Kinetics of the Rapid Modification of Human Serum Albumin with Trinitrobenzenesulfonate and Localization of Its Site

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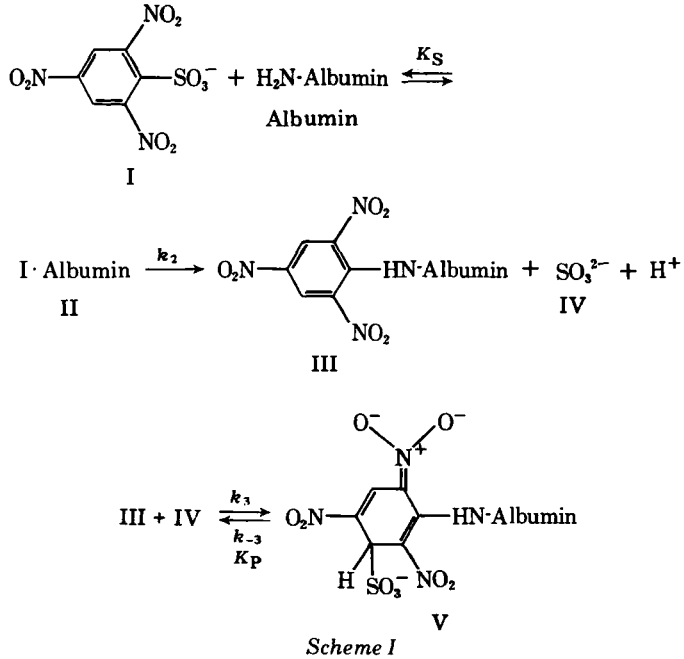
Abstract □ The rapid reaction of human serum albumin with trinitrobenzenesulfonate (I) and the location of the reactive site were investigated to characterize the chemical modification of albumin by I. The modification proceeds through trinitrophenylation of a lysine residue of albumin and monoaddition of the byproduct, sulfite ion, to the trinitrophenylalbumin, as reported previously. The individual kinetic parameters for both reactions were determined at various pH values and 25°. The ε-amino group of the lysine residue which has a pK_a value of ~8.9 was the reactive group involved in the trinitrophenylation. The dissociation constant of the sulfite monoadduct was about 10-fold smaller than that of the monoadduct of the model compound trinitrophenyl α-acetyllysine. The modification of albumin by I reduced the fluorescence intensity of the tryptophan-214 residue in the albumin amino acid sequence. Acetylation of the lysine-199 residue with aspirin and 5-nitroaspirin decreased the trinitrophenylation rate of albumin with I. These results on the fluorescence spectroscopy and the effect of the acetylation suggest that the reactive group for I is the lysine-199 residue located near the tryptophan-214 residue.

Keyphrases □ Kinetics—of reaction of trinitrobenzenesulfonate with human serum albumin □ Albumin, human serum—kinetics of reaction with trinitrobenzenesulfonate, location of drug binding site, fluorescence spectroscopy, acetylation with aspirins □ Trinitrobenzenesulfonate—kinetics of reaction with human serum albumin □ Aspirin—acetylation of human serum albumin

Characterization of drug binding sites on human serum albumin and knowledge of drugs affecting these sites are important to predict displacement of one drug by another, when two or more drugs are administered concurrently (1). Studies on the drug binding sites of the albumin molecule have been carried out by various methods (2–5), one of which is chemical modification (4). Trinitrobenzenesulfonate (I) has been used as a reagent for amino group modification in proteins (6). Kinetic data of the reactions of human and bovine serum albumin with excess I were presented by Goldfarb (7) and Andersson *et al.* (8), respectively. Under their conditions (an excess of I over albumin), multiple reactive sites for I appear to be modified, so that it may be difficult to investigate in detail the kinetics and mechanism for the modification of a single specific site on albumin with I.

In our previous study (9), it was reported that the chemical modification of human serum albumin by I proceeds rapidly and specifically through the trinitrophenylation of a lysine residue in albumin and the sulfite monoaddition, as shown in Scheme I. Abbreviations in Scheme I are as follows: II, the Michaelis–Menten type complex of I and albumin; III, trinitrophenylalbumin; IV, sulfite ion; and V, sulfite monoadduct of III.

The present study is concerned with determination of the individual kinetic parameters in Scheme I at various pH values and with identification of the primary reactive site on albumin for I.



