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Esterase-like Activity of Human Serum Albumin. V.[1] Reaction with 2,4-Dinitrophenyl Diethyl Phosphate

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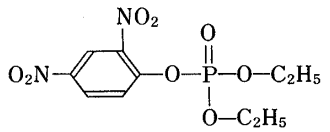
The reaction of 2,4-dinitrophenyl diethyl phosphate (DDP) with human serum albumin (HSA) was investigated kinetically at various pHs and 25 °C. The pH profile of the catalytic rate constant indicated the involvement of an ionizable group with pK_a 7.5 in the reaction. Ethoxycarbonylation of about two histidine residues (imidazole groups) per mol of HSA by diethylpyrocarbonate inactivated the reaction of DDP with HSA by about 70%, suggesting the existence of more than two reactive histidine residues towards DDP. Among these residues, one has higher activity than the others and it appears to be located near the tyrosine-411 residue (R site), because the reaction with DDP was inhibited most strongly by drugs which bind to the R site of HSA.

Keywords——human serum albumin; esterase-like activity; protein binding; phenylbutazone; clofibric acid; flufenamic acid; organophosphate; diethylpyrocarbonate; imidazole catalysis; human serum albumin neutral-base transition

In our previous papers[1–4] it was reported that human serum albumin (HSA) has esterase-like activity towards esters[1–3] and amides.[4] The reactive site towards phenyl acetates[2] and cinnamoylimidazoles[4] was found to be located close to the tyrosine-411 residue (named the R site),[5] while the reactive site towards substituted aspirins[1,3] was close to the lysine-199 residue (the U site)[1,3,5] in the HSA amino acid sequence.[6] These reactive sites were found to correspond to the specific and important drug binding sites on HSA reported elsewhere.[1–5]

As a part of our continuing studies on the esterase-like activity of HSA, the reaction of phosphate ester with HSA was investigated kinetically. As a model substrate 2,4-dinitrophenyl diethyl phosphate (DDP, see Chart 1) was selected, because the release of the product, 2,4-dinitrophenol (DNP), can be easily followed spectrophotometrically. Although something is known regarding the reversible binding of organophosphates to serum albumin,[7–9] little attention has been paid to the phosphorylation of serum albumin by them.[10]



DDP

Chart 1

Experimental

Materials——HSA (Sigma Chem. Co., Fraction V, lot 12F-0051) was used after purification by Chen's method.[11] The concentration of HSA was determined by use of its molar absorptivity ($\varepsilon = 3.66 \times 10^4\ M^{-1}\,cm^{-1}$) at 278 nm, assuming a molecular weight of 69000.[12,13] According to the method of Gulick and Geske,[14] DDP was

synthesized from DNP and diethylchlorophosphate in ether solution in the presence of triethylamine. DDP was recrystallized from ether–petroleum ether mixture. The melting point was 34—35 °C and the result of elemental analysis was within ±0.3% of the calculated value. Diethylpyrocarbonate (DEP) was purchased from Aldrich Chem. Co. (lot 8320 TJ) and was used without further purification. Phenylbutazone, oxyphenbutazone, azapropazone, clofibric acid, and flufenamic acid were the same as those used in the previous studies.[3,5] All other chemicals obtained commercially were of reagent grade.

Kinetic Procedure——The buffer systems used were as follows: pH 5.0, 0.2 M acetate; pH 6.0—8.0, 0.067 M phosphate; pH 9.0, 0.1 M phosphate–0.05 M borate; pH 9.5—10.5, 0.05 M borate. Ionic strength was adjusted to 0.2 with NaCl. The reaction temperature was 25 °C.

The reactions of DDP (1.00×10^{-5} M) with HSA in the presence and absence of drug were followed spectrophotometrically by monitoring the release of DNP at 360 nm. Since the HSA concentration used is more than 5-fold in excess of that of DDP, DDP preferentially reacts with the primary reactive site and the reaction follows the pseudo first-order kinetics.[1-5,12] The concentration of drug was varied as required. For experimental convenience, the stock solution of DDP was prepared in dioxane so that the reaction solution for rate measurements always contained 0.5% (v/v) dioxane.

