] Calculated by eq 87.

	n	s	r	
$\log K = \log K_n = -2.282 + 0.587 \log P$	9	0.116	0.952	(84)
$\log K = \log K_n = -2.383 + 0.338\Delta \log K_A + 0.638 \log P$ (±0.221) (±0.316) (±0.138)	9	0.086	0.977	(85)
$\log K_n = -1.352 + 0.617 \log P$	7	0.169	0.926	(86)
$\log K_n = -1.474 + 0.471\Delta \log K_A + 0.678 \log P$ (±0.349) (±0.207) (±0.450)	7	0.107	0.977	(87)

from the binding data of 9 barbiturates determined at pH 5.8 by Kakemi and his coworkers.[16] At this pH, practically all of the barbiturate molecules exist as the neutral form so that the experimentally determined binding constant, K, is regarded as being equal to K_n. The fitting of the binding constants of 7 barbiturates at pH 7.4, determined by Goldbaum and his associates,[17] yields eq 86 and 87.

Some of the compounds in the original papers are not included in the analyses because of the lack of physicochemical parameters. In Table III, K is not a mass action equilibrium constant but a somewhat less accurate binding ratio with the use of 1% BSA solution and $\log P$ is the hydrophobic parameter of the whole molecule where P is the 1-octanol-H_2O partition coefficient. The $\Delta \log K_A$ terms in eq 85 and 87 are justified at better than the 0.95 level of significance (eq 85, $F_{1,6} = 6.87$; eq 87, $F_{1,4} = 8.50$, $F_{1,6,0.05} = 5.98$, $F_{1,4,0.05} = 7.71$). Thus, the binding equilibrium of this series of compounds seems to be governed by both the hydrophobicity and the electronic character of the molecule. The increment of the constant term from eq 85 to eq 87 is 0.91 which corresponds to 0.57 per unit pH. This value agrees very well with that obtained for the sulfonamide drugs. Although the binding data come from two different laboratories, the pH-dependent binding equilibrium of this series of drugs with BSA also appears to occur between neutral drug and the hydrophobic fraction of the protein surface.

In our previous analyses for serum protein binding of sulfonamides,[11] we refrained from concluding which form is responsible for the binding, the neutral or the ionized, from binding data determined at a single pH, since correlations of equal quality for these two forms could be obtained as described earlier in this paper for eq 14. From the present discussion, the use of eq 6 for the binding data determined even at a single pH point can be justified.

The binding data of sulfonamides and their N^4-acetyl derivatives with serum proteins and Carbon Black in Tables IV and V from various literature sources are also well correlated, resulting in eq 88-93. The slopes of the π term of eq 88, 89, 91, and 92 for BSA and human serum albumin (HSA) binding are very similar to each other being around 1.8 ± 0.2. The binding with Carbon Black is less susceptible to the hydrophobicity of drugs than proteins as shown in eq 90 and 93. The difference in the constant term between equations for sulfonamides and corresponding N^4-acetyl

Sulfonamides

	n	s	r	
BSA, pH 7.4 at 37°[18] $\log K_n = 2.502 + 2.047\pi$ (±0.674) (±0.495)	10	0.476	0.959	(88)
HSA, pH 7.4 at 10°[15] $\log K_n = 2.414 + 1.675\pi$ (±0.444) (±0.327)	12	0.332	0.964	(89)
Carbon Black, pH 7.4 at 40°[19] $\log K_n = 3.662 + 1.014\pi$ (±0.362) (±0.291)	12	0.243	0.926	(90)

N^4-Acetylsulfonamides

BSA, pH 7.4 at 37°[20] $\log K_n = 3.389 + 1.908\pi$ (±0.698) (±0.512)	10	0.492	0.950	(91)
HSA, pH 7.4 at 22-24°[7] $\log K_n = 3.140 + 2.029\pi$ (±1.126) (±0.651)	5	0.264	0.985	(92)
Carbon Black, pH 7.4 at 40°[21] $\log K_n = 4.630 + 1.075\pi$ (±0.505) (±0.374)	5	0.131	0.983	(93)

