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Esterase-Like Activity of Human Serum Albumin II: Reaction with *N-trans*-Cinnamoylimidazoles

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Abstract □ To elucidate the details of the esterase activity of human serum albumin, the reaction of *N-trans*-cinnamoylimidazoles with albumin was investigated kinetically at various pHs at 25°. The reaction consisted of the acylation of albumin (probably the tyrosine-411 residue) by the substrate and the deacylation of cinnamoyl-albumin. The acylation was ~10–100-fold faster than the spontaneous hydrolysis of the substrate over the pH range examined. The pH profile for the deacylation rate constant indicated the participation of a group having a pK_a of ~9.4. The deacylation was subjected to the effect of deuterium oxide. The electron-withdrawing substituent facilitated the deacylation; the Hammett ρ value was 1.63. These results suggest that the deacylation proceeded *via* general base catalysis by this group.

Keyphrases □ Albumin, human serum—esterase-like activity, acylation with *N-trans*-cinnamoylimidazoles, kinetics □ Cinnamoylimidazoles—acylation of albumin, kinetics □ Kinetics—acylation of albumin with *N-trans*-cinnamoylimidazoles at the tyrosine-411 residue binding site

Studies involving the binding of drugs to human serum albumin are pharmacologically and clinically important, since this binding influences the *in vivo* distribution, availability, and elimination of drugs (1). Localization of drug binding sites on the albumin molecule and the classification of drugs with respect to the binding sites can be used to predict the displacement of one drug by another when two or more drugs are administered concurrently (2). It was reported previously that albumin exhibits esterase activity toward phenyl esters (3, 4). The active site involved when the substrate is *p*-nitrophenyl acetate was found to be one of the most important drug binding sites on albumin, and was named the R site (5–7). The R site is located near the reactive tyrosine-411 residue (5, 8–12). Previous studies (5–7) examined the inhibition of the esterase activity by several drugs, and led to the classification and identification of the various binding sites involved.

The details of the esterase activity of albumin have not been described. The activity of albumin toward amide substrates as well as the mechanisim of the deacylation of the acyl-albumin are not known. Since many drugs possess the amide linkage, we have investigated the activity of

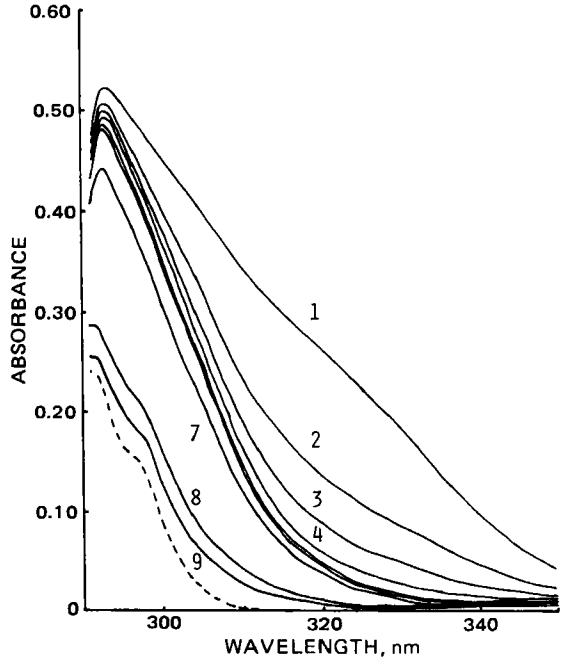

Figure 1—*Periodic difference spectrum of the reaction mixture (albumin + I)* versus *albumin at pH 8.41 and 25°. The dotted line is the spectrum of the mixture of* trans-*cinnamic acid and imidazole. Key: (1) 1.0, (2) 3.0, (3) 5.0, (4) 9.0, (5) 30, (6) 60, (7) 240, (8) 1440, and (9) 2580 min. The initial concentrations of I and albumin are 4.00 × 10^{-5} M and 2.00 × 10^{-4} M, respectively.*