Ethoxycarbonylation of *N*-Acetyl-L-histidine and HSA——Ethoxycarbonylation of *N*-acetyl-L-histidine (NAH) and HSA with DEP was carried out by a modification of Roosemont's method.[15] Fifteen μl of 1.0 M DEP in ethanol was added to 3 ml of various concentrations (3.0×10^{-5} M—2.5×10^{-4} M) of NAH in the pH 7.4 buffer: in the reference cell, 15 μl of ethanol without DEP was used. The difference absorbances at 242 nm were continuously recorded at 25 °C on a Hitachi UV-228 spectrophotometer and the maximum absorbance readings were obtained after about 10 min. By using a calibration line based on the maximum absorbances, the molar absorptivity of ethoxycarbonyl-*N*-acetyl-L-histidine (EAH) was estimated as 3.33×10^3 M^{-1} cm^{-1}.

To 3 ml of 5.0×10^{-5} M HSA in pH 7.4 buffer was added 15 μl of various concentrations (1.0×10^{-2} M—5.0×10^{-2} M) of DEP in ethanol. As in the case of the ethoxycarbonylation of NAH, the maximum absorbances at 242 nm were measured after about 10 min. The number of ethoxycarbonylated histidine residues of HSA was calculated by using the maximum absorbances and the molar absorptivity of EAH (assumed to be equal to the molar absorptivity of the modified histidine residue).

The effects of the ethoxycarbonylation of HSA on the reaction rate with DDP were investigated as follows. After attainment of the maximum absorbances at 242 nm based on the reaction of DEP (5.0×10^{-5} M—2.5×10^{-4} M) with HSA (5.0×10^{-5} M), 15 μl of 2.0×10^{-3} M DDP in dioxane was added to 3 ml of the reaction solution containing the DEP-treated HSA and DEP. The rate of 2,4-dinitrophenol (DNP) release was followed at 360 nm and the pseudo first-order rate constant (k^{m}_{obs}) was calculated.

Results and Discussion

Reaction of DDP with HSA

Figure 1 (a) shows the effects of the HSA concentration on the pseudo first-order rate constant (k_{obs}) for the reaction with DDP. The k_{obs} value increases hyperbolically with the concentration of HSA, suggesting saturation kinetics for the reaction.[2,3] The reaction of

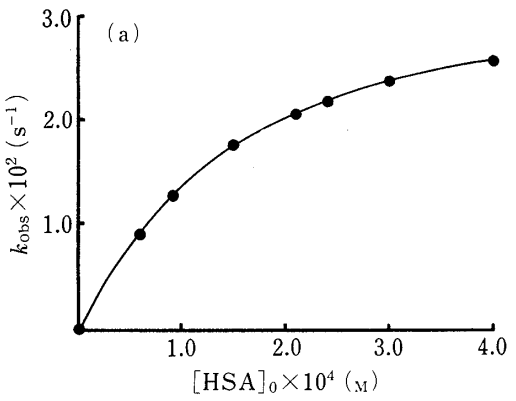


Fig. 1. (a) Effect of HSA Concentration on the Rate of DNP Release at pH 7.4 and 25 °C
$[DDP]_0 = 1.00 \times 10^{-5}$ (M).

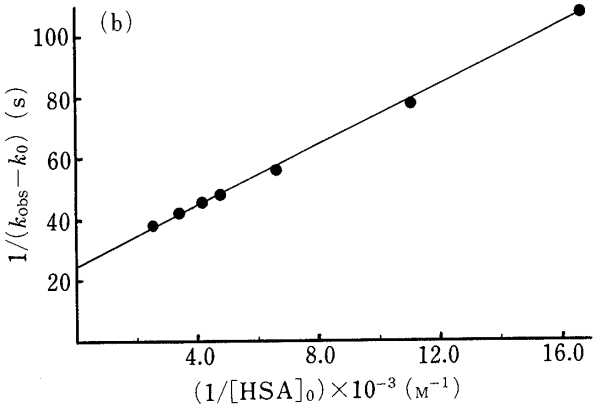


Fig. 1. (b) Plot of $1/(k_{obs}-k_0)$ against $1/[HSA]_0$
Data from Fig. 1(a).