Table IV. Binding Constants of Sulfonamides with Serum Proteins and Carbon Black

				BSA binding			HSA binding			Carbon Black binding		
					Log K_n			Log K_n			Log K_n	
	pK_A[a]	$\Delta \log K_A$[a]	π[a]	Log K	Obsd	Calcd[b]	Log K[c]	Obsd	Calcd[d]	Log K	Obsd	Calcd[e]
Sulfanilamide	10.45	0.00	0.00	2.7	2.7	2.50	1.88	1.88	2.41	3.27	3.27	3.66
N^1-Acetylsulfanilamide	5.40	5.05	1.01				2.30	4.30	4.11	2.83	4.83	4.69
Sulfathiazole	7.10	3.35	0.82	3.1	3.6	4.18	3.22	3.70	3.79	3.95	4.43	4.49
Sulfadiazine	6.15	4.30	1.11	2.9	4.1	4.77	2.65	3.92	4.27	3.56	4.83	4.79
Sulfamerazine	6.93	3.52	0.94	3.7	4.3	4.43	3.30	3.90	3.99	4.23	4.83	4.62
Sulfisomidine	7.38	3.07	0.39	3.1	3.4	3.30	3.34	3.65	3.07	4.09	4.40	4.06
Sulfaphenazole	6.50	3.95	1.59				4.24	5.19	5.08	4.20	5.15	5.27
Sulfamethoxypyridazine	7.05	3.40	1.02	4.8	5.3	4.59	3.69	4.20	4.12	4.23	4.74	4.70
Sulfadimethoxine	6.05	4.40	1.86	5.4	6.7	6.31	4.49	5.86	5.53	4.47	5.84	5.55
Sulfisomezole	5.81	4.64	1.55	3.7	5.3	5.67	3.28	4.88	5.01	3.58	5.18	5.23
Sulfamonomethoxine	6.03	4.42	1.37	3.3	5.7	5.31	3.54	4.93	4.71	3.59	4.98	5.05
Sulfaethidole	6.02	4.43	1.84							3.76	5.16	5.53
Sulfisoxazole	4.62	5.83	2.61	5.0	7.8	7.84	3.68	6.46	6.79			

[a] See footnotes of Table I. [b] Calculated by eq 88. [c] The value of K is in l./mole, recalculated from the original literature[15] where the binding data are expressed by the binding ratio with the use of 5% HSA solution. [d] Calculated by eq 89. [e] Calculated by eq 90.

Table V. Binding Constants of N^4-Acetylsulfonamides with Serum Proteins and Carbon Black

				BSA binding			HSA binding			Carbon Black binding		
					Log K_n			Log K_n			Log K_n	
N^4-Acetyl-	pK_A[a]	$\Delta \log K_A$[b]	π[b]	Log K	Obsd	Calcd[c]	Log K[d]	Obsd	Calcd[e]	Log K	Obsd	Calcd[f]
Sulfanilamide	9.85	0.00	0.00	3.5	3.5	3.39						
Sulfathiazole	6.50	3.35	0.82	3.5	4.4	4.95						
Sulfadiazine	5.55	4.30	1.11	3.3	5.1	5.50	3.41	5.25	5.39	3.79	5.63	5.82
Sulfamerazine	6.33	3.52	0.94	4.3	5.4	5.18						
Sulfisomidine	6.78	3.07	0.39	4.0	4.7	4.13				4.41	5.11	5.05
Sulfamethoxypyridazine	6.45	3.40	1.02	4.7	5.7	5.33	4.38	5.36	5.21			
Sulfadimethoxine	5.45	4.40	1.86	5.5	7.4	6.94	5.30	7.23	6.91	4.71	6.64	6.63
Sulfisomezole	5.21	4.64	1.55	3.8	6.0	6.35	3.87	6.04	6.29	4.15	6.32	6.30
Sulfamonomethoxine	5.43	4.42	1.37	3.3	5.3	6.00				4.25	6.20	6.10
Sulfisoxazole	4.02	5.83	2.61	5.3	8.7	8.37	5.00	8.36	8.44			