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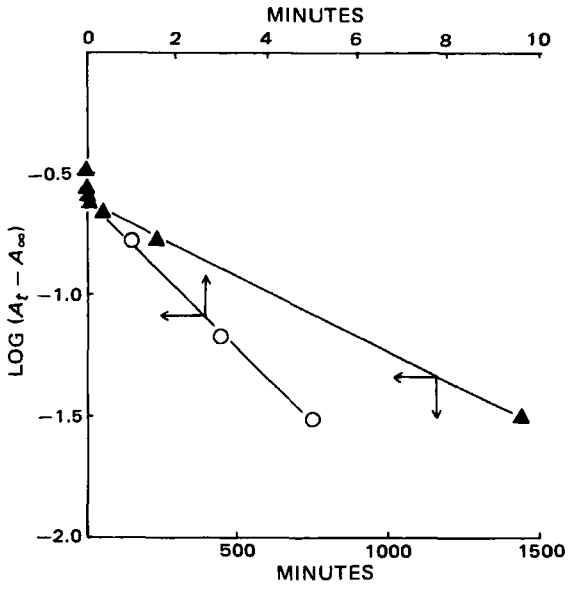


Figure 2—*First-order plots for the spectral changes in Fig. 1.* A_t *and* A_∞ *are the absorbances at time* t *and at completion of the reaction, respectively. Key: (○) at 330 nm and (▲) at 300 nm.*

albumin toward potential model substrates, the substituted *N-trans*-cinnamoylimidazoles. The pH dependence, isotope effect (deuterium oxide), and substituent effects for both the acylation and deacylation were examined using spectrophotometric methods.

EXPERIMENTAL

Materials—Albumin[1] was used after purification by the method of Chen (13). The molecular weight of the albumin was assumed to be 69,000 and the concentration was determained using an extinction coefficient $E^{0.1\%}_{1\,cm}$ of 0.531 at 278 nm (4). *N-trans*-Cinnamoylimidazole (I) was prepared by the reaction of *trans*-cinnamoyl chloride with imidazole in benzene solution (14). *p*-Chloro- (II), *m*-methyl- (III), and *p*-methoxy- (IV) cinnamoylimidazoles were synthesized by the method of Bernhard *et al.* (15) from the respective cinnamic acid and imidazole using dicyclohexylcarbodiimide as the catalyst. Several recrystallizations from cyclohexane gave material with the following melting points: (I) 133–134°, (II) 158–163°, (III) 119–123°, and (IV) 118–120°. Clofibric acid was prepared by the method of Jones *et al.*, mp 116–120° [lit. (16) mp 118–119°]. The ^2H content of the deuterium oxide[2] was >99.75%. All other chemicals purchased were of reagent grade.

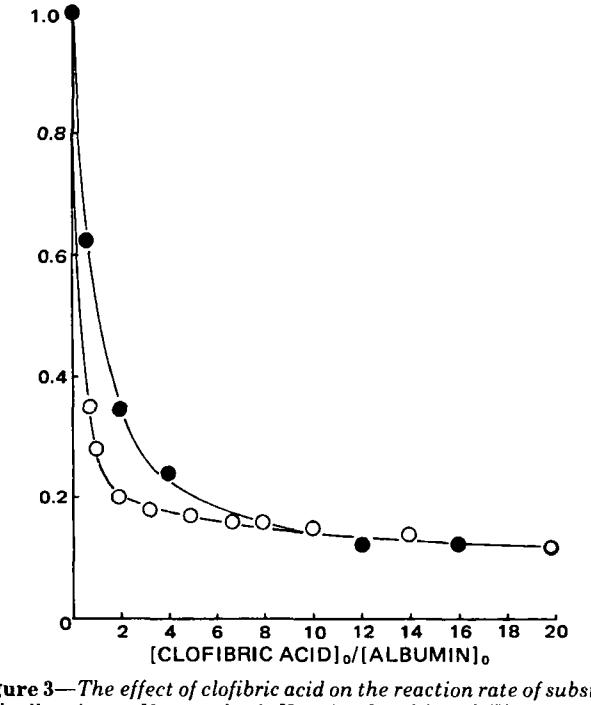


Figure 3—*The effect of clofibric acid on the reaction rate of substrate with albumin at pH 7.4 and 25°. Key: (●) I and (○) V. The initial concentrations of the substrate and albumin are 1.00 × 10^{-5} M and 5.00 × 10^{-5} M, respectively.*

[1] Sigma Chemical Co., Fraction V, lot 18c-0519.