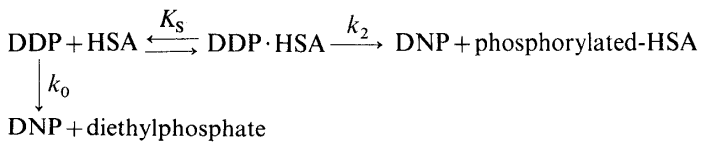


Chart 2

DDP with HSA can thus be expressed as shown in Chart 2. In Chart 2, DDP·HSA is the Michaelis–Menten type complex between DDP and HSA, and K_S is the dissociation constant of DDP·HSA. The catalytic rate constant and the hydrolysis rate constant of DDP are represented by k_2 and k_0, respectively. The K_S and k_2 values are calculated from the slope and intercept of the plot (Fig. 1 (b)) based on Eq. 1.[2,3]

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

Figure 2 shows the pH profiles of k_2 and K_S for the reaction of DDP with HSA, and of k_0 for comparison with k_2. Since the pH-rate profile for hydrolysis of phosphate esters has been reported in the literature,[16–18] the profile of k_0 is not discussed here. The K_S values are slightly dependent on pH. The larger the pH value, the smaller the K_S value (the higher the binding affinity of DDP to HSA). This may be related to the neutral–base (N–B) transition of HSA,[19–23] that is, the conformational change of HSA caused by the pH change.

Up to pH 7.0, the log k_2 values increase linearly with pH, giving a slope of about 1. At higher pH values the log k_2 values increase more slowly. This pH profile indicates the involvement of an ionizable catalytic group of HSA in the reaction. An estimation of pK_a from the region between pH 6 and 9 gave a value of about 7.5, suggesting the imidazole group of histidine residue to be the catalytic group towards DDP. The effect of ethoxycarbonylation of HSA by DEP on the reaction rate with DDP (see the next section) also supports the view that the catalytic group is imidazole. When the imidazole group is the catalytic group, the reaction with DDP may be represented as shown in Chart 3.[24] In Chart 3, K_a and K_a' are the

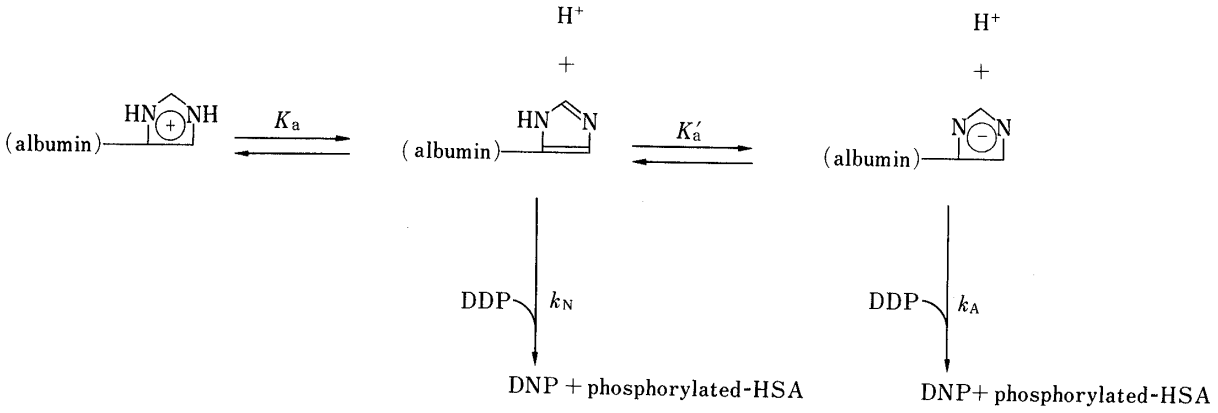


Chart 3

dissociation constants of cationic imidazole and the neutral imidazole group, respectively. The intrinsic rate constants based on the neutral and anionic imidazole groups are expressed by k_N and k_A, respectively. According to Chart 3, k_2 can be represented by Eq. 2.

