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Effects of Drug Binding on the Esterase-Like Activity of Human Serum Albumin. V.[1] Reactive Site towards Substituted Aspirins

Yukihisa Kurono,* Hideto Yamada, and Ken Ikeda

Faculty of Pharmaceutical Sciences, Nagoya City University
Tanabe-dori, Mizuho-ku, Nagoya, 467, Japan

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To distinguish the reactive site on human serum albumin (HSA) towards aspirin derivatives from that towards *p*-nitrophenyl acetate (NPA), the effect of the acetylation of HSA by aspirin and also the effect of several drugs on the reaction rates of substituted aspirins and NPA with HSA were investigated in pH 7.4 phosphate buffer at 25°C. The substituted aspirins examined were 5-nitroaspirin and 3,5-dinitroaspirin. The acetylation by aspirin affected the reaction rate with 5-nitroaspirin but not that with NPA. The inhibition patterns of the reactions with the aspirins caused by clofibric acid, flufenamic acid and phenylbutazone were different from those with NPA. The complex formation between 5-nitroaspirin and the reactive site of HSA decreased the fluorescence intensity due to the only tryptophan residue (Trp-214) of HSA. These results indicate that the reactive site towards the aspirins (near Trp-214) is different from that towards NPA which was found previously to be near Tyr-411. From the pH profiles of the kinetic parameters for the reaction of 5-nitroaspirin with HSA, the pK_a value of the catalytic group constituting the reactive site towards the aspirins was estimated as about 9.5.

Keywords——human serum albumin; esterase-like activity of human serum albumin; enzyme kinetics; substituted aspirins; *p*-nitrophenyl acetate; protein binding; distinction and identification of drug binding sites; clofibric acid; flufenamic acid; phenylbutazone

In our previous study on structure-activity relationships for the esterase-like activity of human serum albumin (HSA) on phenyl acetates including aspirin, a linear relation was found between the logarithms of the catalytic rate constants and pK_a values of the leaving phenols.[2] From this relation, these substrates were presumed to react at an indentical reactive site on HSA. The reactive site towards a typical substrate, *p*-nitrophenyl acetate (NPA), was found to be near Tyr-411 of the HSA amino acid sequence.[3–5] However, Hawkins *et al.*[6] and Walker[7] reported that aspirin acetylates the ε-amino group of Lys-199 on HSA. Thus, it is necessary to resolve the inconsistency regarding the reactive sites of HSA towards NPA and aspirin. Substituted aspirins, 5-nitroaspirin (NA) and 3,5-dinitroaspirin (DA), instead of aspirin itself, were employed here as substrates, since the reaction of aspirin with HSA is too slow for convenient rate measurement.

This study is concerned with the effects of the acetylation by aspirin and also with the effects of some drugs on the reaction rates of the aspirin derivatives and NPA with HSA. Furthermore, the kinetic parameters, the catalytic rate constant and dissociation constant, for the reaction of NA with HSA were determined and the pH profiles of these parameters were obtained in order to characterize the reactive site.

Experimental

Materials and Apparatus——HSA (Fraction V, lot 47C-04423, Sigma Chem. Co.) was used after the purification by Chen's method.[8] The molecular weight of HSA was assumed to be 69000 and the concentration was determined from the extinction coefficient $E_{1cm}^{0.1\%}$ of 0.531 at 278 nm.[9] NA and DA were synthesized by the method of Ciampa.[10] Since the hydrolysis of DA seemed to occur even in the solid state, it was sometimes recrystallized from ether before use. Aspirin and NPA were obtained commercially. Clofibric acid (CA), ibuprofen (IP), flufenamic acid (FA) and phenylbutazone (PB) were the same materials as those used in the previous study.[3] All other chemicals purchased were of reagent grade.

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A Hitachi spectrophotometer (UV-124) and a Union Giken stopped-flow spectrophotometer (RA-401) equipped with a data processor (RA-405) were used for the measurements of the reaction rates. The fluorescence spectra of HSA were measured with a Shimadzu spectrofluorophotometer (RF-510).

Kinetic Runs——The buffer systems used in this study were as follows: pH 6—8, 1/15 M phosphate; pH 8—10.5, 1/20 M borate. The ionic strength was adjusted with NaCl to 0.2. The temperature was 25°C unless otherwise stated.

