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# Effects of Drug Binding on the Esterase-Like Activity of Human Serum Albumin. III.[1] Evaluation of Reactivities of the Two Active Sites by Using Clofibric Acid as an Inhibitor

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The reactivities of the two esterase-like active sites on human serum albumin (HSA) towards *p*-nitrophenyl acetate (NPA) were evaluated in pH 7.4 phosphate buffer ($\mu=0.2$) and at 25°C. Clofibric acid (CA) was used to distinguish between the two sites, the primarily reactive site (R site) being inhibited by CA and the secondarily reactive site (T site) not. The kinetic parameters for the two sites were determined. The dissociation constant between CA and the R site of HSA determined kinetically was consistent with the reciprocal of the binding constant obtained from the equilibrium dialysis experiment. The contribution of the T site to the overall activities of HSA was estimated from the specificity constants (the ratio of the catalytic rate constant to the dissociation constant) and was 11.1%.

**Keywords**——human serum albumin; esterase-like activity of human serum albumin; enzyme kinetics; protein binding; equilibrium dialysis; Scatchard plot; competitive inhibition; Clofibric acid; *p*-nitrophenyl acetate

Studies on drug binding to human serum albumin (HSA) are very important, since the pharmacological activity of a drug is affected by protein binding.[2] In the cource of studies on the effects of drug binding on the esterase-like activity of HSA,[1,3] it was found that the activity of HSA towards *p*-nitrophenyl acetate (NPA) was due to a primarily reactive site (R site) and a secondarily reactive site (T site). Both sites participated in drug bindings.[1] Although Means *et al.*[4–6] also reported that HSA has multiple reactive sites for NPA, the reactivities were not studied in detail. Therefore, it is of interest to distinguish between the above two reactive sites and to evaluate the individual reactivities. In the previous study,[1] it was suggested that clofibric acid (CA) could conveniently be used to distinguish between the two sites.

In the present study, equilibrium dialysis was carried out to determine the basic binding parameters of CA to HSA. Then, the reactivity of the T site in addition to the R site of HSA towards NPA was evaluated by using CA as an inhibitor. Furthermore, the contribution of the T site to the overall esterase-like activities of HSA was estimated.

## Experimental

**Materials**——HSA (Sigma Chem. Co., Fraction V, Lot 18C-0515) was used after purification by Chen's method.[7] The molecular weight of HSA was assumed to be 69000 and the concentration was determined by using an extinction coefficient $E^{0.1\%}_{1\,cm}$ of 0.531 at 278 nm.[4] CA was synthesized by the method of Jones *et al.*, mp 116—120°C; literature, mp 118—119°C.[8] All other chemicals used were obtained commercially. Visking® cellulose tubing (17/32) was used for the equilibrium dialysis. For all experiments, 1/15 M phosphate buffer of pH 7.4 ($\mu=0.2$, adjusted with NaCl) was used at 25°C.

**Equilibrium Dialysis**——Visking® cellulose tubing was used after pretreatment (boiling it for 30 minutes twice in distilled water and once in the phosphate buffer). The compartments for the dialysis consisted of 10 ml of HSA solution at one of three concentrations inside and 40 ml of a variety of CA concentrations ($1.70\times10^{-5}$ M—$1.02\times10^{-3}$ M) outside. After 15 hours under constant shaking at 25°C, the concentration of CA outside was determined spectrophotometrically at 228 nm.

**Kinetic Procedures**——The reaction of NPA ($1.00\times10^{-5}$ M) with HSA ($>4.00\times10^{-5}$ M) in the presence and absence of CA was followed spectrophotometrically by monitoring the appearance of the product, *p*-

nitrophenol (P), at 400 nm. Pseudo first-order analysis could be applied to the reaction, as described in the previous papers.[1,3,9] The pseudo first-order rate constant was calculated from the plot of log ($Ab_\infty$—Ab) against time, where $Ab_\infty$ and Ab are the absorbances at the completion of the reaction and at time $t$, respectively. A Hitachi UV-124 spectrophotometer equipped with a constant temperature (25°C) apparatus was used.

The deacylation rate of acetyl-HSA, based on the release of acetic acid, was followed by a pH-stat apparatus (Toa Dempa HS-2A). The deacylation was very slow compared with the acylation (the release of *p*-nitrophenol).

## Results and Discussion

### Binding of CA to HSA

Since it is known that the binding parameters for the interaction of a drug with HSA often vary with the experimental conditions, such as buffer components, ionic strength, source of HSA, *etc.*, equilibrium dialysis was carried out to obtain the binding parameters for CA to HSA under the conditions used in the kinetic study. Figure 1 shows the Scatchard plot for the binding of CA to HSA, where $\bar{r}$ represents moles of bound CA per mole of HSA and $C_f$ is the free concentration of CA. The curvature of the plot indicates the presence of more than one class of binding sites. When two classes of binding sites on HSA for CA were assumed to be present,[10] the following binding parameters were obtained: $n_1=0.861$, $K_1=1.73\times10^5$ ($M^{-1}$), $n_2=5.86$ and $K_2=1.57\times10^3$ ($M^{-1}$), where $n$ and $K$ denoted the number of the binding sites and the association constant, respectively. Subscripts 1 and 2 indicated the high and low affinity binding sites, respectively. The number of high affinity sites ($n_1$) appears to be near unity. The high affinity binding site has an approximately one hundered times larger binding constant than the low affinity sites. These binding parameters of CA to HSA are not very different from those reported in the literature.[11]

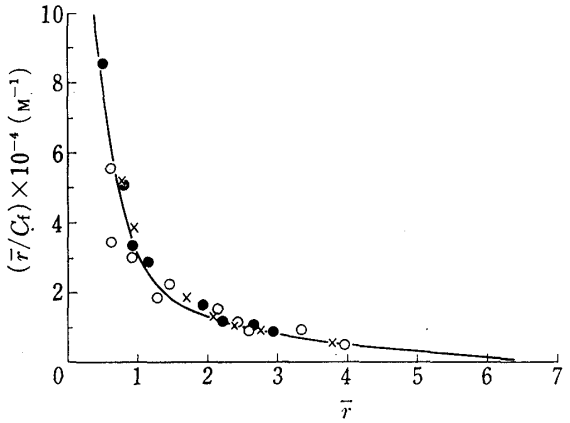


Fig. 1. Scatchard Plot for the Binding of CA to HSA

$[HSA]_0$: ○, $5.63\times10^{-5}$ (M); ●, $1.03\times10^{-4}$ (M); ×, $2.06\times10^{-4}$ (M).
The solid curve was calculated by using the values: $n_1=0.861$, $K_1=1.73\times10^5$ ($M^{-1}$), $n_2=5.86$ and $K_2=1.57\times10^3$ ($M^{-1}$).

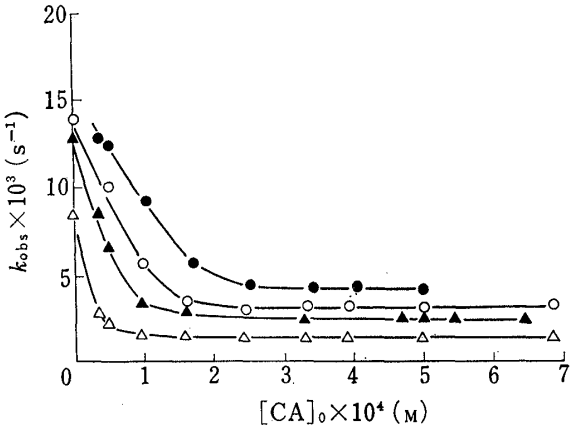


Fig. 2. Effect of CA Concentration on the Apparent First-Order Rate Constant for the Reaction of NPA with HSA at a Variety of Concentrations

$[HSA]_0$: ●, $1.99\times10^{-4}$ (M); ○, $1.49\times10^{-4}$ (M); ▲, $1.01\times10^{-4}$ (M); △, $4.95\times10^{-5}$ (M).
$[NPA]_0=1.00\times10^{-5}$ (M).

### Inhibition of the Esterase-Like Activity of HSA by CA and Reaction Scheme

Figure 2 shows the effect of CA concentration on the apparent first-order rate constant ($k_{obs}$) for the reaction of NPA with HSA at various concentrations. Strong inhibition is observed at low concentrations of CA. CA approximately equimolar with HSA (for example, $1.0\times10^{-4}$ M) inhibits the reaction about 73% (inferred from the ratio ($r\fallingdotseq0.27$) of $k_{obs}$ in the presence of CA to that in its absence: $(1-r)\times100\fallingdotseq73\%$). This strong inhibition is due to binding ($K_1$) of CA to the high affinity site of HSA in Fig. 1, because the percent of CA bound

to the high affinity site is 79%, as calculated from the $K_1$ value for the equimolar solution ($1.0\times10^{-4}$ M) of CA and HSA. The slightly smaller percent (73%) of inhibition compared with that (79%) of binding may be due to the approximate calculation neglecting the contribution of the secondary activity to the overall activities (see a later section). Therefore, it may rationally be concluded that the high affinity site for CA is identical with the primarily reactive site (R site) for NPA.

In the previous paper,[1] it was reported that the R site was located near Tyr-411 in Brown's HSA sequence and corresponded to the Site II of the HSA proposed by Sudlow *et al.*[12] Furthermore, the remaining reactivity at high concentrations of CA was found to be due to the secondarily reactive site (T site), rather than a result of non-competitive inhibition or mixed-type inhibition by CA. This was inferred from the results on the reactivity with diisopropylfluorophosphate and on the inhibition by other drugs such as flufenamic acid and ethacrynic acid.[1]

The remaining reactivities at high concentrations of CA depend on the concentrations of HSA employed. The $k_{obs}$ values at high CA concentrations (*e.g.* $5.00\times10^{-4}$ M) are not proportional to the HSA concentrations used, but increase hyperbolically with the HSA concentrations. This dependence of $k_{obs}$ on the concentration of HSA suggests that the reaction of NPA with the T site proceeds *via* Michaelis-Menten type complex formation.

$$\mathrm{NPA} + \mathrm{HSA_R} \overset{K_{S,R}}{\rightleftharpoons} \mathrm{NPA\cdot HSA_R} \xrightarrow{k_{2,R}} \mathrm{P_R} + \mathrm{Acetyl\text{-}HSA_R}$$

$$\mathrm{CA} + \mathrm{HSA_R} \overset{K_{I,R}}{\rightleftharpoons} \mathrm{CA\cdot HSA_R}$$

$$\mathrm{NPA} + \mathrm{HSA_T} \overset{K_{S,T}}{\rightleftharpoons} \mathrm{NPA\cdot HSA_T} \xrightarrow{k_{2,T}} \mathrm{P_T} + \mathrm{Acetyl\text{-}HSA_T}$$

$$\mathrm{NPA} \xrightarrow{k_0} \mathrm{P_S} + \mathrm{Acid}$$

Chart 1

TABLE I. Glossary for Chart 1

| Abbreaviation | Explanation |
|---|---|
| $HSA_R$ | R site ot HSA |
| $HSA_T$ | T site of HSA |
| $NPA\cdot HSA_R$ | Complex between NPA and R site |
| $NPA\cdot HSA_T$ | Complex between NPA and T site |
| $CA\cdot HSA_R$ | Complex between CA and R site |
| Acetyl-$HSA_R$ | R site acetylated with NPA |
| Acetyl-$HSA_T$ | T site acetylated with NPA |
| Acid | Acetic acid |
| $P_R$ | Product of the reaction of $NPA\cdot HSA_R$, *p*-nitrophenol |
| $P_T$ | Product of the reaction of $NPA\cdot HSA_T$, *p*-nitrophenol |
| $P_S$ | Product of the spontaneous reaction of NPA, *p*-nitrophenol |
| $K_{S,R}$ | Dissociation constant of $NPA\cdot HSA_R$, $[NPA]\cdot[HSA_R]/[NPA\cdot HSA_R]$ |
| $K_{S,T}$ | Dissociation constant of $NPA\cdot HSA_T$, $[NPA]\cdot[HSA_T]/[NPA\cdot HSA_T]$ |
| $K_{I,R}$ | Dissociation constant of $CA\cdot HSA_R$, $[CA]\cdot[HSA_R]/[CA\cdot HSA_R]=1/K_1$ |
| $k_{2,R}$ | 1st order rate constant of $NPA\cdot HSA_R$ |
| $k_{2,T}$ | 1st order rate constant of $NPA\cdot HSA_T$ |
| $k_0$ | 1st order rate constant of NPA, hydrolysis rate constant |

When the R and T sites are assumed to be independent of each other, the entire reactions including the inhibition of the esterase-like activities by CA may be represented as shown in Chart 1. The abbreviations of the reaction species and the kinetic parameters used in Chart 1 are explained in Table I. The above assumption implies that one HSA molecule

acts on NPA (substrate) and CA (inhibitor) as if it were different molecules as regards the reactive sites.

**Determination of the Individual Kinetic Parameters**

According to Chart 1, the apparent first-order rate constant ($k^0_{obs}$) of product ($P=P_R+P_T+P_S$) formation in the absence of CA can be represented as follows:

$$k^0_{obs} = \frac{(k_{2,R}K_{S,T}+k_{2,T}K_{S,R})[HSA]_0}{K_{S,T}K_{S,R}+(K_{S,T}+K_{S,R})[HSA]_0} \qquad \text{(Eq. 1)}$$

where the term $k_0$ is neglected, because $k_0$ is very small compared with $k_{2,R}$ and $k_{2,T}$. In Eq. 1 the initial concentration of HSA, $[HSA]_0$, is used instead of the free concentration of HSA, since an excess of HSA compared with NPA was employed in the present study.[1,3] Inversion of Eq. 1 gives Eq. 2.

$$\frac{1}{k^0_{obs}} = \frac{K_{S,T}K_{S,R}}{(k_{2,R}K_{S,T}+k_{2,T}K_{S,R})} \cdot \frac{1}{[HSA]_0} + \frac{K_{S,T}+K_{S,R}}{(k_{2,R}K_{S,T}+k_{2,T}K_{S,R})} \qquad \text{(Eq. 2)}$$

In the presence of CA, on the other hand, the rate constant ($k_{obs}$) of the reaction is expressed by the following equation.

$$k_{obs} = \frac{\left\{k_{2,R}K_{S,T}+k_{2,T}K_{S,R}\left(1+\frac{[CA]}{K_{I,R}}\right)\right\}[HSA]_0}{K_{S,T}K_{S,R}\left(1+\frac{[CA]}{K_{I,R}}\right)+\left\{K_{S,T}+K_{S,R}\left(1+\frac{[CA]}{K_{I,R}}\right)\right\}[HSA]_0} \qquad \text{(Eq. 3)}$$

In the presence of excess CA in terms of $[HSA]_0$ and $K_{I,R}$, Eq. 3 can be transformed as follows:

$$k_{obs} = \frac{k_{2,R}K_{S,T}K_{I,R}[HSA]_0}{K_{S,R}(K_{S,T}+[HSA]_0)} \cdot \frac{1}{[CA]_0} + \frac{k_{2,T}[HSA]_0}{K_{S,T}+[HSA]_0} \qquad \text{(Eq. 4)}$$

The second term of the right-hand side in Eq. 4 is considered as the contribution of the T site alone to the esterase-like activities of HSA. This term is denoted by $k^T_{obs}$, that is,

$$k^T_{obs} = \frac{k_{2,T}[HSA]_0}{K_{S,T}+[HSA]_0} \qquad \text{(Eq. 5)}$$

The reciprocal form of Eq. 5 is as follows:

$$\frac{1}{k^T_{obs}} = \frac{K_{S,T}}{k_{2,T}} \cdot \frac{1}{[HSA]_0} + \frac{1}{k_{2,T}} \qquad \text{(Eq. 6)}$$

The individual kinetic parameters for the reactions of NPA with HSA can be determined as follows. First, the values of $k^T_{obs}$ at different concentrations of HSA are obtained from the intercepts of plots based on Eq. 4, as shown in Fig. 3. Secondly, $K_{S,T}$ and $k_{2,T}$ are calculated from the slope and intercept of the plot according to Eq. 6 (Fig. 4). Finally, the values of $K_{S,R}$ and $k_{2,R}$ are calculated from the slope and intercept of the plot based on Eq. 2 (Fig. 4) by substituting the $K_{S,T}$ and $k_{2,T}$ values already determined. Moreover, once the kinetic parameters for the reactions of NPA with HSA are determined, $K_{I,R}$ values can also be calculated from the slopes of plots according to Eq. 4 (Fig. 3) by substitution of the kinetic parameters ($k_{2,R}$, $K_{S,R}$ and $K_{S,T}$) into the slopes.

Table II lists the individual kinetic parameters and the $K_{I,R}$ value thus obtained. The $K_{I,R}$ value in Table II is the average value of $K_{I,R}$ estimated from the slopes of several lines as shown in Fig. 3. Because $K_{I,R}$ should be equal to the reciprocal of $K_1$ in Fig. 1, the $K_{I,R}$ value was compared with the $1/K_1$ value ($5.78\times10^{-6}$ M). The ratio of $K_{I,R}$ to $1/K_1$ was 0.804. Since the two values, $K_{I,R}$ and $K_1$, were originally obtained by completely different methods (kinetic and equilibrium dialysis methods), this slight discrepancy may be inevitable. This small difference between $K_{I,R}$ and $1/K_1$, therefore, supports the validity of the theory and the analytical method for the determination of the kinetic parameters in this study.

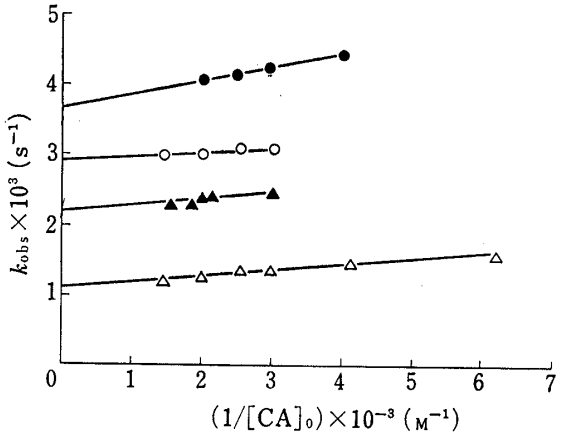


Fig. 3. Plots of $k_{obs}$ against $1/[CA]_0$ for the Determination of $k^{T}_{obs}$

$[HSA]_0$: ●, $1.99\times10^{-4}$ (M); ○, $1.49\times10^{-4}$ (M); ▲, $1.01\times10^{-4}$ (M); △, $4.95\times10^{-5}$ (M). $[NPA]_0=1.00\times10^{-5}$ (M).

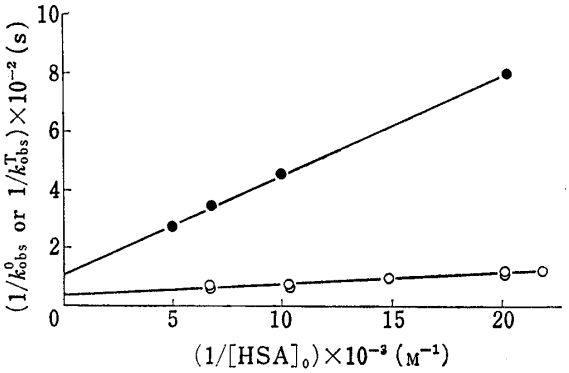


Fig. 4. Plots of $1/k^{0}_{obs}$ and $1/k^{T}_{obs}$ against $1/[HSA]_0$ for the Determination of the Kinetic Parameters of the Reactions of NPA with HSA

○, for $k^{s}_{obs}$; ●, for $k^{T}_{obs}$

Table II. Kinetic Parameters in Chart 1 and Specificity Constants for R and T Sites

| Kinetic parameter | Value |
|---|---|
| $k_{2,R}$ ($s^{-1}$) | $3.14\times10^{-2}$ |
| $k_{2,T}$ ($s^{-1}$) | $9.07\times10^{-3}$ |
| $k_0$ ($s^{-1}$) | $1.67\times10^{-5}$ |
| $K_{S,R}$ (M) | $1.33\times10^{-4}$ |
| $K_{S,T}$ (M) | $3.09\times10^{-4}$ |
| $K_{I,R}$ (M) | $4.65\times10^{-6}$ |
| $k_R=k_{2,R}/K_{S,R}$ ($M^{-1}$ $s^{-1}$) | $2.36\times10^{2}$ |
| $k_T=k_{2,T}/K_{S,T}$($M^{-1}$ $s^{-1}$) | $2.94\times10$ |
| $100\ k_T/(k_R+k_T)$ (%) | 11.1 |

## Contribution of the T Site to the Overall Esterase-Like Activities of HSA

For a simple expression of the reactivity of each site the ratios of the rate constants ($k_{2,R}$ and $k_{2,T}$) to the dissociation constants ($K_{S,R}$ and $K_{S,T}$) may be convenient, because the larger the rate constant and the smaller the dissociation constant, the faster is the reaction. These ratios are expressed by $k_R$ and $k_T$ for the R and T sites, respectively, and are listed in Table II. The ratio in this study seems to be equivalent to the specificity constant proposed by Brot and Bender,[13] since the deacylation rate of acetyl-HSA is very small compared with the acylation rates (see "Experimental"). The contribution of the T site to the overall esterase-like activities of HSA may be expressed as $100\ k_T/(k_R+k_T)$, which is also included in Table II. This value (11.1%) reflects the remaining reactivity in inhibition curves obtained from plots of $k_{obs}/k^{0}_{obs}$ against $[CA]_0/[HSA]_0$ based on the data in Fig. 2.

Since many drugs bind to different sites on HSA in complicated ways,[1,12] it is difficult to determine the precise binding constants between the drug and the different sites by the conventional kinetic method. HSA carries two reactive sites for NPA, one of which is simultaneously the high affinity binding site for CA. From the analysis of the reaction system described above, we could determine the individual kinetic parameters for NPA as listed in Table II. Using these parameters, calculation of the binding constants between the multiple sites on HSA and a variety of drugs by means of analog computer simulation is now in progress.

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