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Esterase-like Activity of Human Serum Albumin. IV.[1] Reactions with Substituted Aspirins and 5-Nitrosalicyl Esters

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To elucidate the reactivity of the lysine-199 residue of human serum albumin (HSA) with ester-type drugs and to characterize the region near the lysine, the reactions of substituted aspirins and 5-nitrosalicyl esters with HSA were investigated kinetically at 25 °C. The Michaelis constant (K_S in M) and the catalytic rate constant (k_2 in s^{-1}) were determined, and the relationship between these constants and the structure of the substrates was explored. For the reactions with substituted aspirins at pH 9.9, the logarithm of k_2 is correlated with the pK_a value of the hydroxyl group in the parent salicylic acid. The regression line is as follows: $\log k_2 = -0.843pK_a + 6.75$ ($r = -0.988$). The K_S value is influenced by the number of substituents on the phenyl ring rather than the nature of the substituents. For the reactions with 5-nitrosalicyl esters at pH 7.4, the k_2/K_S values were correlated with Hansch's hydrophobic substituent constant π and Taft's steric constant E_S as follows: $\log(k_2/K_S) = 0.423\pi + 0.386E_S + 1.35$ ($r = 0.908$).

Keywords——human serum albumin; esterase-like activity; quantitative structure–activity relationship; kinetics; aspirin; 5-nitrosalicyl ester; amino acid sequence

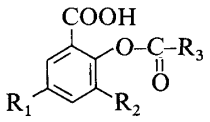
In the previous papers[1-3] it was reported that human serum albumin (HSA) has esterase-like activity toward phenyl acetates, *p*-nitrophenyl esters, and cinnamoylimidazoles. The quantitative structure–activity relationships for the reactions with the ester substrates were examined in order to characterize the reactive site of HSA.[2] The reactive site toward these substrates was found to be located near the tyrosine-411 (Tyr-411) residue of the HSA amino acid sequence,[4] and was named the R site.[5] However, it was found that the reactive site towards some substituted aspirins (which are phenyl acetates with a carboxyl group at the *ortho* position) is located near the lysine-199 (Lys-199) residue, away from the R site.[6] The site near the Lys-199 residue, referred to the U site, is one of the important drug-binding sites.[5-7] It is of importance not only to characterize the U site but also to elucidate the reactivity with substituted aspirins, since HSA may affect the cleavage of these ester-type drugs *in vivo*.

In this study the reactions of substituted aspirins and 5-nitrosalicyl esters with the U site of HSA were investigated kinetically in order to characterize the U site and to elucidate the reactivity of HSA with ester-type drugs. The Michaelis constants and the catalytic rate constants for the reactions were determined, and the relationships between these constants and the structures of the substrates were explored.

Experimental

Materials——HSA (Fraction V, lots 47C-04421, 37F-02271, and 100F-02061, Sigma Chem. Co.) was used after purification by Chen's procedure.[8] The molecular weight of HSA was assumed to be 69000 and the concentration was determined based on an extinction coefficient $E^{0.1\%}_{1\,cm}$ of 0.531 at 278 nm.[9] According to the method of Zaugg *et al.*,[10] substituted aspirins and 5-nitrosalicyl esters were synthesized from the corresponding salicylic acid and acid

TABLE I. Substrates Used and Their Melting Points



Substrates	R_1	R_2	R_3	mp (°C)	Literature values, mp (°C)
1	H	H	CH_3	132—134	135[11]
2	NO_2	H	CH_3	163—165	164—165[12] 153.5—154.5[13]
3	NO_2	NO_2	CH_3	93—94	92.5—94.0[14]
4	Br	Br	CH_3	153—154	155—157[10]
5	Cl	Cl	CH_3	138—141	
6	NO_2	H	CH_2CH_3	104—105	
7	NO_2	H	$CH_2CH_2CH_3$	95—97	
8	NO_2	H	$CH_2(CH_2)_2CH_3$	74—76	
9	NO_2	H	$CH_2(CH_2)_3CH_3$	86—88	
10	NO_2	H	$CH(CH_3)_2$	119—124	
11	NO_2	H	$CH_2CH(CH_3)_2$	91—93	