Acetylation of HSA by aspirin was carried out by the method of Hawkins *et al.*[6] Namely, a mixture of HSA (1.00×10^{-3} M) and aspirin (5.00×10^{-3} M) in pH 7.4 phosphate buffer was incubated at 37°C for 24 h and then dialyzed at 4°C for 48 h with multiple changes of the same buffer. After the dialysis, salicylic acid produced and aspirin unreacted were not detectable by the UV assay. In the control experiment, aspirin was omitted and HSA was treated as above.

The reactions of NA and NPA with HSA in the absence and presence of the drug were followed spectrophotometrically by monitoring the appearance of the phenols at the appropriate wavelengths. The reaction was initiated by the addition of 15 μl of substrate acetonitrile solution into 3 ml of the buffer solution containing HSA and the drug in the UV cell. The concentration of acetonitrile was always 0.5% (v/v). In the presence of excess HSA compared with the substrate, pseudo first-order analysis could be applied to the reaction as discussed previously.[2,3,11] The reaction of DA with HSA was followed with the stopped-flow apparatus, since the reaction was very fast.

Fluorescence Measurements——The fluorescence spectra of HSA solution (1.00×10^{-5} M) in the presence and absence of NA (2.00×10^{-6} M) at pH 7.4 were measured. The excitation wavelength was 300 nm.[3]

Results and Discussion

Effects of Acetylation by Aspirin on the Reaction Rates of NA and NPA with HSA

Table I shows the values of the pseudo first-order rate constant, k_{obs}, for the reaction of the substrate (NA or NPA) with the acetylated HSA and the control HSA. In the case of NPA, there seems to be no significant change in k_{obs} between the control HSA and the acetylated HSA. In contrast, the k_{obs} value for the reaction of NA with the acetylated HSA is about half of that with the control HSA. The remaining activity ($k_{obs}=2.43\times10^{-4}$ s^{-1}) is probably due to secondarily reactive site (s) towards NA, since according to the method of Hawkins *et al.*, about 1.2 acetyl groups are covalently bound per mole of HSA.[6a] Therefore, the results shown in Table I indicate that the reactive site on HSA towards NA is different from that towards NPA but identical with that towards aspirin itself.

Table I. Effects of Acetylation of HSA by Aspirin on the Reaction Rates of NA and NPA with HSA

	k_{obs} (s^{-1})[a]	
	NA	NPA
Control HSA[b]	4.94×10^{-4}	7.92×10^{-3}
Acetylated HSA[b]	2.43×10^{-4}	7.32×10^{-3}

a) pH 7.4 phosphate buffer ($\mu=0.2$) containing 0.5% (v/v) acetonitrile and at 25°; $[NA]_0=[NPA]_0=1.00\times10^{-5}$ (M); $[HSA]_0=5.00\times10^{-5}$ (M).
b) See "Experimental" for details of these HSA preparations.

Effects of Some Drugs on Reaction Rates of Substituted Aspirins with HSA

To further distinguish the reactive site towards the substituted aspirins from that towards NPA, the effects of some drugs on the reaction rates of the aspirins and NPA with HSA were examined. The results for CA are shown in Fig. 1, where k°_{obs} on the ordinate is the rate constant for the reaction of the substrate with HSA in the absence of CA and the concentrations with subscript 0 on the abscissa represent the initial concentrations of CA and HSA. CA does not inhibit the reactions of NA and DA with HSA at low ratios of $[CA]_0/[HSA]_0$. The slight inhibition in the case of NA at the high ratio may result from the binding of CA to the secondary low affinity sites of HSA.[12,13] On the other hand, the reaction with NPA is strongly inhibited at low ratios of $[CA]_0/[HSA]_0$. Similar results were also obtained for

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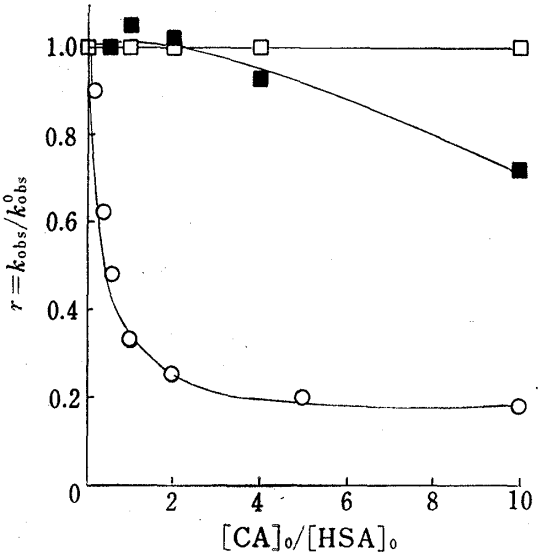

Fig. 1. Effect of CA on the Reaction Rates of NA, DA and NPA with HSA

■, NA; □, DA; ○, NPA.
pH 7.4 phosphate buffer containing 0.5% (v/v) acetonitrile and at 25°C; $[Substrate]_0=1.00\times10^{-5}$ (M); $[HSA]_0=5.00\times10^{-5}$ (M).