[a]The pK_A values of N^4-acetylsulfonamides are estimated as 0.6 unit lower than those of the corresponding sulfonamides. The Hammett ρ values of pK_A of substituted benzenesulfonamides and benzenesulfonanilides have been shown by Willi,[32] as nearly equal to each other being -1.10 ± 0.10. This would suggest that, regardless of variation in the N^1 substituents, the pK_A difference between sulfonamide drug and its N^4-acetyl derivative can be calculated as $\rho(\sigma_{p\text{-}NH_2} - \sigma_{p\text{-}AcNH}) = 0.6 \pm 0.1$. [b]The same values as those of the corresponding sulfonamides are used. [c]Calculated by eq 91. [d]The values of K expressed in l./mole, recalculated from the original paper[7] where they are in l./μmole. [e]Calculated by eq 92. [f]Calculated by eq 93.

Table VI. K_m and V_{max} Values of Sulfonamides for Pigeon Liver Acetyltransferase [a,b]

		Log $(1/K_m) + \log\left(\frac{K_A + [H^+]}{[H^+]}\right)$			Log $V_{max} - \log\left(\frac{K_A + [H^+]}{[H^+]}\right)^f$	
	Log $(1/K_m)$[c]	Obsd	Calcd[d]	Log V_{max}[e]	Obsd	Calcd[g]
Sulfanilamide	3.93	3.93	3.81	−5.38	−5.38	−5.38
Sulfathiazole	3.77	4.25	4.39	−6.15	−7.08	−7.11
Sulfadiazine	3.76	5.03	4.60	−6.00	−7.84	−7.65
Sulfamerazine	3.78	4.38	4.48	−6.22	−7.31	−7.26
Sulfisoxazole	3.05	5.83	5.66	−5.74	−9.10	−9.29
Sulfisomidine	3.80	4.11	4.09	−5.80	−6.50	−6.70
Sulfadimethoxine	3.64	5.01	5.13	−6.52	−8.45	−8.25
Sulfisomezole	3.40	5.00	4.91	−6.10	−8.27	−8.10
Sulfamonomethoxine	3.25	4.64	4.78	−5.82	−7.77	−7.89
Sulfaethidole	3.58	4.97	5.11	−6.22	−8.18	−8.25
Sulfamethoxypyridazine	3.83	4.34	4.53	−5.33	−6.31	−7.18[h]

[a]For π and $\Delta \log K_A$ values, see Table IV. [b]Of compounds studied in the original paper, ref 24, N^1-acetylsulfanilamide is not included, since this compound may be anticipated to behave as an acetyl donor as well as an acetyl acceptor. [c]The K_m values are expressed in moles/l., converted from the original paper, where they are in mmoles/l. [d]Calculated from eq 96. [e]The V_{max} values are expressed in moles/(l. min), converted from the originally reported values in mmoles/(l. min). [f]The K_A values are those of the N^4-acetyl derivatives, see Table V. [g]Calculated from eq 102. [h]Calculated from eq 101.

derivatives is nearly constant, indicating that the acetyl group contributes similarly to the binding with various polymers. Some compounds in the original literature, the physicochemical parameters of which are not available, and a few, the binding data of which are only poorly correlated on preliminary calculations, are not included in the analyses. Our overall results seem to require careful reexaminations of the binding constants for the poorly predicted compounds.