anhydride in the presence of a small amount of sulfuric acid. The esters were recrystallized from ether or benzene. The esters used and their melting points are listed in Table I. The results of elemental analyses of the synthesized esters are not listed, because the analytical values obtained for carbon, hydrogen, and nitrogen were all within the range of the calculated value $\pm 0.3\%$. The salicylic acids, acid anhydrides, aspirin (**1**), and all other chemicals used were obtained commercially. The numbers in Table I are those used in this paper to refer to the substrates.

Kinetic Runs——The reaction rate of the substrate with HSA was followed spectrophotometrically at an appropriate wavelength for monitoring the appearance of the corresponding salicylic acid. A Hitachi spectrophotometer (UV-124) and a Union Giken stopped-flow spectrophotometer (RA-401) were used for the measurements of the rates. The reaction was carried out in the presence of excess HSA over the substrate.[1-3] The concentrations of the substrates ranged from 2.0×10^{-6} to 2.0×10^{-5} M, depending on the molar absorptivities of the salicylic acids and also on the binding affinities of the substrates to HSA. The pseudo-first order rate constant (k_{obs}) was determined from a plot of $\log(A_\infty - A)$ against time, where A_∞ and A are the absorbances at completion of the reaction and at time t, respectively. The reaction solution always contained 0.5% (v/v) acetonitrile for experimental convenience, and the temperature was 25 °C. The buffer solutions used were pH 7.4, 1/15 M phosphate ($\mu = 0.2$, adjusted with sodium chloride) and pH 9.9, 2/5 M carbonate for the reactions with 5-nitrosalicyl esters and with substituted aspirins, respectively.

Determination of Kinetic Parameters for the Reaction of Substrates with HSA——The reaction of a substrate (S) with HSA can be expressed as shown in Chart 1.[2,6] Abbreviations in Chart 1 are: S·HSA, the Michaelis–Menten type

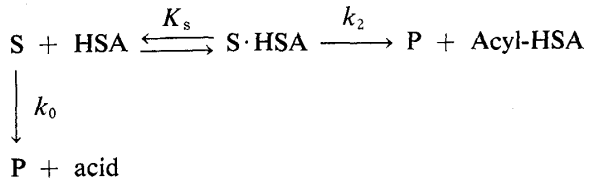


Chart 1

complex between S and HSA; P, the corresponding salicylic acid released; Acyl-HSA, HSA acylated with S; K_S, dissociation constant of S·HSA; k_2, catalytic rate constant; k_0, hydrolysis rate constant of S. According to Chart 1, the k_{obs} value determined experimentally can be represented by equation (1).[1-3,6]

$$k_{obs} = \frac{k_0 K_S + k_2 [HSA]_0}{K_S + [HSA]_0} \quad (1)$$

where $[HSA]_0$ is the initial concentration of HSA. The K_S and k_2 values can be calculated from the slope and intercept of the double-reciprocal plot based on equation (2).

$$\frac{1}{k_{obs}-k_0}=\frac{K_S}{(k_2-k_0)}\cdot\frac{1}{[HSA]_0}+\frac{1}{k_2-k_0} \quad (2)$$

Determination of pK_a for Salicylic Acids——The pK_a value of the hydroxyl group in salicylic acids was determined spectrophotometrically based on equation (3).[15]

$$\log\frac{A-A_{P^-}}{A_P-A}=pK_a-pH \quad (3)$$

In equation (3), A_P, A_{P^-}, and A are the absorbances at an appropriate wavelength for the free form, ionic form, and their mixture, respectively.

