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Effects of Drug Binding on the Esterase-like Activity of Human Serum Albumin. VI.[1] Reaction with Di-*p*-nitrophenyl Adipate

YUKIHISA KURONO,* HITOMI SUGIURA, and KEN IKEDA

Faculty of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori, Mizuho-ku, Nagoya 467, Japan

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The binding of R type drugs, which have high affinity to a site (R site) near the tyrosine-411 residue, remarkably accelerated the reaction of di-*p*-nitrophenyl adipate (D) with human serum albumin (HSA). The reaction was investigated kinetically at various pHs and 25 °C. The reaction in the presence of an excess of HSA over D proceeded in two steps. The pH-profiles for both rate constants were sigmoidal, indicating the participation of two tyrosine residues having pK_a values of about 9.3 in the reactions. The two residues of HSA involved seem to be separated by a distance corresponding to the length of the adipoyl group.

Keywords——human serum albumin; esterase-like activity; kinetics; analog computer; tyrosine residue; protein binding; flufenamic acid; ibuprofen; diazepam; clofibric acid

Human serum albumin (HSA) has esterase-like activity towards esters[1-4] and amides.[5] The reactive sites towards typical substrates, *p*-nitrophenyl acetate and 5-nitroaspirin, were found to be located close to the tryosine-411 (Tyr-411) (named the R site[6]) and Lys-199 (U site[1,6]) residues of the HSA amino acid sequence,[7] respectively. From studies on the inhibition of the activities caused by several drugs, these sites were concluded to be important drug binding sites on HSA.[1,3-6]

When the reaction of di-*p*-nitrophenyl adipate (D) with HSA was examined to elucidate the details of the esterase-like active sites (also the drug binding sites), R type drugs[6] which bind to the R site of HSA were found to accelerate the reaction remarkably. In this paper we describe the kinetics and mechanism of the reaction, and the characterization of the reactive site of HSA towards D.

Experimental

Materials and Apparatus——HSA (Sigma Chem. Co., Fraction V, lot 12F-0051) was used after purification by Chen's method.[8] The molecular weight of HSA was assumed to be 69000 and the concentration was estimated based on an extinction coefficient $E^{0.1\%}_{1\,cm}$ of 0.531 at 278 nm.[2] According to a modified method of Wood *et al.*,[9] D was synthesized from *p*-nitrophenol and adipoyl chloride in the presence of *N*,*N*-dimethylaniline. The results of elemental analysis for carbon, hydrogen, and nitrogen of D were within the range of the calculated values $\pm 0.3\%$, and the melting point was 113—116 °C. Clofibric acid (CA), ibuprofen (IP), flufenamic acid (FA), phenylbutazone (PB), and warfarin (WF) were the same products as those used in the previous studies.[1,6] Diazepam (DA) and azapropazone (AP) were gifts from Takeda Pharmaceutical Co. and Nippon Chemiphar Co., Ltd., respectively. All other chemicals were commercial reagent-grade products.

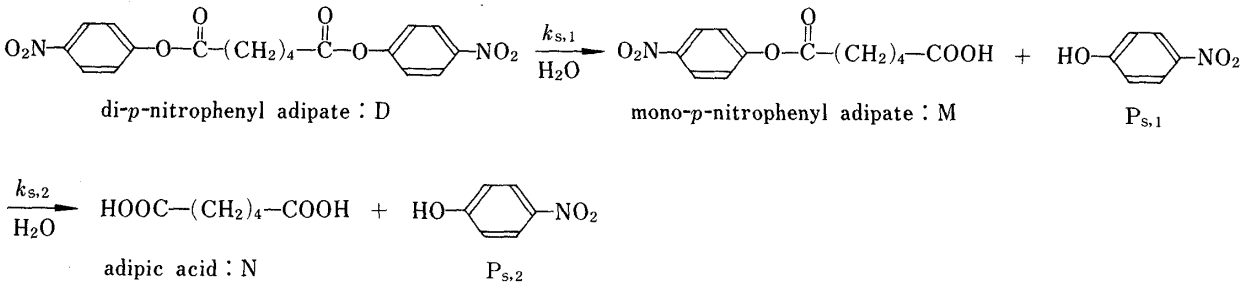
Hitachi UV-124 and UV-228 spectrophotometers were used for the measurements of the reaction rates. The analog computer used was a Hitachi ALS-20M (Hitachi-Denshi Co., Ltd.).