Scheme I

EXPERIMENTAL

Materials—Human serum albumin[1] was used after purification as previously described (10). The concentration of albumin was determined by use of its molar absorbance ($\epsilon_{albumin} = 3.66 \times 10^4\ M^{-1}\ cm^{-1}$) at 278 nm (11), assuming a molecular weight of 69,000. 2,4,6-Trinitrobenzenesulfonic acid sodium salt[2] and sodium bisulfite[3] were used without further purification. All other chemicals were reagent grade and were used without further purification.

Kinetic Data—The buffer systems used were: pH 6–8.3, 0.067 *M* phosphate; pH 8.3–9, 0.1 *M* phosphate and 0.05 *M* borate; pH 9–11, 0.05 *M* borate and 0.05 *M* carbonate. Ionic strength was adjusted to 0.2 *M* with sodium chloride. The temperature was kept at 25° in all experiments unless otherwise stated.

The UV spectral changes based on the reactions shown in Scheme I are presented in our previous paper (9). The trinitrophenylation rate of albumin with I was followed at a wavelength of 360 nm (showing an isosbestic point of III and V) with a stopped-flow spectrophotometer[4]. Pseudo first-order analysis was applied under the condition of excess albumin concentration relative to that of I.

For the analysis of the reversible reaction (III + IV ⇄ V) shown in Scheme I, the UV spectrum of either III or V (not of the mixture of III, IV, and V) is necessary. Since neither III nor V was isolated, each spectrum of III and V could not be measured independently, and thus the spectrum was unknown. Trinitrophenyl α-acetyllysine (VI) was chosen as a model compound for III for the following reason. The absorbance at 360 nm (isosbestic point of III and V), after completion of the spectral change due to the trinitrophenylation of albumin with I, was essentially equal to that of VI found in the literature (12), when the original absorbance based on the excess albumin over I was corrected. Therefore, the UV spectrum of III at wavelengths >360 nm could be assumed to be identical with the spectrum of VI.

[1] Sigma Chemical Co., Fraction V, lots 30F-02271 and 100F-02061.
[2] Wako Chemical Co., lot SDL 8630.
[3] Katayama-kagaku, lot 800805.
[4] Model RA-401, Union-Giken, Osaka, Japan.

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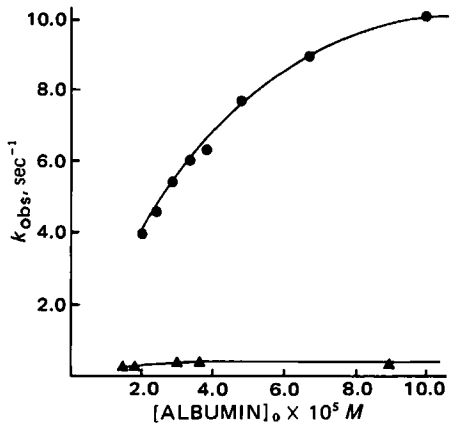

Figure 1—*Effect of albumin concentration on the rate of trinitrophenylation with I at 25°. The concentration of I was 1.0 × 10^{-5} M. Key: (●) pH 10.7; (▲) pH 7.4.*

Since it was indicated from preliminary experiments that the dissociation constant (K_P = [III][IV]/[V]) was relatively small, an elaborate method (13) was used for the determination of K_P, without employing the usual spectral method [*e.g.*, Benesi–Hildebrand method (14)]. The usual method is applicable only to the equilibrium system in which one component is much larger than the other.

Two reaction solutions were prepared (13): one was composed of an initial concentration of III ($[III]_0$) and of IV ($[IV]_{0,1}$), and the other of $[III]_0$ and a concentration $[IV]_{0,2}$. It was found that I was completely converted into III in the presence of excess albumin, and thus $[III]_0$ was equal to the initial concentration of I. The above two solutions were equilibrated together and their absorbances at 415 nm were measured. The increments of absorbances from the sum of the intact (initial) absorbances of III and IV, which are represented by ΔA_1 and ΔA_2, are proportional to the equilibrium concentrations of V, $[V]_{e,1}$ and $[V]_{e,2}$, respectively. For actual calculations of ΔA_1 and ΔA_2 the absorbance of the model compound VI was used instead of the absorbance of III, because the absorbance of III was reasonably assumed to be identical with that of VI as stated earlier. The relationship between the increment of absorbance and the equilibrium concentration is as follows: $[V]_{e,1} = \alpha\Delta A_1$ and $[V]_{e,2} = \alpha\Delta A_2$. The coefficient α is equal to $1/(\epsilon_V - \epsilon_{III} - \epsilon_{IV})$, where ϵ with subscript III, IV, or V is the molar absorbance of each species. Then, K_P can be represented as follows (13):

$$K_P = \frac{[III]_e[IV]_e}{[V]_e} \qquad \text{(Eq. 1)}$$

$$= \frac{([III]_0 - \alpha\Delta A_1)([IV]_{0,1} - \alpha\Delta A_1)}{\alpha\Delta A_1} = \frac{([III]_0 - \alpha\Delta A_2)([IV]_{0,2} - \alpha\Delta A_2)}{\alpha\Delta A_2}$$