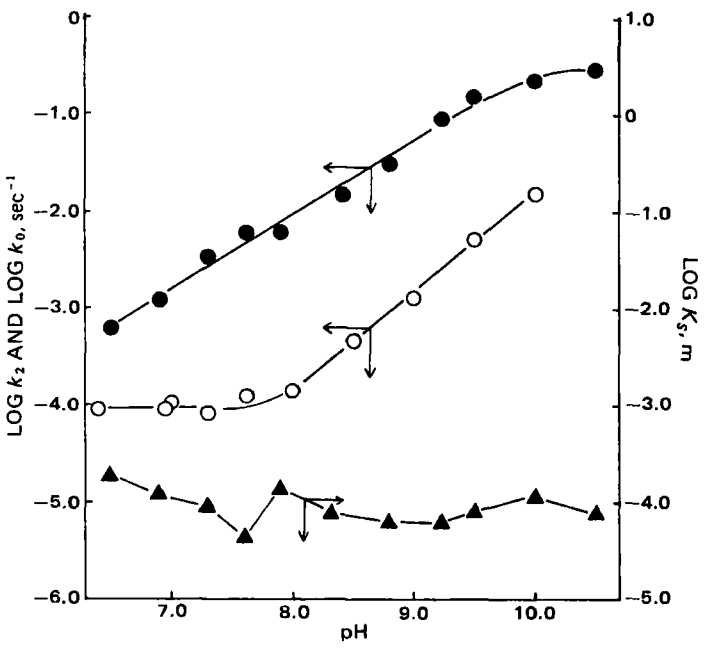


Figure 4—*Plots of log* k_2, *log* k_0, *and log* K_s *versus pH at 25°. Key: (●) log* k_2, *(○) log* k_0, *and (▲) log* K_s.

Kinetic Procedures—The reactions of the substrates with albumin were carried out in the presence of an albumin concentration more than fourfold in excess of the substrates, so that the substrates preferentially reacted with the primary reactive site (3, 4). The reactions were initiated by mixing 15 μl of the substrate in acetonitrile with 3 ml of buffered albumin solution preincubated in the spectrophotometer[3] cell kept at 25°. The acylation rate of albumin with I was followed at 330 nm to preclude interference due to the subsequent deacylation. The pseudo first-order rate constant was determined as described in a previous paper (3).

The deacylation of cinnamoyl-albumin was followed at 300 nm (Figs. 1 and 2). Sörensen buffer (0.067 *M* phosphate, pH 7.0–8.0) and Kolthoff

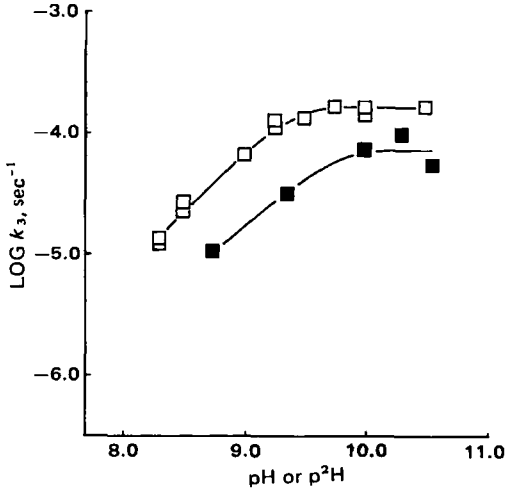
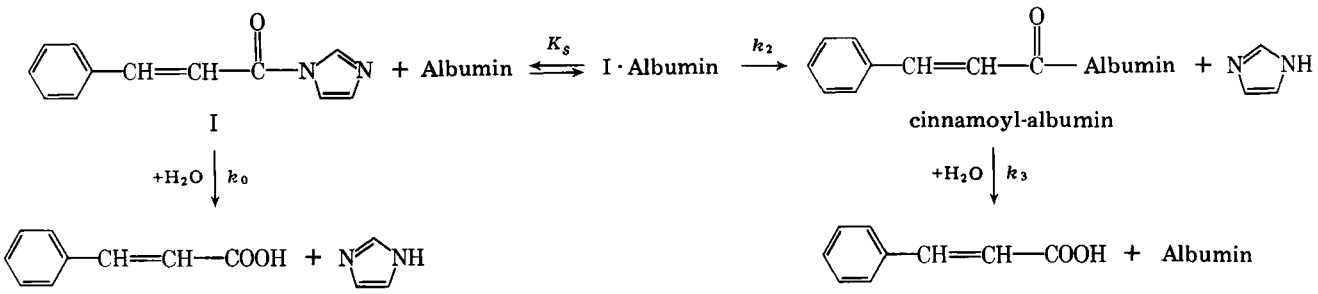