$$k_2=\frac{k_N K_a[H^+]+k_A K_a K_a'}{[H^+]^2+K_a[H^+]+K_a K_a'} \quad (2)$$

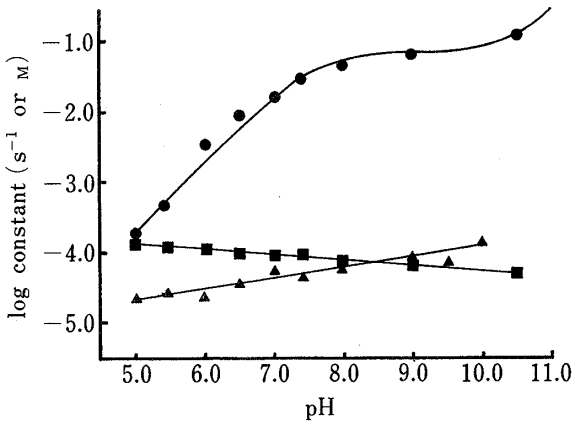


Fig. 2. The pH Profiles of k_2, k_0, and K_S for the Reaction of DDP with HSA at 25 °C

●, k_2; ▲, k_0; ■, K_S.
The solid curve for k_2 was simulated on the basis of Eq. 2 employing the parameters determined (see the text).

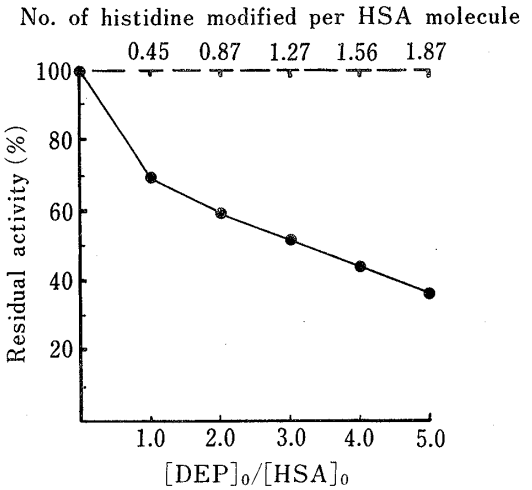


Fig. 3. Effects of Ethoxycarbonylation on the Reaction Rate of DDP with HSA

pH 7.4 phosphate buffer containing 0.5% (v/v) ethanol and 0.5% (v/v) dioxane and 25 °C; $[DDP]_0 = 1.00 \times 10^{-5}$ (M); $[HSA]_0 = 5.00 \times 10^{-5}$ (M); lower scale on abscissa, $[DEP]_0/[HSA]_0$; upper scale on abscissa, number of histidine residues modified per HSA molecule.

When K_a' is assumed to be 1×10^{-13} M,[24,25] the other parameters k_N, K_a, and k_A can be estimated respectively as $6.80 \times 10^{-2}\,s^{-1}$, 2.88×10^{-8} M ($pK_a = 7.54$), and $1.66 = 10\,s^{-1}$ from the pH profile of k_2 shown in Fig. 2. Although the estimation of the k_A value depended on the K_a' value employed for the calculation, the anionic imidazole moiety was about 240-fold more reactive than the neutral imidazole moiety. For the determination of a more reliable k_A value the k_2 value in a more alkaline region is necessary. Such an experiment could not be carried out, however, since HSA would be denatured above pH 11.[26]