From Eq. 1 a quadratic equation with respect to α is obtained, and the solution is (13):

$$\alpha = \frac{([IV]_{0,1} - [IV]_{0,2})\Delta A_1\Delta A_2 \pm \sqrt{\{([IV]_{0,1} - [IV]_{0,2})\Delta A_1\Delta A_2\}^2 - 4[III]_0\Delta A_1\Delta A_2(\Delta A_1 - \Delta A_2)(\Delta A_2[IV]_{0,1} - \Delta A_1[IV]_{0,2})}}{2\Delta A_1\Delta A_2(\Delta A_1 - \Delta A_2)} \qquad \text{(Eq. 2)}$$

Because α is calculated from Eq. 2, we can estimate K_P from Eq. 1. Furthermore, applying ϵ_{III} presumed from VI to the equation $\alpha = 1/(\epsilon_V -$ $\epsilon_{III} - \epsilon_{IV}$) we can obtain ϵ_V also, since ϵ_{IV} is determined independently (negligible). The K_P and α thus obtained were reproducible in other reaction solutions equilibrated from the different initial concentrations of IV.

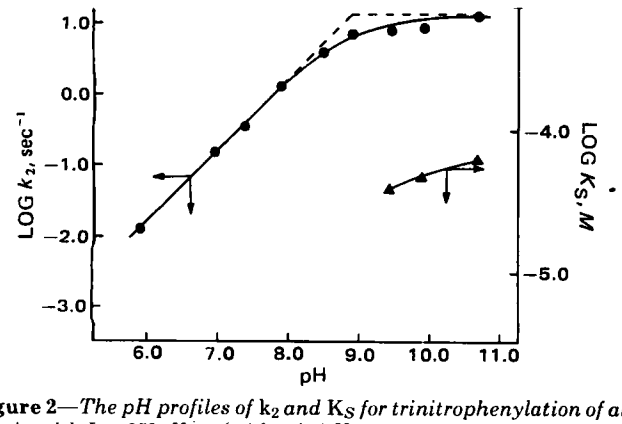

Figure 2—*The pH profiles of* k_2 *and* K_S *for trinitrophenylation of albumin with I at 25°. Key: (●)* k_2*; (▲)* K_S*.*

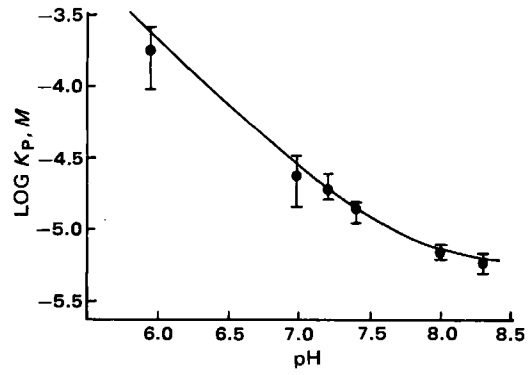

Figure 3—*The pH profile for* K_P *at 25°. The bars in this figure indicate the standard deviations. The solid curve was calculated using Eq. 6.*

The rate of the sulfite monoaddition was also followed at 415 nm. As the molar absorbance (ϵ_{III}, ϵ_{IV}, ϵ_V, and $\epsilon_{albumin}$) of each component in the reaction solution is known, the concentration of V at each time interval is calculated from the absorbance of the reaction solution. The rate constant (k_3) was determined from the slope of the plot based on Eq. (3), which is derived for the reversible reaction (15):

$$\frac{1}{E - F}\log\frac{F([V] - E)}{E([V] - F)} = \frac{k_3}{2.303}t \qquad \text{(Eq. 3)}$$

where,

$$E, F = \frac{([III]_0 + [IV]_0 + K_P) \pm \sqrt{([III]_0 + [IV]_0 + K_P)^2 - 4[III]_0[IV]_0}}{2}$$

The rate constant (k_{-3}) for the release of IV from V was calculated from K_P and k_3 already determined: $k_{-3} = K_P \cdot k_3$.