Figure 5—*Plots of log* k_3 *versus pH or* p^2H *at 25°. Key: (□) in water, and (■) in deuterium oxide.*

[2] Merck & Co., Inc.
[3] Hitachi UV-124 Spectrophotometer.



Scheme I

buffer (0.05 *M* borate, pH 8.0–10.5) were used as the reaction medium. The ionic strength was adjusted to 0.2 *M* with sodium chloride. In deuterium oxide, a p^2H value was estimated from p^2H = pH-meter reading + 0.4 (17).

RESULTS AND DISCUSSION

Reaction of *N-trans*-Cinnamoylimidazole with Albumin—The absorptivity changes noted during the reaction between albumin and I at pH 8.41 are shown in Fig. 1. Each spectrum is the difference between the reaction mixture and an equimolar albumin solution. The changes with time consist of a fast reaction followed by a slow one. The spectra eventually approach the sum of the spectra of equimolar *trans*-cinnamic acid and imidazole (represented by the dotted line). The first-order plot of the absorbance changes at 300 nm is obviously composed of the two steps (Fig. 2). The rate constant for the fast reaction obtained from the plot at 330 nm is ~300-fold larger than that obtained from the second (slow reaction) phase at 300 nm. It is probable that the fast reaction reflected by the decrease in the absorbance of I is the acylation of albumin (formation of cinnamoyl-albumin), and the subsequent slow reaction is the deacylation of the cinnamoyl-albumin.

To localize the reactive site toward I on albumin, the effect of clofibric acid on the acylation rate of albumin was examined. Clofibric acid was found previously (5–7) to bind singly at the R site on albumin and to inhibit the reaction of albumin with *p*-nitrophenyl acetate (V). The results are shown in Fig. 3. The *r* value in the ordinate of Fig. 3 represents the ratio of the rate constant in the presence of clofibric acid to that in its absence. The acylation of albumin is inhibited by clofibric acid, in a manner similar to its reaction with V. This inhibitory effect of clofibric acid indicates that both substrates I and V react with the same active site (R site, near tyrosine-411) on albumin. The tyrosine-411 residue of the R site, therefore, is considered to be acylated by I.

The overall reaction of I with albumin can be represented as in Scheme I. In this case, I·albumin is the Michaelis–Menten-type complex between I and albumin and K_s is the dissociation constant of the complex. The first-order rate constants of I and I·albumin are expressed by k_0 and k_2, respectively. The first-order rate constant for the deacylation of the cinnamoyl-albumin is given by k_3.