Kinetic Runs——The buffer systems employed were as follows: pH 6.5—7.4, 0.067 M phosphate; pH 8.0—9.0, 0.05 M borate–0.10 M phosphate; pH 10.0—11.0, 0.05 M borate–0.05 M carbonate. Ionic strength was adjusted to 0.2 with NaCl. The reaction temperature was 25 °C.

The reactions of D (1.00×10^{-5} M) with HSA in the presence and absence of drug were followed spectrophotometrically by monitoring the appearance of *p*-nitrophenol (P) at 400 nm. Since a concentration of HSA more than threefold in excess of D was employed, D practically reacts with the primary reactive site and the reaction follows

pseudo first-order kinetics.[1-5] The drug concentration was varied as required. For experimental convenience, the stock solution of D was prepared in acetonitrile so that the reaction solution for rare measurements always contained 0.5% (v/v) acetonitrile.

Determination of the Rate Constant by Using an Analog Computer——One mol of D releases two mol of P, as shown in Chart 1, where M and N are mono-*p*-nitrophenyl adipate and adipic acid, respectively. The first-order rate



di-*p*-nitrophenyl adipate : D　　mono-*p*-nitrophenyl adipate : M　　$P_{s,1}$

adipic acid : N　　$P_{s,2}$

Chart 1

constants for the formations of M and N are represented by $k_{s,1}$ and $k_{s,2}$, respectively. $P_{s,1}$ and $P_{s,2}$ are *p*-nitrophenol (P) produced in the reactions characterized by $k_{s,1}$ and $k_{s,2}$, respectively.

The analog computer was used for the determinations of $k_{s,1}$ and $k_{s,2}$ because the experimentally measurable products, $P_{s,1}$ and $P_{s,2}$, are identical (*p*-nitrophenol) and thus the analytical method is complicated.[10] The following rate and conservation equations can be derived according to the reaction scheme in Chart 1.

$$d[D]/dt = -k_{s,1}[D] \quad (1)$$

$$d[M]/dt = k_{s,1}[D] - k_{s,2}[M] \quad (2)$$

$$d[P_{s,1}]/dt = k_{s,1}[D] \quad (3)$$

$$[N] = [P_{s,2}] = [D]_0 - [D] - [M] \quad (4)$$

$$[P] = [P_{s,1}] + [P_{s,2}] \quad (5)$$

In Eq. 4 $[D]_0$ is the initial concentration of D. After amplitude and time scaling for Eqs. 1—5, an analog circuit diagram was prepared (Fig. 1). The details of the amplitude and time scaling, and of the programming are described in the literature.[11,12] The accuracy of the rate constants determined by the computer is generally ±5%.[13]

When $k_{s,1}$ was more than 50-fold larger than $k_{s,2}$, the computer was found to be unnecessary for the determination, since ordinary first-order analysis could be applied to the individual steps.

When necessary, the analog computer was also used to determine the rate constants for the reaction of D with HSA, as in the case of the spontaneous hydrolysis of D.