Acetylation of Albumin with Aspirin and 5-Nitroaspirin—Acetylation of albumin with aspirin (VII) was carried out by a previous method (16). A mixture of albumin (1.00 × 10^{-4} *M*) and VII (5.00 × 10^{-4} *M*) in pH 7.4 buffer was incubated at 37° for 24 hr and dialyzed at 4° for 48 hr against multiple changes of the same buffer. In the control experiment, VII was omitted and albumin was treated as above.

In the case of the acetylation by 5-nitroaspirin (VIII), the absorbance at 370 nm due to 5-nitrosalicylic acid (IX) released from the reaction of albumin (1.00 × 10^{-4} *M*) with VIII in pH 7.4 and at 25° was measured to estimate the degree of the acetylation. Three concentrations of VIII were used: 1.25 × 10^{-4} *M*, 2.50 × 10^{-4} *M*, and 3.75 × 10^{-4} *M*. When the absorbances reached corresponded to 1.0, 2.0, and 3.0 moles of IX per mole of albumin, respectively, the reaction solutions were cooled at 4° and dialyzed as described above. These albumins were denoted as monoacetyl-, diacetyl-, and triacetylalbumin, respectively.

Fluorescence Measurements—The fluorescence spectra of albumin solution (1.00 × 10^{-5} *M*) in the presence and absence of I (2.50 × 10^{-6} *M*) were measured. The excitation wavelength was 300 nm (17, 18).

RESULTS AND DISCUSSION

Kinetics on Trinitrophenylation of Albumin with I—Figure 1 shows the effect of the initial concentration of albumin ($[Albumin]_0$) on the apparent first-order rate constant (k_{obs}) for the trinitrophenylation at pH 7.4 and 10.7. At pH 10.7 the k_{obs} value increases hyperbolically with the albumin concentration, indicating saturation by trinitrophenylation (5, 19). According to Scheme I, k_{obs} can be represented as (5, 19):

$$k_{obs} = \frac{k_2[albumin]_0}{K_S + [albumin]_0} \qquad \text{(Eq. 4)}$$

where K_S and k_2 are the dissociation constant of II (= [I][albumin]/[II]) and the first-order rate constant of II for trinitrophenylation, respectively. The k_2 and K_S values were calculated from the intercept and slope of a plot based on the following, which is rearranged from Eq. 4 (5, 19):

$$\frac{1}{k_{obs}} = \frac{K_S}{k_2}\cdot\frac{1}{[albumin]_0} + \frac{1}{k_2} \qquad \text{(Eq. 5)}$$

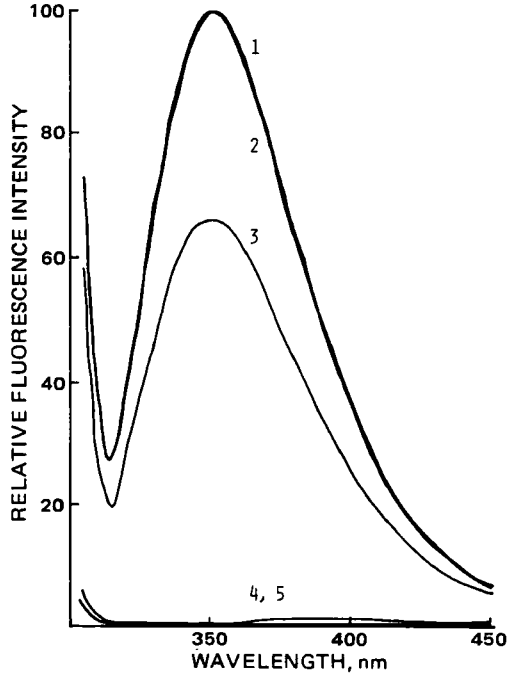

Figure 4—*Fluorescence emission spectra excited at 300 nm. Key: (1) 1.0 × 10⁻⁵ M albumin; (2) 1.0 × 10⁻⁵ M albumin and 5.0 × 10⁻⁶ M sodium bisulfite; (3) 1.0 × 10⁻⁵ M albumin and 2.5 × 10⁻⁶ M I; (4) 2.5 × 10⁻⁶ M I; and (5) 5.0 × 10⁻⁶ M sodium bisulfite.*

At pH 7.4, in contrast, the values of k_{obs} are almost independent of the albumin concentration. This independence suggests that K_S in Eq. 4 is far less than $[albumin]_0$ employed, and thus the k_{obs} value obtained under these conditions was assumed to be equal to k_2.