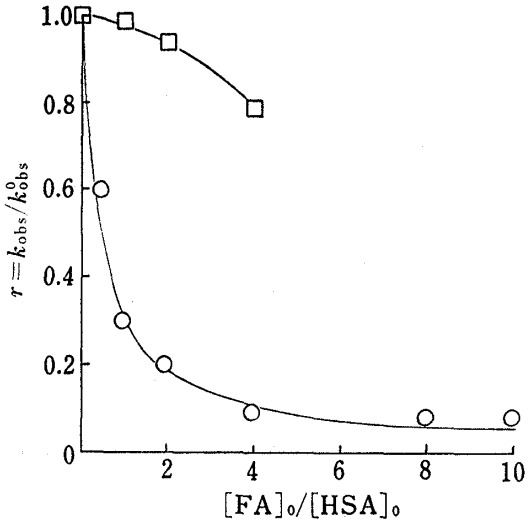

Fig. 2. Effect of FA on the Reaction Rates of DA and NPA with HSA

□, DA; ○, NPA.
pH 7.4 phosphate buffer containing 0.5% (v/v) acetonitrile and at 25°C; $[Substrate]_0=1.00\times10^{-5}$ (M); $[HSA]_0=5.00\times10^{-5}$ (M).

IP (not shown here). The high affinity binding site of CA and IP was shown previously[1,3,13] to be near Tyr-411 of the HSA sequence, which is the primarily reactive site (R site) towards NPA. These results also support the view that the reactive site towards the aspirins is different from that towards NPA.

It is reported that FA binds to three high affinity binding sites on HSA.[14] It was concluded previously[1,3] from the inhibition pattern of FA for the reaction of NPA with HSA (○, as shown in Fig. 2) that two binding sites of HSA for FA are the R site and the secondarily reactive site (T site) towards NPA, with this order of binding affinity. However, the location of the T site in the HSA amino acid sequence is not yet known. To examine whether or not the T site is the reactive site towards the aspirins, the effect of FA on the reaction rate of DA with HSA was investigated. Fig. 2 shows the results. Up to the ratio $[FA]_0/[HSA]_0$ of about 1, FA hardly inhibits the reaction and it is bound primarily to the R site in this region. From values of 1 to 4[15] of the ratio, an inhibition of about 20% ($r\fallingdotseq 0.8$) is observed, approximately in parallel with the decrease for NPA in this range. From this result it may be considered that the T site is the reactive site towards DA. However, the dissociation constant of the substrate-HSA complex for the reaction of DA with HSA would be different from that for NPA and the order of binding affinity between the T site and the third binding site for FA is unknown. The results shown in Fig. 2, therefore, do not unequivocally demonstrate that the reactive site towards DA is the T site.

Figure 3 shows the effect of PB on the reaction rates of the substituted aspirins and NPA with HSA. In the previous studies,[1,3] two high affinity binding sites of HSA for PB were identified. The primary binding site is near Trp-214 (U site), which is not involved in the reaction of NPA with HSA, and the secondary site is the R site. These binding properties of PB to HSA are reflected by the inhibition curve (○) for NPA in Figure 3, that is, the curve for NPA shows a slight shoulder at low ratios of $[PB]_0/[HSA]_0$ and at higher ratios the r values decrease.[3] In contrast, PB directly inhibits (without the shoulders) the reactions of the aspirins with HSA. The reactive site on HSA towards the aspirins, therefore, appears to be the U site and not the T site. The difference in the inhibition curves between NA and DA probably arises from the difference in the dissociation constants (K_S) of the substrate-HSA

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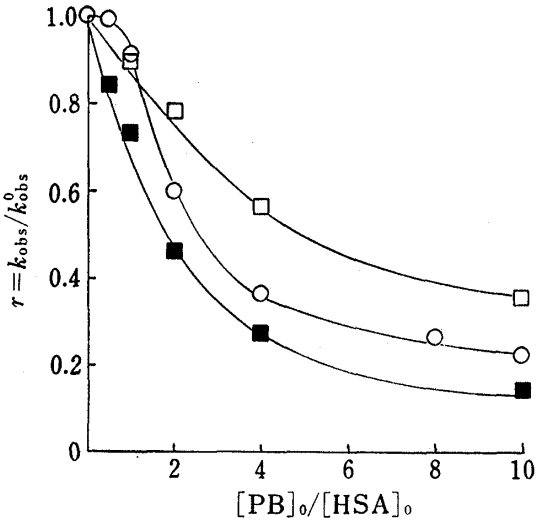