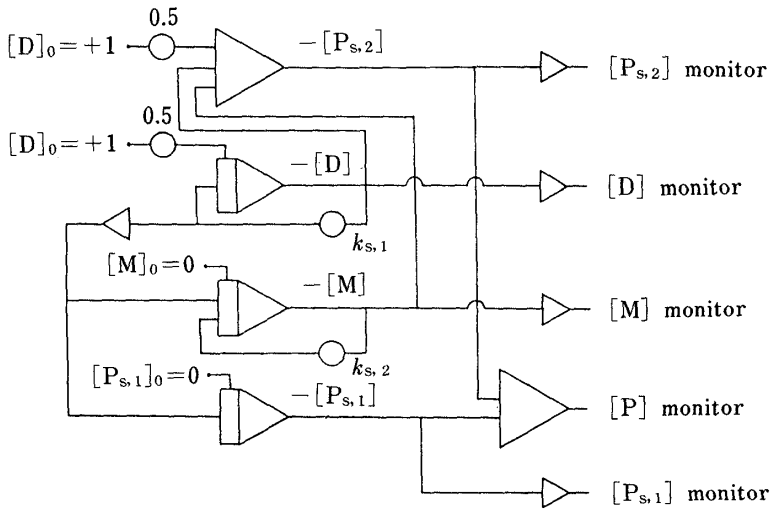


Fig. 1. Analog Circuit Diagram Applicable to Spontaneous Hydrolysis of D

Results and Discussion

Spontaneous Hydrolysis of D

Figure 2 shows the release of *p*-nitrophenol (●) from D as a function of time at pH 10.0 and 25 °C, where the ordinate shows the ratio of product concentration ([C]) to 2 $[D]_0$. Curves 1—5 in Fig. 2 are the time courses of hydrolysis products simulated by the analog computer. From the curve 5 that gave the best fit to the experimental points (●), the values of $k_{s,1}$ and $k_{s,2}$ were estimated as 8.91×10^{-5} and 2.29×10^{-5} (s^{-1}), respectively.

Figure 3 shows the pH-profiles of $k_{s,1}$ and $k_{s,2}$. The rate constants increase with increasing pH values, indicating the involvement of hydroxide ion in both reactions. The smaller effect of OH^- concentration on $k_{s,2}$ (□) than on $k_{s,1}$ (○) may be due to the carboxylate ion on the M molecule, both anions repelling each other.

Effects of Drug Binding on the Reaction of D with HSA

The reaction of D (1.00×10^{-5} M) with HSA (5.00×10^{-5} M) at pH 7.4 and 25 °C, for example, initially released about 1×10^{-5} M P with pseudo first-order kinetics. The subsequent release of about 1×10^{-5} M P occurred at about 100-fold smaller rate than the initial release of about 1×10^{-5} M P. The rate constants for the former and latter reactions are designated as $k_{obs,1}$ and $k_{obs,2}$, respectively, and were found to be 2.38×10^{-3} and 1.75×10^{-5} (s^{-1}), respectively. The values of $k_{obs,1}$ and $k_{obs,2}$ seem to be larger than that of $k_{s,1}$ assessed by extrapolation from the alkaline region to pH 7.4 in Fig. 3.[14]

To localize the reactive site towards D, the effects of several drugs, whose binding sites on HSA are already known, on $k_{obs,1}$ were examined. Figure 4 shows the results for PB, WF, and AP, which bind primarily to the U site.[1,6,7,15,16] In Fig. 4, $k'_{obs,1}$ on the ordinate represents the rate constant in the presence of a drug, and the concentrations with subscript 0 on the abscissa are the initial concentrations of drug and HSA. AP hardly affected the reaction rate of D with HSA up to a drug-to-HSA ratio of 4. At a $[Drug]_0/[HSA]_0$ ratio of more than 2, PB and WF slightly accelerated and inhibited the reactions, respectively. These results with three drugs indicate that the primary binding site (U site) of the drugs is not involved in the reaction of D with HSA.

Figure 5 shows the effects of R type drugs,[1,6,7,15,16] on $k_{obs,1}$. All of these drugs markedly accelerated the reaction. The reactive site of HSA towards D is, therefore, considered to be a site which is conformationally altered by the binding of an R type drug, leading to rate

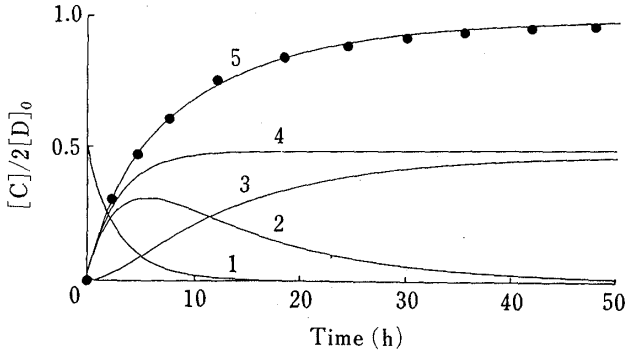


Fig. 2. Time Course of *p*-Nitrophenol Release from D at pH 10.0 and Analog Computer Simulation Curves of Hydrolysates of D

●, experimental results; 1, D; 2, M; 3, $P_{s,2}$; 4, $P_{s,1}$; 5, P.