Results and Discussion

Substituted Aspirins

Table II lists the kinetic parameters for the reactions of substituted aspirins with HSA, and also the pK_a values of the hydroxyl group in the parent salicylic acid molecules. Although the number of substituted aspirins used is small ($n=5$), the logarithm of k_2 was correlated well with the pK_a value of the hydroxyl group. The regression line is expressed by equation (4).

$$\log k_2=-0.843\ (\pm0.246)\ pK_a+6.75\ (\pm2.59) \quad (4)$$

$$n=5,\ s=0.313,\ r=-0.988$$

In equation (4), the values in parentheses are the 95% confidence intervals; n, the number of aspirin derivatives used; s, the standard deviation; r, the correlation coefficient. In the previous study,[6] it was found that the catalytic group of HSA for the reactions with **1** and **2** is the lysine-199 (Lys-199) residue. The negative slope (-0.843) hence suggests that the reaction of the substrate with HSA occurs through the nucleophilic attack of the free ε-amino group of Lys-199 at the carbonyl carbon atom of the substrate. It is of interest to compare the correlation for the aspirin derivatives with that for phenyl acetates obtained previously.[2] The relationship for phenyl acetates excluding aspirin was expressed by equation (5).

$$\log k_2=-0.751\ (\pm0.169)\ pK_a+5.55\ (\pm1.51) \quad (5)$$

$$n=5,\ s=0.122,\ r=-0.993$$

Although many differences exist between the two reactions, *e.g.* in the catalytic group of HSA, *ortho* substituent effect, and charge of the substrates, the differences in the slope and intercept between equations (4) and (5) are small. This phenomenon may be explained as follows. The nucleophilicity of a catalyst is related to pK_a of the catalyst.[17,18] The pK_a values of both Lys-199 and Tyr-411 of HSA were found previously[2,6] to be about 9.5, and thus the nucleophilicities of both groups are presumed to be identical.[17,18] Moreover, the *ortho* effect

Table II. Rates and Dissociation Constants for Reactions of Substituted Aspirins with HSA[a]

Substrates	k_2 (s^{-1})	K_S (M)	k_0 (s^{-1})	pK_a[b]
1	4.0×10^{-4}	2.2×10^{-3}	2.1×10^{-5}	12.4[c]
2	4.2×10^{-2}	1.6×10^{-4}	6.4×10^{-4}	9.9
3	6.1	7.3×10^{-5}	1.9×10^{-3}	7.1
4	9.8×10^{-4}	3.6×10^{-5}	1.3×10^{-5}	11.2
5	1.2×10^{-3}	7.3×10^{-5}	2.5×10^{-5}	11.2

a) pH 9.9, 2/5 M carbonate buffer containing 0.5% (v/v) acetonitrile and 25 °C.
b) pK_a of the hydroxyl group of the corresponding salicylic acid.
c) Literature value.[16]

TABLE III. Rates and Dissociation Constants for Reactions of 5-Nitrosalicyl Esters with HSA[a)]

Substrates	k_2 (s^{-1})	K_S (M)	k_0 (s^{-1})	k_2/K_2 ($M^{-1}s^{-1}$)	$\pi^{b)}$	$E_S^{c)}$
2	8.4×10^{-4}	2.6×10^{-5}	7.6×10^{-6}	3.2×10	0.50	0.00
6	4.4×10^{-4}	8.5×10^{-6}	1.0×10^{-5}	5.2×10	1.00	−0.07
7	1.2×10^{-3}	1.5×10^{-5}	5.9×10^{-6}	8.0×10	1.50	−0.36
8	9.3×10^{-4}	9.1×10^{-6}	5.2×10^{-6}	1.0×10^{2}	2.00	−0.39
9	1.8×10^{-4}	—[d)]	6.0×10^{-6}	—	2.50	−0.40
10	2.5×10^{-3}	3.7×10^{-5}	8.3×10^{-6}	6.8×10	1.30	−0.47
11	6.2×10^{-4}	1.3×10^{-5}	1.8×10^{-6}	4.8×10	1.80	−0.93

a) pH 7.4, 1/15 M phosphate buffer ($\mu=0.2$) containing 0.5% (v/v) acetonitrile and 25 °C.
b) Hansch's hydrophobic substituent constant.[20)]
c) Taft's steric substituent constant.[21)]
d) Could not be determined accurately.

and the charge difference of the substrates seem to be reflected in the individual pK_a values of the hydroxyl groups in salicylic acids and phenols. The correlations expressed by equations (4) and (5) were obtained using such pK_a values. Hence, the similarity of the two correlations, obtained for substituted aspirins and phenyl acetates, may be reasonable.