Effects of Ethoxycarbonylation of HSA on the Reaction Rate with DDP

Since DEP has been used commonly as a histidine-modifying (ethoxycarbonylation) reagent for proteins,[15,27,28] we examined the effects of the ethoxycarbonylation on the reaction rate of DDP with HSA (Fig. 3). The abscissa shows the ratio of the initial concentration of DEP used to that of HSA (lower scale), and also the number of histidine residues modified per HSA molecule (upper scale). The residual activity on the ordinate was calculated by means of equation (3), where k^m_{obs} is the pseudo first-order rate constant for the reaction of DDP with the DEP-treated HSA as described in the experimental section.

$$\text{residual activity} = \frac{k_{obs} - k^m_{obs}}{k_{obs} - k_0} \times 100 \qquad (3)$$

When 1 and 2 mol of histidine residues per mol of HSA were modified by DEP, the residual activities were about 50% and 30%, respectively. These results support the view that the catalytic group towards DDP is imidazole of histidine residues in HSA. There should be more than two (three or more) reactive histidine residues towards DDP, because about 30% residual activity still exists after the modification of about 2 mol of histidines per mol of HSA.

Effects of Drug Binding on the Reaction Rate of DDP with HSA

To localize the reactive sites towards DDP, the effects of several drugs, whose binding sites on HSA are already known, on k_{obs} were examined. Figure 4 shows the results for phenylbutazone, oxyphenbutazone, and azapropazone, which bind primarily to the U site.[1,5,6,29,30] In this figure k'_{obs} on the ordinate is the rate constant in the presence of drug, and

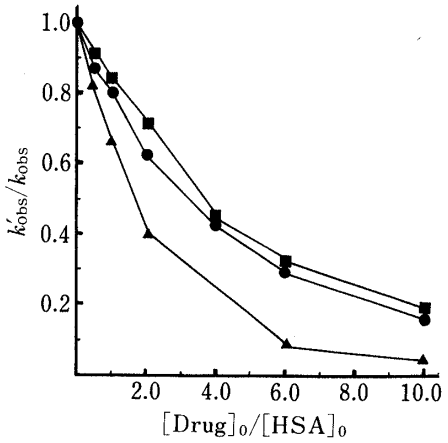


Fig. 4. Effects of Drugs on the Reaction Rate of DDP with HSA at pH 7.4 and 25 °C

$[DDP]_0 = 1.00 \times 10^{-5}$ (M); $[HSA]_0 = 5.00 \times 10^{-5}$ (M); ▲, phenylbutazone; ●, oxyphenbutazone; ■, azapropazone.

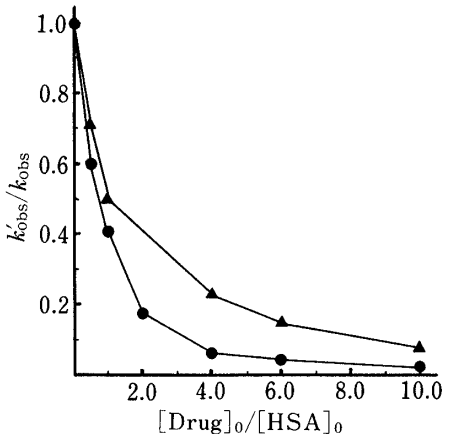


Fig. 5. Effects of Drugs on the Reaction Rate of DDP with HSA at pH 7.4 and 25 °C

$[DDP]_0 = 1.00 \times 10^{-5}$ (M); $[HSA]_0 = 5.00 \times 10^{-5}$ (M); ▲, clofibric acid; ●, flufenamic acid.