Recently, Hansch and his coworkers analyzed the binding equilibrium of a set of miscellaneous compounds including phenols, anilines, hydrocarbons, and alcohols with BSA under conditions where phenols and anilines exist as neutral molecules and found that the binding data are well correlated by eq 94, where C is the molar concentration of

$$\log\left(\frac{1}{C}\right) = 2.301 + 0.751 \log P \quad \begin{matrix} n & s & r \\ 42 & 0.159 & 0.960 \end{matrix} \quad (94)$$

compounds required for a "1-to-1" binding with the protein and P is the 1-octanol–H_2O partition coefficient.[22] The coefficients of the π term of eq 85 and 87 for the barbiturate drugs agree well with the slope of eq 94. They also found that eq 95 holds between log P (i-BuOH–H_2O) and log P (1-octanol–H_2O).[23] The slope in eq 72 which is derived

$$\log P_{i\text{-BuOH}} = 0.373 + 0.719 \log P_{1\text{-octanol}} \quad \begin{matrix} n & s & r \\ 67 & 0.136 & 0.992 \end{matrix} \quad (95)$$

with π(i-BuOH–H_2O) is thus converted to $1.3 \times 0.72 \cong 0.94$ with the scale of π(1-octanol–H_2O), which is fairly close to the value in eq 94. Similarly, the slopes in eq 88 and 91 are equivalent to $1.9 \times 0.72 \cong 1.37$ with the 1-octanol–H_2O scale. The reason why these equations show somewhat higher dependence on the hydrophobicity of sulfonamide drugs is not certain.

The same procedure can be also applied in analyzing the enzymatic N^4-acetylation of sulfonamides which is considered to be one of their main metabolic pathways in liver. The Michaelis constant, K_m, and maximum acetylation velocity, V_{max}, of a series of sulfonamides were determined by Kakemi and his coworkers with a preparation of the pigeon liver acetyl transferase at pH 7.4.[24] The data are shown in Table VI. Without considering the variation in the degree of ionization and hydrophobicity among compounds in the set, they postulated that the K_m value is related to log K_A and the V_{max} value is determined by the reactivity of the N^4-amino group expressed in terms of the superdelocalizability.

The value of $1/K_m$ corrected to the fraction of neutral

form corresponds to K_n, which is the binding equilibrium constant between neutral substrate and enzyme, and can be analyzed by eq 6 to yield eq 96. The slope is equivalent

$$\log 1/K_m + \log\left(\frac{K_A + [H^+]}{[H^+]}\right) = \underset{3.813 + 0.707\pi}{\underset{(\pm 0.277)\ (\pm 0.196)}{}} \quad \begin{matrix} n & s & r \\ 11 & 0.200 & 0.938 \end{matrix} \quad (96)$$

to $0.7 \times 0.72 \cong 0.5$ in the 1-octanol-H_2O scale. The addition of a $\Delta \log K_A$ term to eq 96 does not improve the correlation. This result can be compared with a recent work of Martin and Hansch who analyzed log $1/K_m$ values of a set of miscellaneous drugs with log P for the mixed function oxidases of rat liver microsomes.[25] For the ionizable drugs, the correction for ionization was found to considerably improve the correlation.

The V_{max} value would reflect processes such as the desorption from the enzyme-product complex, EP, and the diffusion into the bulk phase of the N^4-acetylation product as shown in eq 97. The desorption step is considered to be

$$\begin{array}{ccc} EP \underset{}{\overset{K}{\rightleftharpoons}} C_F(1-\alpha) & \xrightarrow{k} & C(1-\alpha) \\ \Big\uparrow\Big\downarrow K_A & & \Big\uparrow\Big\downarrow K_A \\ C_F\alpha + [H^+] & \xrightarrow{k} & C\alpha + [H^+] \end{array} \quad (97)$$

enzymic environment bulk phase

C = concentration of product in bulk phase
C_F = concentration of product in equilibrium with enzyme

reversible with an equilibrium constant, K. A similar enzyme preparation was found to catalyze the acetyl migration from acetanilide derivatives to acetyl acceptors reversibly.[26] Since the desorption equilibrium is just the reverse of the adsorption, it is reasonable to consider that the molecular form which is responsible for this step is the neutral N^4-acetylsulfonamides. The degree of dissociation, α, in the bulk phase would not be varied from that before diffusion occurs so that the rate constant of the diffusion process, k, is common for both the neutral and ionized forms. Thus, the maximum rate of acetylation can be expressed by eq 98, where the maximum concentration of EP can be taken as that of the total enzyme which is held constant and k is not greatly changed in the set of sulfonamides of rather narrow range of molecular weight. The logarithmic form of eq 98 is, then, turned into eq 99 and