The binding affinity of the substrate to HSA, which is reflected by K_S, is affected in a complicated manner by the substituents. The K_S values are influenced by the number of the substituents rather than the nature of the substituents, since the values of K_S decrease in the order **1**>**2**>**3** and are identical for **3** and **5**.

Since the kinetics and mechanism of the hydrolysis of substituted aspirins have been reported in the literature,[13,14,19)] the k_0 values given in Table II will not be discussed here.

5-Nitrosalicyl Esters

Table III lists the kinetic parameters for the reactions of 5-nitrosalicyl esters with HSA, and also gives k_0 for comparison. The K_S value for **9** was too small to be determined accurately under the experimental conditions employed. For the same carbon number in the acyl group, the K_S value of a compound with a branched chain is larger than that of the compound with a normal chain (K_S for **7**<K_S for **10** and K_S for **8**<K_S for **11**). As regards k_2 and k_0, we could not find any clear correlations.

For the reactions of *p*-nitrophenyl esters with α-chymotrypsin, Milstien and Fife[22)] found that a plot of $\log(k_2/K_S)$ against Taft's steric substituent constant E_S gives a fair correlation. Moreover, Hansch and Coats[20)] reported that the addition of the hydrophobic substituent constant π to the above correlation results in a much better correlation. Hence, similar regression analyses were tried for the k_2/K_S values obtained for the reactions of 5-nitrosalicyl esters with HSA. The plot of $\log(k_2/K_S)$ *versus* π is shown in Fig. 1 (●). With the exception of **11**, a good relationship was obtained, as expressed by equation (6).

$$\log(k_2/K_S)=0.335\ (\pm0.109)\pi+1.37\ (\pm0.148) \quad (6)$$

$$n=, s=0.0384, r=0.985$$

Since **11** has a relatively bulky side chain, the steric factor E_S was added for the analysis for all compounds including **11**. The result was as follows:

$$\log(k_2/K_S)=0.423\ (\pm0.374)\pi+0.386\ (\pm0.616)E_S+1.35\ (\pm0.388) \quad (7)$$

$$n=6, s=0.0955, r=0.908$$

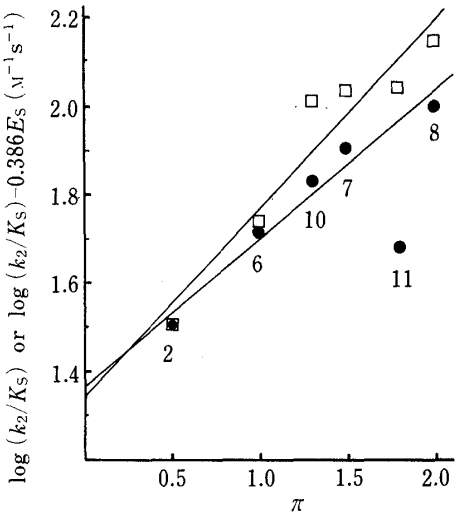


Fig. 1. Plots of $\log(k_2/K_S)$ *versus* π, and of $\log(k_2/K_S)-0.386E_S$ *versus* π for 5-Nitrosalicyl Esters

●, $\log(k_2/K_S)$ *vs.* π; □, $\log(k_2/K_S)-0.386E_S$ *vs.* π.
Numbers are the same as those in Table I.

Although the effect of addition of the E_S term is not statistically significant at the 0.95 level of significance, the point for **11** appears to be better fitted than in the case of the $\log(k_2/K_S)$ *versus* π plot, as shown in Fig. 1 (□).

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