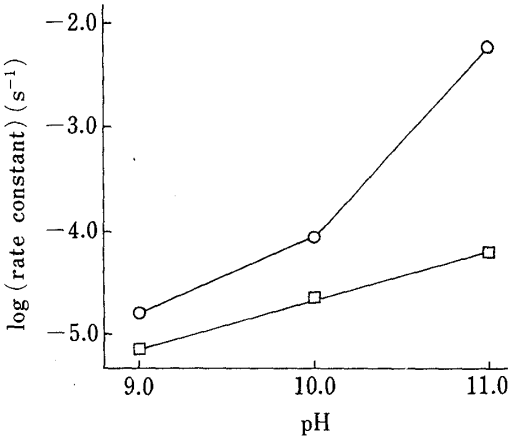


Fig. 3. pH–Profiles of $k_{s,1}$ and $k_{s,2}$

○, $k_{s,1}$; □, $k_{s,2}$.

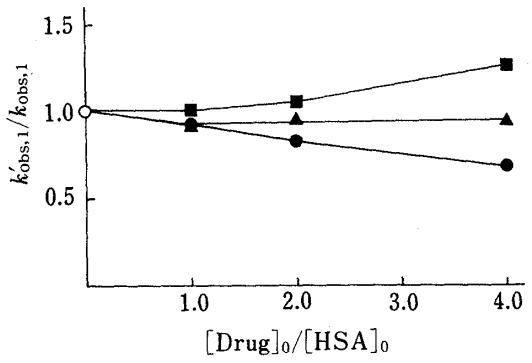


Fig. 4. Effects of U-Type Drugs on $k_{obs,1}$ at pH 7.4 and 25 °C

$[HSA]_0 = 5.00 \times 10^{-5}$ (M); $[D]_0 = 1.00 \times 10^{-5}$ (M). ■, phenylbutazone (PB); ●, warfarin (WF); ▲, azapropazone (AP).

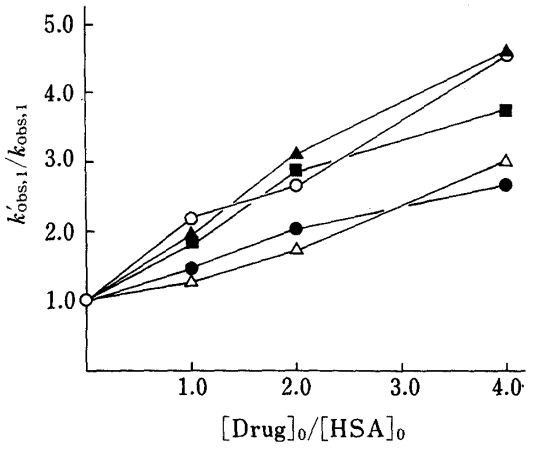


Fig. 5. Effects of R-Type Drugs on $k_{obs,1}$ at pH 7.4 and 25 °C

$[HSA]_0 = 5.00 \times 10^{-5}$ (M); $[D]_0 = 1.00 \times 10^{-5}$ (M). ●, clofibric acid (CA); ▲, octanoic acid; ■, flufenamic acid (FA); ○, ibuprofen (IP); △, diazepam (DA).