$$V_{max} = \left(\frac{dC}{dt}\right)_{max} = \left(\frac{dC(1-\alpha)}{dt}\right)_{max} + \left(\frac{dC\alpha}{dt}\right)_{max}$$

$$= C_F(1-\alpha)k + C_F\alpha k = [EP]_{max}Kk + [EP]_{max}\left(\frac{\alpha}{1-\alpha}\right)Kk$$

$$= [E]_{total}Kk/(1-\alpha) \quad (98)$$

$$\log V_{max} - \log\left(\frac{K_A + [H^+]}{[H^+]}\right) = \log K + \text{constant} \quad (99)$$

$$= a\pi + b\Delta \log K_A + c \quad (100)$$

further to eq 100 modified with free energy related parameters where a, b, and c are constants. The fitting of the data of V_{max} into eq 100 yields eq 101. If the value for the sulfamethoxypyridazine is not included for the analysis, the correlation is much improved as shown in eq 102. The $\Delta \log K_A$ term in eq 101 is significant at better than the 0.90 level of confidence ($F_{1,8} = 3.88$, $F_{1,8,0.10} = 3.29$), suggesting that

$$\log V_{max} - \log\left(\frac{K_A + [H^+]}{[H^+]}\right)$$

$$\begin{matrix} & n & s & r & \\ = \underset{(\pm 0.795)}{-5.249} - \underset{(\pm 0.781)}{0.799\pi} - \underset{(\pm 0.387)}{0.330\Delta \log K_A} & 11 & 0.363 & 0.954 & (101) \\ = \underset{(\pm 0.387)}{-5.381} - \underset{(\pm 0.376)}{0.757\pi} - \underset{(\pm 0.186)}{0.333\Delta \log K_A} & 10 & 0.171 & 0.990 & (102) \end{matrix}$$

the value of V_{max} would describe, besides desorption and diffusion steps, a process where an electron migration occurs to lead the EP complex. The negative sign indicates that the greater the electron-donating character of the N^1 substituents, the easier the N^4 lone-pair electrons are attacked by an acetyl donor. The slope of the π term in eq 101 and 102 is exactly opposite in sign to that in eq 96, indicating that the hydrophobic bonding slows down the desorption of the products and the desorption is, in effect, the reverse of the adsorption equilibrium. The susceptibility of adsorption to the hydrophobicity of the acetylation products is very similar to that of parent sulfonamides, being quite compatible with the results obtained for the serum protein binding.

When the effect of ionization is not taken into account and the values of log $1/K_m$ and log V_{max} are directly analyzed, eq 103–108 are obtained. Although these equations show smaller standard deviations than eq 96 and 101 so that the correction for the ionization seems to be insignificant statistically, it is very difficult to uncover their physicochemical meaning.

$$\begin{matrix} & n & s & r & \\ \log 1/K_m & & & & \\ = 3.991 - 0.305\pi & 11 & 0.169 & 0.810 & (103) \\ = 4.143 - 0.140\Delta \log K_A & 11 & 0.192 & 0.749 & (104) \\ = 4.035 - 0.256\pi - 0.028\Delta \log K_A & 11 & 0.179 & 0.813 & (105) \\ \log V_{max} & & & & \\ = -5.691 - 0.198\pi & 11 & 0.352 & 0.397 & (106) \\ = -5.498 - 0.116\Delta \log K_A & 11 & 0.339 & 0.468 & (107) \\ = -5.481 - 0.134\Delta \log K_A - 0.041\pi & 11 & 0.359 & 0.470 & (108) \end{matrix}$$