Fig. 3. Effect of PB on the Reaction Rates of NA, DA and NPA with HSA

□, DA; ■, NA; ○ NPA.
pH 7.4 phosphate buffer containing 0.5% (v/v) acetonitrile and at 25°C; $[Substrate]_0=1.00\times10^{-5}$ (M); $[HSA]_0=5.00\times10^{-5}$ (M).

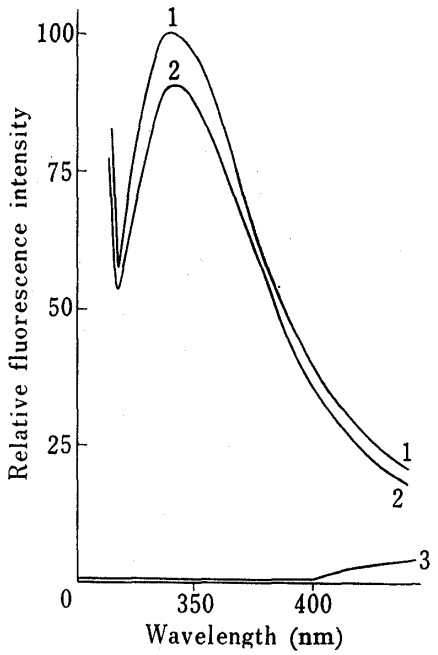

Fig. 4. Fluorescence Emission Spectra excited at 300 nm

1, 1.00×10^{-5} M HSA;
2, 1.00×10^{-5} M HSA + 2.00×10^{-6} M NA;
3, 2.00×10^{-6} M NA.

complexes, that is, K_S for the reaction of DA with HSA may be smaller than that for NA (see the next section).

To confirm that the reactive site towards the aspirins is the U site, the fluorescence spectra due to the single tryptophan residue (Trp-214) of HSA were measured (Fig. 4). The intensity of the emission spectrum (spectrum 2) for the mixture of NA and HSA is smaller than that (spectrum 1) for HSA. It took about one and half minutes to prepare the solution (mixing of NA and HSA) and then to measure the spectrum of the solution. Since the half-life for the reaction of NA with HSA under the experimental conditions used is about 20 minutes, the extent of reaction during about 1.5 minutes is slight. Thus, the decreased emission spectrum is mainly due to the complex formation between NA and the reactive site near Trp-214.

According to Walker[7] and Hawkins *et al.*,[6] Lys-199 on HSA is acetylated with aspirin itself. Since the acetylation of HSA with aspirin by their method affected the reaction rate with NA, as shown in Table I, the lysine residue (Lys-199) is also involved in the reaction of HSA with the substituted aspirins. Thus, in connection with the identification of the reactive site towards the aspirins, it can be concluded that the binding site of the substrates, NA and DA, is the U site and that the catalytic group for the reactions is the ε-amino group of Lys-199, which is located sterically near Trp-214.

The pH Profiles of Kinetic Parameters for the Reaction of NA with HSA

Figure 5a shows the effect of HSA concentration on the k_{obs} value for the reaction of NA with HSA. The k_{obs} value increases hyperbolically with the concentration of HSA, indicating saturation kinetics for the reaction.[2,16] Thus, the reaction of NA with HSA can be expressed as shown in Chart 1.[2] In this chart, the catalytic rate constant and the hydrolysis rate constant of NA are represented by k_2 and k_0, respectively. The K_s and k_2 values are calculated from the intercept and slope of the plot (Fig. 5b) based on equation (1).[2,16]

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

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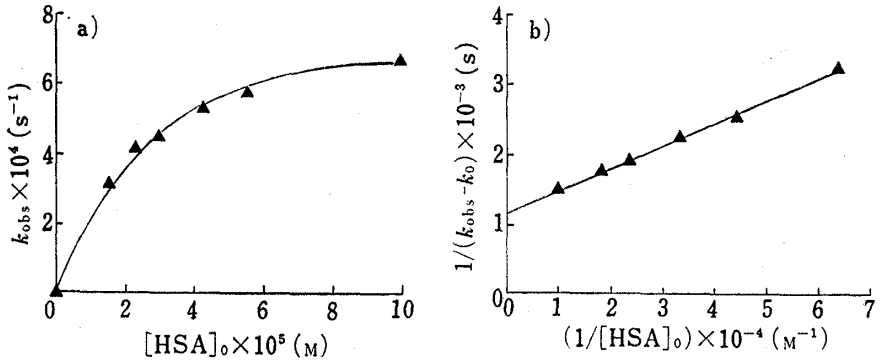