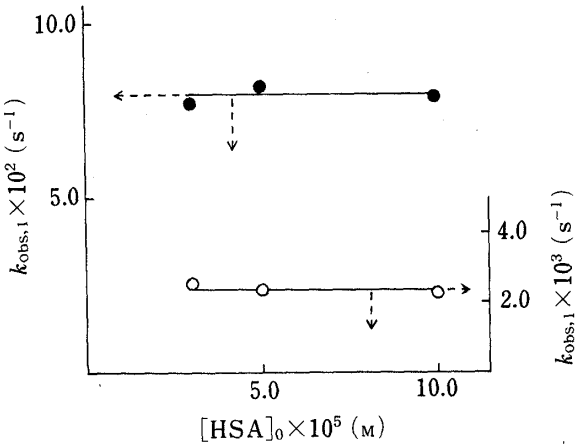


Fig. 6. Effect of HSA Concentration on $k_{obs,1}$ at 25 °C

$[D]_0 = 1.00 \times 10^{-5}$ (M); ○, pH 7.4; ●, pH 10.0.

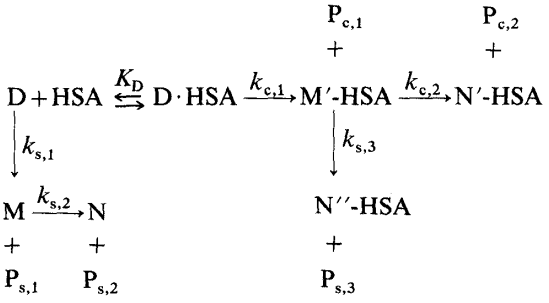


Chart 2

acceleration.

Characterization of the Reactive Site towards D

Figure 6 shows the effect of the HSA concentration employed for the reaction with D on $k_{obs,1}$ at pH 7.4 and 10.0. At both pHs $k_{obs,1}$ is independent of the HSA concentration. This independence suggests that the initial reaction of D with HSA proceeds *via* a Michaelis–Menten type complex (D·HSA).[1-3,17] The overall reactions of D with HSA, therefore, may be as shown in Chart 2. In Chart 2, $P_{c,1}$, $P_{c,2}$, and $P_{s,3}$ are all *p*-nitrophenol. M′-HSA, N′-HSA, and N″-HSA are mono-*p*-nitrophenyl adipoyl-HSA, adipoyl-HSA, and hemi-adipoyl-HSA (produced by hydrolysis of M′-HSA), respectively. The first-order rate constants for the formations of M′-HSA, N′-HSA, and N″-HSA are designated as $k_{c,1}$, $k_{c,2}$, and $k_{s,3}$, respectively. The dissociation constant of the complex D·HSA is designated as K_D.

Figure 7 illustrates the pH-profile of $k_{obs,1}$ which is, for the following reasons, approximately equal to $k_{c,1}$. According to Chart 2, $k_{obs,1}$ can be represented as follows:[1-3,17]

$$k_{obs,1} = \frac{k_{s,1}K_D + k_{c,1}[HSA]_0}{K_D + [HSA]_0} \qquad (6)$$

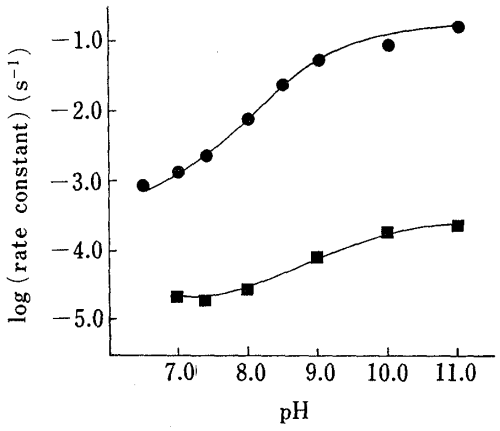


Fig. 7. pH–Profiles of $k_{obs,1}$ and $k_{obs,2}$ at 25 °C

●, $k_{obs,1}$ ($k_{c,1}$); ■, $k_{obs,2}$ ($k_{c,2}$). Solid lines were calculated from Eq. 7 using the parameters listed in Table I.