the concentrations with subscript 0 on the abscissa represent the initial concentrations of drug and HSA. When $[Drug]_0/[HSA]_0$ is unity, for example, the per cent of the inhibition caused by these drugs is about 20% to 30% (the value of k'_{obs}/k_{obs} in Fig. 4 is about 0.8 to 0.7). The binding constants of the drugs to HSA ($K=[Drug \cdot HSA]/([Drug][HSA])$ were reported to be $1 \times 10^5\ M^{-1}$ to $1 \times 10^6\ M^{-1}$ [31,32] The following estimation of unbound U site concentration ($[HSA_U]$) of HSA leads to the inference that the U site is the secondary (not primary) reactive site towards DDP. Assuming $K=1 \times 10^5\ M^{-1}$, the lowest affinity of a drug to the U site of HSA, and $[Drug]_0=[HSA_U]_0=5 \times 10^{-5}$ M,

$$K=[Drug \cdot HSA_U]/([Drug][HSA_U])$$
$$=(5 \times 10^{-5}-[HSA_U])/[HSA_U]^2=1 \times 10^{-5} \quad (4)$$

From Eq. 4 $[HSA_U]$, the free (non-inhibited) U site concentration, can be calculated as 1.79×10^{-5} M. If the U site is the primary reactive site, the remaining activity (k'_{obs}/k_{obs} in Fig. 4) at the $[Drug]_0$-to-$[HSA]_0$ ratio of unity should be 0.358 ($[HSA_U]/[HSA]_0=1.79 \times 10^{-5}/5 \times 10^{-5}=0.358$). However, the remaining activity in Fig. 4 was 0.7 to 0.8. The U site of HSA is, hence, concluded to be the secondary reactive site towards DDP and the primary reactive site may be located elsewhere.

Figure 5 shows the results for clofibric acid and flufenamic acid, which are classified as R type drug.[3,5,6,29-32] At the drug-to-HSA concentration ratio of 1 the reaction of DDP with HSA is inhibited about 50% to 60% ($k'_{obs}/k_{obs}=0.5$—0.4) by the drugs. The drug-unbound R site concentration calculated similarly to the case of the U site indicates that the primary reactive site towards DDP is the R site of HSA. The remaining activities may arise mainly from the secondary reactive site(s) including the U site and partly from the free (drug-unbound) R site.

There are 16 histidine residues in HSA.[6,33] Similarly to the case of bovine serum albumin, a three domain model for HSA has been considered, that is, Domains 1, 2, and 3, which consist of the amino acid sequences 1—198, 199—390, and 391—585, respectively.[6,33] Domains 1, 2, and 3 contain 7, 5, and 4 histidine residues, respectively. The R and U sites belong to Domains 3 and 2, respectively. It is, thus, reasonable that at least one histidine residue in the R and U sites (in Domains 3 and 2) participates in the reaction with DDP.

Recently, it has been shown that the N–B transition occurring around neutral pH affects

the drug binding on the specific binding sites of HSA,[19-23] and the transition is probably related to the histidine residues of HSA.[20,34] The difference in binding affinity between the N and B forms of HSA, however, seems to be small. For instance, in the case of the warfarin-binding site[30,35] (corresponding to the U site and Site I[29]), the B form of HSA binds warfarin three times more strongly than the N form,[19-21] while in the case of the diazepam-binding site[30,35] (corresponding to the R site and Site II[29]), the B from binds diazepam only 1.4 times more strongly than the N form.[22,23] As described in the section on the log K_S–pH profile, the binding affinity for DDP (reflected by the K_S value) was affected slightly by pH change, in agreement with the diazepam binding data in the literature.[22,23] On the other hand, the k_2 value at pH 9.0 is about 30-fold larger than that at pH 6.0, as shown in Fig. 2. In connection with the k_2 value, therefore, DDP may be a good probe for studying the N–B transition of the R site (diazepam site and Site II).

It is noticeable that in the R site of HSA there are two kinds of catalytic groups dependent upon the substrates used, that is, the imidazole group of the histidine residue is reactive towards DDP and the hydroxyl group of tyrosine-411 residue is reactive towards phenyl acetates[2] and cinnamoylimidazoles.[4] Further characterization of the R site seems to be required.

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is the reason why no experiment was carried out above pH 11.
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