Acylation of Albumin with *N-trans*-Cinnamoylimidazole—The apparent first-order rate constants (k_{obs}) for the acylation of albumin were measured with several concentrations of albumin. The kinetic parameters k_2 and K_s for the acylation can be calculated from the intercept and slope of the double reciprocal plot based on the following (3):

$$\frac{1}{k_{obs} - k_0} = \frac{K_s}{(k_2 - k_0)} \frac{1}{[\text{albumin}]_0} + \frac{1}{k_2 - k_0}$$

The pH dependencies of k_2, K_s, and k_0 (for a comparison with k_2) are shown in Fig. 4. The slope of the log k_2–pH profile between pH 6.5 and 9.0 is approximately unity; at pH >9.5 k_2 becomes independent of pH. From this profile the pK_a value of the catalytic group is estimated as ~9.7. This pK_a value supports the theory that the acylation occurs at the tyrosine-411 residue of the R site, since the tyrosine-411 residue involved for the reaction with V has pK_a of ~9.5 (3, 5, 8). In addition, the absence of a deuterium oxide effect in the fast step also supports this theory for the acylation of albumin.

The value of K_s is almost independent of pH in the region examined. The spontaneous hydrolysis of I proceeds through the usual base catalysis in the alkaline region. From the comparison of k_0 with k_2, it is obvious that albumin accelerates the cleavage of amide substrate I as well as ester substrates (3, 4).

Deacylation of Cinnamoyl-Albumins—The pH dependence of k_3 for cinnamoyl-albumin is illustrated in Fig. 5. Up to pH ~9, the log k_3–pH profile shows a slope of approximately unity. However, the rate constant reaches the limiting value at higher pH. This pH dependence implies the participation of a group having pK_a of ~9.4 for the deacylation. In deuterium oxide the deacylation rate is about three–fourfold smaller than that in water, as shown in Fig. 5. This deuterium effect indicates that the water molecule also plays a role in the deacylation. Therefore, general acid or base catalysis by the group rather than nucleophilic catalysis predominates in the deacylation of cinnamoyl-albumin (18).

The substituent effect for the deacylation rate was examined to distinguish between the general acid and base catalysis. The log k_3–pH profiles for *p*-chloro-, *m*-methyl-, and *p*-methoxycinnamoyl-albumins are shown in Fig. 6. A pH variation of ~0.5 units seems to exist in the

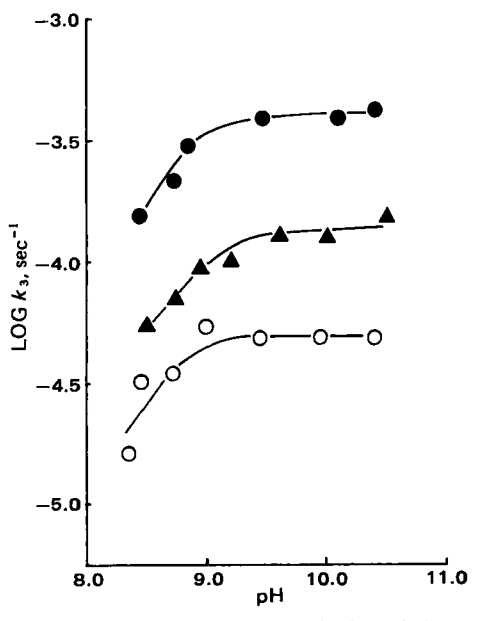


Figure 6—*pH dependence of* k_3 *for the substituted cinnamoyl-albumins at 25°. Key: (●) p-chlorocinnamoyl-albumin, (▲) m-methylcinnamoyl-albumin, and (○) p-methoxycinnamoyl-albumin.*