Fig. 5. a) Effect of HSA Concentration on the Rate of 5-Nitrosalicylic Acid Release. b) Plot $1/(k_{obs}-k_0)$ against $1/[HSA]_0$ (data from Fig. 5a).

pH 7.4 phosphate buffer containing 0.5% (v/v) acetonitrile and at 25°C; $[NA]_0=1.00\times10^{-5}$ (M).

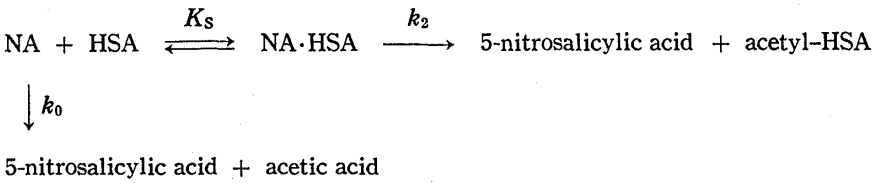

Chart 1

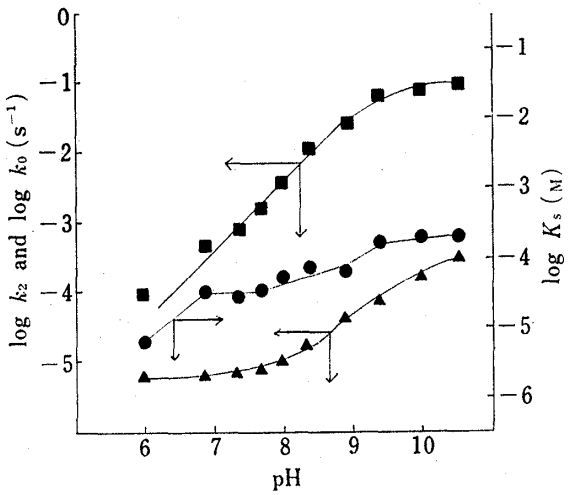

Fig. 6. The pH Profiles of k_2, k_0 and K_S for the Reaction of NA with HSA at 25°C

■, k_2; ▲, k_0; ●, K_s.

The numerical analysis for the reaction of DA with HSA could not be carried out in the same way as for NA, because under conditions identical with those shown in Fig. 5a, k_{obs} (about $2.0\times10^{-1}\,s^{-1}$) was independent of the HSA concentration, implying that K_S is less than about 2×10^{-5} M.

Figure 6 shows the pH profiles of k_2 and K_s for the reaction of NA with HSA, and also of k_0 for a comparison with k_2. Since the shape of the pH profile of k_0 for NA is similar to those for DA[17,18] and for aspirin[19] in the literature, it is not discussed here. The k_2 values markedly depend on pH. The slope of the profile between pH 6.0 and 8.5 is about unity and above pH 9.5 k_2 becomes approximately independent of pH. The pK_a value of the catalytic group (ε-amino group of Lys-199 in HSA) was estimated as about 9.5 from this log k_2-pH profile.

The dependence of the K_S value on pH is complicated. The higher the pH value, the larger the K_S value (the lower the binding affinity of NA to HSA). This may be qualitatively interpreted as follows. The deprotonation of basic groups (for example, ε-amino group of lysine and/or guanidino group of arginine[20]) constituting the binding site (U site) of NA on HSA increases with increasing pH value. Since NA exists as an anion (due to the carboxyl group on NA) in the range of pH studied, the ionic interaction[20] between NA and the U site of HSA may decrease with increasing pH value. Recently, it was reported[21] that the binding of warfarin (which is bound to the U site[1,3]) to HSA at pH 9.3 is about 3 times stronger than that at pH 6.1. The reason for the difference in the binding properties between NA and warfarin is now under study.

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We have been classifying and identifying the drug binding sites on HSA in relation to the inhibition types of the reaction of NPA with HSA caused by various drugs.[1,3] However, the use of only NPA as a substrate gives little information on drug binding sites (*e.g.* the U site) not involved in the reaction. As described in this paper, since the reactive site towards substituted aspirins is different from that towards NPA, NA and DA can be used as new substrates for the distinction and identification of drug binding site (s) on HSA.

References and Notes

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