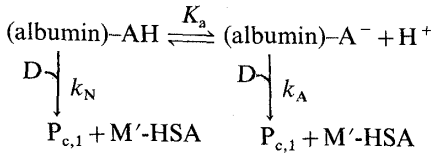


Chart 3

TABLE I. Intrinsic Rate Constants and Dissociation Constants for $k_{c,1}$ and $k_{c,2}$ at 25 °C

	k_N (s^{-1})	k_A (s^{-1})	K_a (M) (pK_a)
$k_{c,1}$	3.58×10^{-4}	1.59×10^{-1}	4.77×10^{-10} (9.32)
$k_{c,2}$	1.69×10^{-5}	2.25×10^{-4}	5.23×10^{-10} (9.28)

As shown in Fig. 6 the independence of $k_{obs,1}$ from $[HSA]_0$ implies that $[HSA]_0 \gg K_D$. Furthermore, $k_{obs,1}$ is much larger than $k_{s,1}$ (see Figs. 3 and 6). The independence and the difference in the rate constants indicate that $k_{obs,1}$ in Eq. 6 approximates to $k_{c,1}$. The pH-profile of $k_{c,1}$ is sigmoidal, and may indicate the involvement of an ionizable catalytic group of HSA, as shown in Chart 3, in the reaction. In Chart 3, (Albumin)–AH and (Albumin)–A^- represent the neutral and anionic forms of the catalytic group of HSA, respectively, and K_a is the dissociation constant of (Albumin)–AH. The intrinsic rate constants based on (Albumin)–AH and (Albumin)–A^- are designated as k_N and k_A, respectively. According to Chart 3, $k_{c,1}$ can be represented by Eq. 7.

$$k_{c,1} = \frac{k_N[H^+] + k_A K_a}{[H^+] + K_a} \tag{7}$$

Each parameter was estimated from the pH-profile of $k_{c,1}$, and is listed in Table I. From the pK_a value obtained, the catalytic group of HSA is considered to be either the hydroxyl group of a tyrosine residue or the ε-amino group of a lysine residue of HSA. The ratio of k_A to k_N ($k_A/k_N = 4.44 \times 10^2$) may indicate the tyrosine residue to be the catalytic group, since the nucleophilicity of the ammonium ion of the lysine residue is much smaller than that of the amino group and thus the slope of the profile below pH about 8 should be unity (the profile should not be sigmoidal).

There may be another possible interpretation of this $k_{c,1}$-pH profile, that is, the conformational change of HSA caused by the pH change (*e.g.*, N–B transition[18-21]). However, the midpoint (pH_{50}) of the changes of various properties due to the N–B transition of HSA in the literature[18-21] seems to be one to two orders of magnitude smaller than the

pK_a obtained in this study. Interpretation of our profile in terms of the N–B transition, therefore, does not seem reasonable.

Figure 7 also includes the pH-profile of $k_{obs,2}$ which is the rate constant for the later reaction after the completion of the initial reaction ($k_{c,1}$) of D with HSA. According to Chart 2, $k_{obs,2}$ is considered to be the sum of $k_{c,2}$ and $k_{s,3}$. The $k_{obs,2}$ value, however, is not proportional to the hydroxide ion concentration in the alkaline region, and the pH-profile of $k_{obs,2}$ is sigmoidal. This profile, therefore, suggests that $k_{s,3}$ (hydrolysis of M′-HSA by hydroxide ion catalysis) is small compared with $k_{c,2}$ and that the mono-*p*-nitrophenyl adipoyl group of M′-HSA may be buried inside the protein. As a consequence, $k_{obs,2}$ approximated to $k_{c,2}$. This approximation may be supported by the finding that $k_{obs,2}$ at pH 11.0 is smaller than $k_{s,1}$ at the same pH (see Figs. 7 and 3).

As in the case of $k_{c,1}$, Eq. 7 was applied to the sigmoidal profile of $k_{obs,2}$ ($\fallingdotseq k_{c,2}$) and each parameter in Eq. 7 was determined (Table I). The ratio of k_A to k_N was 1.33×10. The ratio and the pK_a value again suggest the catalytic group is another tyrosine residue, different from that for $k_{c,1}$. It is of interest to note that the two tyrosine residues of HSA are separated by a distance corresponding to the length of the adipoyl group ($-\overset{\overset{O}{\|}}{C}-(CH_2)_4-\overset{\overset{O}{\|}}{C}-$).

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