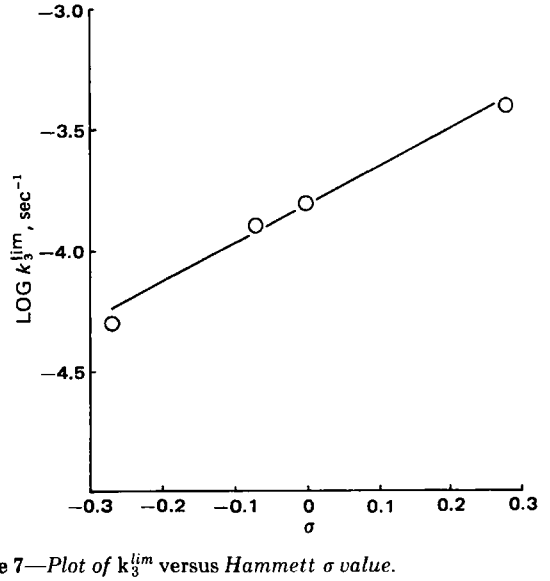


Figure 7—*Plot of* k_3^{lim} versus *Hammett σ value.*

apparent pK_a of the group participating in the deacylation. This variation may be attributable to the perturbation of the pK_a induced by the substituted cinnamoyl group (15, 19). A plot of the logarithm of the rate constants in the pH-independent region (k_3^{lim}) against the Hammett σ values gives a straight line (correlation coefficient = 0.994), as shown in Fig. 7. The electron-withdrawing substituent facilitates the reaction and the Hammett ρ value is 1.63. This ρ value suggests that the deacylation proceeds *via* general base catalysis rather than general acid catalysis, because the latter is, in general, independent of polar effects (20). The requirement of the limited conformation around the catalytic site is demonstrated by the finding that the deacylation of denatured cinnamoyl-albumin in 8 *M* urea was retarded.

Although the acylation of albumin with I (k_2) was faster than the spontaneous hydrolysis of I (k_0), as shown in Fig. 4, the deacylation of the cinnamoyl-albumin (k_3 in Fig. 5) is slower than the hydrolysis of I (k_0 in Fig. 4). The deacylation rate is related to the molecular activity (in the past the term "turn over number" has been used) which gives an indication of efficiency of an enzyme (21, 22). In this context, the esterase-like activity rather than the intrinsic esterase may be an appropriate expression for the activity of albumin toward the amide and ester substrates.

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Stability Constants for Complex Formation Between α-Cyclodextrin and Some Amines

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Received March 18, 1982 from the *School of Pharmacy, University of Wisconsin, Madison, WI 53706.* Accepted for publication August 9, 1982.

Abstract □ Complex formation of α-cyclodextrin with 15 amines (including seven 4-substituted anilines) was studied by the potentiometric method, supplemented by direct UV spectrophotometry and a competitive indicator spectrophotometric method. The data were analyzed in terms of 1:1 and 1:2 complexes (amine–cyclodextrin ratios) and the stability constants K_{11a}, K_{12a}, K_{11b}, and K_{12b} were evaluated; the subscripts indicate the stoichiometry and conjugate acid–base form. For all amines K_{11b} was greater than K_{11a} and K_{12a} was 0. On the basis of the relationship of complex stability to amine structure, it was concluded that the primary binding site in anilines is the 4-substituent.

Keyphrases □ Complex formation—of α-cyclodextrin with amines, determination of the stability constants and binding sites □ α-Cyclodextrin—complex formation with amines, determination of stability constants and binding sites □ Amines—complex formation with α-cyclodextrin, determination of stability constants and binding sites

Cycloamyloses (also called cyclodextrins) are cyclic oligomers containing six or more D-glucose units linked 1 → 4; they are produced by the action of *Bacillus macerans* amylase on starch. The six- and seven-unit substances are called cyclohexaamylose (α-cyclodextrin) and cycloheptaamylose (β-cyclodextrin), respectively. These molecules are doughnut shaped, and their possession of a cavity of fixed size and shape has led to considerable interest in their chemical properties. The production, purification, and chemistry of the cycloamyloses have been reviewed (1–4).

Any molecule smaller than the cavity of a cyclodextrin can enter the cavity and there undergo noncovalent interaction with the atoms lining and rimming the cavity. The resulting association product is called an inclusion complex. The cyclodextrin is thus a host for the smaller (guest) molecule. The dimensions of the α-cyclodextrin cavity permit the inclusion of many mono- and disubstituted benzene derivatives. A 1:1 stoichiometry is commonly observed (and often assumed in experimental studies), but it has now been well established that 1:2 complexes (*i.e.*, 1 substrate:2 cyclodextrins) may exist in some systems[1] (5–9).

The present paper is one of a series that describes

[1] The substrate (S) is the guest; the cyclodextrin (ligand, L) is the host. Stoichiometric ratios are given in the form SL (1:1) and SL_2 (1:2).

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