With eq 96 and 102, the observable pseudo-first-order rate constant of acetylation, k_{Ac} (in min^{-1}), which can be taken as V_{max}/K_m, can be expressed as shown in eq 109. The contributions from the π terms are compensated for in this equation. The log K_A of any N^4-acetyl derivative, log K_A^{Ac}, is considered to be not much different from that of the parent sulfonamide being only about 0.6 log unit higher, so that the first term of right side of eq 109 could

$$\log k_{Ac} = \log V_{max} + \log 1/K_m$$

$$\cong \log\left[(K_A^{Ac} + [H^+])/(K_A + [H^+])\right] - 0.333\Delta \log K_A - 1.568 \quad (109)$$

$$\cong -0.333\Delta \log K_A - 1.568 \quad (110)$$

be ignored to result in eq 110. Thus, the overall rate of enzymic acetylation is governed mostly by the electronic effect of N^1 substituent and the correction for ionization is not required. Our earlier analyses showed that eq 111 holds for the first-order rate constant of *in vivo* acetylation, k_{Ac} (in hr^{-1}), determined by means of the method developed

$$\log k_{Ac} + \log\left(\frac{K_A + [H^+]}{[H^+]}\right)$$

	n	s	r	
$= -1.255 + 0.876\pi$	6	0.222	0.971	(111)
$(\pm 0.432)\ (\pm 0.299)$				

by Nelson[27] with the use of rats.[12] Here, with the correction term, the log k_{Ac} values are well correlated only with the hydrophobicity of the N^1 substituent. Although the test objects are different from each other, the slope in eq 96 for the value of $1/K_m$ with the pigeon liver enzyme is very similar to that in eq 111 for the k_{Ac} with rats. This would suggest that a step of hydrophobic bonding equilibrium of the neutral form of drugs with liver components is critical for *in vivo* acetylation mechanism.

The present work underlines the importance of hydrophobic bonding instead of the electrostatic interaction for the binding of sulfonamide drugs with serum protein as well as enzyme preparations. A critical assumption that the drug is bound in the neutral form seems to be justified by correlations which are statistically as well as physicochemically significant. Unless the effect of ionization is separated from other physicochemical effects, a true correlation of physicochemical significance is not obtained. The results also show that the extrathermodynamic approach, considering the free energy related hydrophobic parameter of drugs, is capable of improving earlier points of view of structure-activity relationship in certain series of drugs.

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Cycloprop[16α,17α]androstanes

Kenneth E. Fahrenholtz,* Kenneth P. Meyers, and R. W. Kierstead

Chemical Research and Pharmacology Departments, Hoffmann-La Roche Inc., Nutley, New Jersey. *Received January 10, 1972*

A number of 17β-acyloxycycloprop[16α,17α]androstanes were prepared, some of which had substantial levels of oral androgenic activity. The corresponding 17β-alkoxy-3-keto derivatives were also prepared and converted to androstano[2,3-*d*]isoxazoles **23a-d** by standard procedures. These latter compounds combine significant levels of oral anabolic activity with diminished androgenicity.

The preparation of orally active anabolic agents has been pursued by chemists for many years.[1,2] This goal is complicated by the observation that, with a few exceptions, useful levels of oral anabolic activity are found only in steroids containing a 17α-alkyl substituent. Unfortunately this group also causes the steroid to exert undesirable side effects, including reversible hepatotoxicity.[3]

In the search for types of compounds which might circumvent the problems of liver toxicity, it occurred to us that the steric and electronic effects of a 17α-methyl group would not be altered greatly by its incorporation into a cycloprop[16α,17α] steroid. At the beginning of this work, numerous examples of this type of substitution in pregnanes and corticoids had been reported, but no reports of cycloprop[16α,17α]androstanes had appeared. After the conclusion of our synthetic work, Johns and Salamon[4] described the preparation of some of the intermediates used in our work. Therefore, we are prompted to report our results of chemical and biological interest.

When 3′*H*-cycloprop[16α,17α]-5α-androstan-17β-ol-3-one acetate (1)[4] was found to have a high level of oral androgenic activity, several ring A analogs were prepared in an effort to increase the anabolic activity. Dehydrogenation of 1 with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ)