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In order to investigate the reactivity of human serum albumin (HSA) with ester-type drugs and to characterize the site of the esterase-like activity, the Michaelis constants (Ks) and the catalytic rate constants (kcat) were determined for the reactions of phenyl acetates and p-nitrophenyl esters with HSA at 25°. A linear relationship between log kcat and Hammett σ− values was found for phenyl acetates at pH 9.9; its slope was +1.52. It is suggested that aspirin also reacts with HSA by the same mechanism. The effects of aromatic substituents on the Ks values were small. The Ks values for p-nitrophenyl esters at pH 7.0 were correlated with Hansch's π and Taft's Eπ values as follows: log Ks = −0.578 π − 0.184 Eπ − 3.566 (r = 0.963). The hydrophobic interaction was predominant in the binding of the substrates to HSA. The log kcat–pH profile obtained for p-nitrophenyl acetate indicates the participation of a single catalytic group, pKα = 9.5, in this reaction.

Keywords—human serum albumin; esterase-like activity of human serum albumin; structure-activity relationship; regression analysis; hydrophobic interaction; reactive site of human serum albumin; enzyme kinetics; reaction of aspirin with human serum albumin; phenyl acetates; p-nitrophenyl esters

In the previous paper it was reported that the reaction rate of p-nitrophenyl acetate (6) with human serum albumin (HSA) decreased in the presence of drugs such as N-arylanthranilic acids, coumarin derivatives and prostaglandins, and these inhibitions were analyzed kinetically. The kinetic method is useful for studies on drug-HSA interactions, since information relating to the drug binding sites and to the binding affinity of the drugs for the reactive site of HSA can be obtained. Recently, Koh and Means have studied the interaction of small fatty acid anions with HSA by a similar kinetic method, and suggested that the anion binding site appeared to be a relatively small, uniform apolar cavity with one or more cationic groups located near one end. It is important not only to characterize the drug binding site of HSA but also to investigate the reactivity of HSA with the esters, since HSA may affect the cleavage of ester-type drugs in vivo.

To elucidate the reactivity of HSA with the ester drugs and to characterize the reactive site, the reactions of phenyl acetates and p-nitrophenyl esters with HSA were investigated kinetically in this study. The Michaelis constants and catalytic rate constants for the reactions were determined. The relationships between the structure of the substrates and the reactivity of HSA were examined.

Experimental

Materials—HSA (Sigma Chem. Co., Fraction V, lot 47c-04421; the same lot number as that employed in the previous study) was used after purification by Chen's method. The molecular weight of HSA was

2) Location: Tanabe-dori, Mizuho-ku, Nagoya, 467, Japan.
Table I. Substrates Used, and Melting or Boiling Points of Substrates synthesized by the Method of Spasov

<table>
<thead>
<tr>
<th>Substrates</th>
<th>mp or bp (°C)</th>
<th>Literature values</th>
<th>mp or bp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(°C/mmHg)</td>
<td></td>
<td>(°C/mmHg)</td>
</tr>
<tr>
<td>1 aspirin</td>
<td>32–33</td>
<td>31–32b</td>
<td></td>
</tr>
<tr>
<td>2 p-methoxyphenyl acetate</td>
<td>76–80/4</td>
<td>108/12.52a</td>
<td></td>
</tr>
<tr>
<td>3 p-chlorophenyl acetate</td>
<td>72–73/2</td>
<td>116.5/21b</td>
<td></td>
</tr>
<tr>
<td>4 m-chlorophenyl acetate</td>
<td>53–55</td>
<td>55–58b</td>
<td></td>
</tr>
<tr>
<td>5 m-nitrophenyl acetate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 p-nitrophenyl butyrate</td>
<td>37–38</td>
<td>36.5–3711</td>
<td></td>
</tr>
<tr>
<td>7 p-nitrophenyl propionate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 p-nitrophenyl valerate</td>
<td>123/1.5</td>
<td>158–160/611</td>
<td></td>
</tr>
<tr>
<td>9 p-nitrophenyl capronate</td>
<td>93–95</td>
<td>94–9511</td>
<td></td>
</tr>
<tr>
<td>11 p-nitrophenyl iso-butyrate</td>
<td>93–95</td>
<td>94–9511</td>
<td></td>
</tr>
</tbody>
</table>

The procedures for kinetic runs—The reaction of the substrate with HSA was carried out in the presence of excess HSA to avoid complications due to the multiple reactive sites of HSA,9 as described in the previous paper. Sörensen buffer (1/15 m phosphate for pH 6.0 to 8.0 and 1/20 m borate for pH 8.0 to 10.5) was used to investigate the pH profiles of the reaction parameters of 6 with HSA. The ionic strength of each buffer was not fixed, because the addition of NaCl to the buffer increased the reaction rates in a complicated manner. For the fast reaction of 6 with HSA in the alkaline region, a stopped flow apparatus (Union Giken RA-1100) was employed. In the neutral region, a Hitachi 124 UV spectrophotometer equipped with a thermostated cell was used. The initial concentration of 6 was about 2.0×10^{-5} M and the HSA concentration was varied from about 7.0×10^{-4} to 4.0×10^{-4} M.

The reactions of phenyl acetates with HSA were carried out in 2/5 m carbonate buffer, pH 9.9, since the phenolate anions released had larger molar absorptivities (e) than the free phenols. The initial concentrations of phenyl acetates were about 1.0×10^{-4} M, depending on the e values of the phenolate anions. The initial HSA concentrations corresponded to a three to six-fold excess relative to those of the substrates.

The reactions of p-nitrophenyl esters with HSA were followed in pH 7.0 phosphate buffer. The initial concentrations of the substrates were about 3.0×10^{-4} M and those of HSA were from 1.5×10^{-5} to 1.0×10^{-4} M.

Determination of Reaction Parameters—On the basis of the study by Means and Bender9 and our previous results, the reactions of the substrates with HSA were assumed to proceed through the pathway

\[
\text{EST} + \text{HSA} \rightleftharpoons K_S \text{EST-HSA} \xrightarrow{h_0} \text{phenol} + \text{Acyl-HSA} \xrightarrow{\text{Acyl}} \text{acid} + \text{HSA}
\]

\[\text{Chart 1}\]

7) A. Spasov, Chem. Abstr., 34, 2343 (1940).
9) a) Beilstein’s Handbuch, Hauptwerk, Bd. VI, p. 187; b) ibid., p. 185.
shown in Chart 1. In this chart, EST is the substrate and EST-HSA is the Michaelis-Menten type complex between EST and HSA. Acyl-HSA is HSA acylated by the substrate. $K_d$ denotes the dissociation constant of the complex. The rate constants of EST-HSA and EST are represented by $k_{cat}$ and $k_o$, respectively. $k_{dest}$ is the rate constant of Acyl-HSA.

To determine the effect of $k_{dest}$ on the apparent rate of phenol release, acetic acid formation rates in the reaction of 6 with HSA at pH 7.0 and 10.0 were preliminarily measured by means of a pH-stat apparatus (Toa-Dempa HS-20A). The rates of acetic acid formation were very small compared with those of the p-nitrophenol release. Thus, the influence of the decylation rate on the observed rate of phenol appearance could be neglected under the experimental conditions employed.

The pseudo first order rate constant of phenol release, $k_{obs}$, can be represented as follows.

$$k_{obs} = \frac{k_a K_S + k_{cat}[HSA]}{K_S + [HSA]}$$  \hspace{1cm} (1)

Equation (1) can be rearranged to give the following equation (2), which is the same as that in the previous report.\(^3\)

$$\frac{1}{k_{obs} - k_o} = \frac{K_S}{(k_{cat} - k_o)[HSA]} + \frac{1}{k_{cat} - k_o}$$  \hspace{1cm} (2)

Here, the concentration of HSA in equation (1) is approximated to $[HSA]_o$, the initial concentration of HSA, because the concentration of HSA is much higher than that of the substrate. A linear relationship between $1/(k_{obs} - k_o)$ and $1/[HSA]_o$ was obtained in all cases. The $K_S$ value was obtained from the slope divided by the intercept, and the value of $k_{cat}$ was calculated from the intercept and $k_o$.

**Results and Discussion**

**The pH Profiles of the Reaction Parameters of p-Nitrophenyl Acetate with HSA**

Means and Bender presented a pH-rate profile for the reaction of 6 with HSA and suggested the participation of a basic group with a $pK_a$ value near 8.7 in the reaction.\(^6\) The second-order rate constants in their report, however, seem to be values of $k_{cat}/K_S$ (m$^{-1}$ sec$^{-1}$). In contrast, the pH dependencies of the individual kinetic parameters, i.e. $K_S$ and $k_{cat}$, were separately examined in the present work.

Figure 1 shows the pH profiles of $K_S$ and $k_{cat}$ for the reaction of 6 with HSA. The $K_S$ values are slightly dependent on pH over the range from 6 to 10. This indicates that the binding is little affected by pH changes. The value of $K_S$ at pH about 10.5 was larger than that in the neutral region. This large $K_S$ value may be related to the effect of an ionic group(s) near the reactive site of HSA on the binding, or to the slight conformation change of HSA above pH 10.\(^12\)

The values of $k_{cat}$ were markedly dependent on pH. The slope of the log $k_{cat}$-pH profile between pH 6 and 9 was about unity, and over pH 9 a plateau gradually appeared with increasing pH. This profile suggests the involvement of a single catalytic group in HSA for the reaction, as in the mechanism proposed by Means and Bender.\(^6\) The $pK_a$ value of the catalytic group appears to be around 9.5, which is rather different from the value, 8.7, presented by Means and Bender. This discrepancy in the $pK_a$ values might arise from the different pH profiles, that is, log ($k_{cat}/K_S$) versus pH and log $k_{cat}$ versus pH.

**Structure-Activity Relationships for the Reaction of Phenyl Acetates with HSA**

The rates and dissociation constants for the reactions of phenyl acetates with HSA are listed in Table II. Table II also shows the spontaneous rate constants ($k_o$) of the substrates, the $pK_a$ values of the corresponding phenols\(^13\) and the Hammett $\sigma$ values.\(^14\)

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Figure 2 shows the relationships between the rate constants and the Hammett $\sigma^-$ values. For both $k_{\text{cat}}$ and $k_0$ values, good linear relationships were obtained. A plot of log $k_{\text{cat}}$ versus $\sigma^-$ gave a slope of +1.52. The positive value of the slope suggests that the reaction of the substrate with HSA proceeds through nucleophilic attack of the catalytic group of HSA to the carbonyl carbon atom of the substrate. The finding that the slope is steeper than that for the spontaneous reaction ($k_0$) indicates that the reaction occurs in a nonpolar region of HSA. The slope of the log $k_0$ versus $\sigma^-$ plots was +0.82, for comparison.

To correlate the result for 1 with those for the other substrates in Table II, the rate constants were plotted against the p$K_a$ values of the corresponding phenols. Here again, there were good linear relationships, as shown in Fig. 3. The correlation implies that the same reactive site of HSA participates in the reaction for all the substrates including 1, since an approximately linear relationship between the p$K_a$ values of the phenols and Hammett $\sigma^-$ values was found.\(^{15}\)

Hawkins and his co-workers\(^{16}\) reported that the lysine residue in HSA was acetylated by aspirin in vivo and in vitro, although kinetic studies were not carried out. The position of this residue was shown to be lysine-199 according to Brown’s completed sequence of

HSA. As shown in Fig. 3, aspirin reacted with HSA by the same mechanism as with other substrates, so it is possible that the catalytic group is the free (unprotonated) ε-amino group of lysine-199 for the substrates 1—6. However, the reaction rates of 6 with HSA acetylated at pH 7.3 and 10.0 according to the method of Hawkins et al. did not significantly alter. This result appears to rule out the lysine residue. Another possible candidate is the hydroxyl group of tyrosine as proposed by Tildon and Ogilvie for the reaction of 6 with bovine mercaptalbumin. Identification of the reactive group of HSA for these substrates is in progress.

Figure 4 illustrates the relationship between log $K_s$ and $pK_a$ of the corresponding phenol. A slight positive correlation between log $K_s$ and $pK_a$ was observed; the deviation of aspirin from the line may be attributed to the carboxyl group at the ortho-position. Thus, the difference between the log $K_s$ values for 2 and 6, i.e. the maximum and the minimum, respectively, in Table II except for aspirin, was only 0.242 log unit. Accordingly, the effects of aromatic substituents on substrate binding to HSA appears to be small.

<table>
<thead>
<tr>
<th>Substrates $^{(b)}$</th>
<th>$K_s$ (M)</th>
<th>$k_{rel}$ (sec$^{-1}$)</th>
<th>$c$</th>
<th>$E_s$</th>
<th>$a$ $e$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>$1.55 \times 10^{-4}$</td>
<td>$2.93 \times 10^{-3}$</td>
<td>0.50</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>7</td>
<td>$8.69 \times 10^{-5}$</td>
<td>$7.15 \times 10^{-2}$</td>
<td>1.00</td>
<td>$-0.07$</td>
<td>$-0.10$</td>
</tr>
<tr>
<td>8</td>
<td>$4.29 \times 10^{-5}$</td>
<td>$1.02 \times 10^{-2}$</td>
<td>1.50</td>
<td>$-0.36$</td>
<td>$-0.12$</td>
</tr>
<tr>
<td>9</td>
<td>$2.57 \times 10^{-5}$</td>
<td>$2.58 \times 10^{-2}$</td>
<td>2.00</td>
<td>$-0.39$</td>
<td>$-0.13$</td>
</tr>
<tr>
<td>10</td>
<td>$1.11 \times 10^{-5}$</td>
<td>$2.22 \times 10^{-2}$</td>
<td>2.50</td>
<td>$-0.40$</td>
<td>$-0.16$</td>
</tr>
<tr>
<td>11</td>
<td>$3.59 \times 10^{-5}$</td>
<td>$1.08 \times 10^{-2}$</td>
<td>1.30</td>
<td>$-0.47$</td>
<td>$-0.19$</td>
</tr>
<tr>
<td>12</td>
<td>$3.85 \times 10^{-5}$</td>
<td>$1.47 \times 10^{-2}$</td>
<td>1.80</td>
<td>$-0.93$</td>
<td>$-0.13$</td>
</tr>
<tr>
<td>13</td>
<td>$4.11 \times 10^{-5}$</td>
<td>$6.42 \times 10^{-4}$</td>
<td>1.98</td>
<td>$-1.54$</td>
<td>$-0.30$</td>
</tr>
</tbody>
</table>

$^{(a)}$ Obtained at pH 7.0 and 37. $^{(b)}$ Numbers refer to those in Table I. $^{(c)}$ Hansch’s hydrophobic substituent constant.$^{19,20}$ $^{(d)}$ Taft’s steric substituent constant.$^{19,20}$ $^{(e)}$ Taft’s polar substituent constant.$^{19,20}$

Structure-Activity Relationships for Reactions of p-Nitrophenyl Esters with HSA

Table III lists the kinetic parameters for the reactions of p-nitrophenyl esters with HSA. The dissociation and rate constants varied widely. Regression analyses in studies of enzymic reactions, for example a-chymotrypsin, using substituent constants such as Hansch's $\pi$-value\(^{20}\) and Taft's $E_g$ and $\sigma^*$-values,\(^{21}\) have been carried out by Hansch and his co-workers,\(^{22,23}\) and Milstien and Fife.\(^{24}\) The values of $\pi$, $E_g$ and $\sigma^*$ were used as measures of hydrophobicity, steric effect and polarity of the substrate, respectively; these are listed in Table III for convenience. Similar regression analyses were attempted for the $K_b$ and $k_{cat}$ values listed in Table III.

The plot of $\log K_b$ against $\pi$-value is shown in Fig. 5. A fairly good relationship was obtained and its regression line can be expressed as

$$\log K_b = -0.501(\pm 0.189)\pi - 3.591(\pm 0.318)$$

$$n = 8, \quad s = 0.130, \quad r = 0.935$$

where the values in parentheses are the 95\% confidence intervals; $n$, $s$ and $r$ represent the number of compounds employed, the standard deviation and the correlation coefficient, respectively. Since the points of 11, 12 and 13 (which have side chains) departed slightly from the line, a steric factor $E_g$ was added to the analysis. The results obtained by the least-squares method can be summarized as

$$\log K_b = -0.578(\pm 0.196)\pi - 0.184(\pm 0.250)E_g - 3.566(\pm 0.281)$$

$$n = 8, \quad s = 0.108, \quad r = 0.963$$

Figure 5 also shows a plot of $(\log K_b + 0.184 E_g)$ versus $\pi$. Although the $E_g$ term in equation (4) was not statistically significant at the 0.05 level of significance, the points for 12 and 13 appear to be better fitted than in the case of equation (3). The effects of aromatic substituents on the $K_b$ values were small (Fig. 4), while the binding of $p$-nitrophenyl esters to the reactive site of HSA was markedly affected by substituents at the acyl portion. These results suggest that the alkyl chains of the acyl group are mainly bound to the HSA reactive site by hydrophobic forces.

We further attempted to correlate the values of $k_{cat}$ in Table III with various factors such as Taft's $\sigma^*$ and $E_g$ values, etc., but without success. Thus, other factors, so far unidentified, may affect the $k_{cat}$ values in the reactions of $p$-nitrophenyl esters with HSA.

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