Figure 2 illustrates the pH dependence of k_2 and K_S thus obtained. The value of k_2 markedly depends on pH. Below pH values of about 8, the slope of the profile is 1, and above pH values of about 9.5, the k_2 value is independent of pH. From this profile the pK_a value of the ϵ-amino group of a lysine residue at the reactive site of albumin was estimated as about 8.9.

As stated earlier the data show that the K_S value at neutral pH is less than that above pH 9.5. The pH dependence of K_S may be explained qualitatively as follows. Deprotonation of basic groups (*e.g.*, ϵ-amino group of lysine and/or guanidino group of arginine) constituting the reactive site for I increases with increasing pH value, thereby decreasing positive-charge localization. Compound I is an anion, therefore, the ionic interaction (20) between I and the reactive site may decrease with increasing pH.

Sulfite Monoaddition to Trinitrophenylalbumin—Figure 3 shows a log K_P–pH profile. The K_P values decrease with increasing pH values. This pH dependence suggests that sulfite ion (IV) rather than bisulfite ion reacts with III. Then, K_P in the pH region examined can be expressed as a function of the hydrogen ion concentration $[H^+]$ by:

$$K_P = K'_P\left(\frac{[H^+] + K_2}{K_2}\right) \qquad \text{(Eq. 6)}$$

where K_2 and K'_P are the dissociation constant of bisulfite ion and the intrinsic dissociation constant of V, respectively. From a plot of K_P against $[H^+]$, K'_P and K_2 were estimated as 5.0×10^{-6} *M* and 2.2×10^{-8} *M*, respectively. The K'_P value for V is about 10-fold smaller (tight binding) than that (4.8×10^{-5} *M*) for the sulfite monoadduct of VI found in the literature (12). The difference in these K'_P values may be explained in terms of the distribution of sulfite ion between albumin and the aqueous phases. For III, the concentration of sulfite ion in the microenvironment of the trinitrophenylated group is probably higher than that in water for VI, since albumin generally has a strong affinity for anionic small ligands. The microenvironment of albumin may also affect the dissociation ($K_2 = 2.2 \times 10^{-8}$ *M*) of bisulfite ion, since in water the literature value for K_2 is about 6.2×10^{-8} *M* (21).

There was some difficulty in determining accurately the rate constant (k_3) for the sulfite monoaddition, because the absorbance changes at 415 nm due to trinitrophenylation seemed to be incomplete at the initial stage of the sulfite monoaddition; that is, the changes based on both the trinitrophenylation and the monoaddition could not be separated clearly.

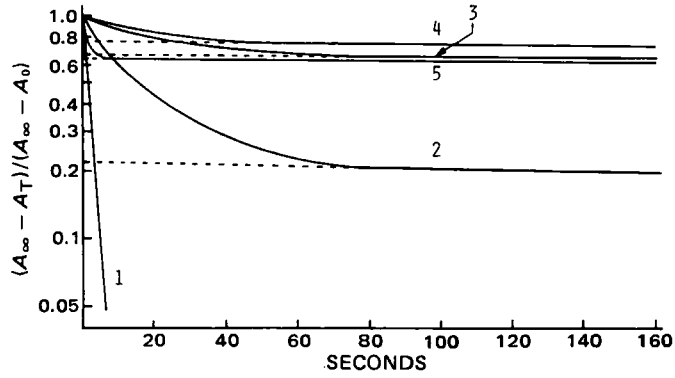

Figure 5—*Kinetics for the trinitrophenylation of albumin and acetylalbumins with I at pH 7.4 and 25° plotted as log $[(A_\infty - A_t)/(A_\infty - A_0)]$ against reaction time. Key: (1) 5.0 × 10⁻⁵ M control albumin and 1.0 × 10⁻⁵ M I; (2) 5.0 × 10⁻⁵ M monoacetylalbumin and 1.0 × 10⁻⁵ M I; (3) 5.0 × 10⁻⁵ M diacetylalbumin and 1.0 × 10⁻⁵ M I; (4) 5.0 × 10⁻⁵ M triacetylalbumin and 1.0 × 10⁻⁵ M I; (5) 3.3 × 10⁻⁵ M albumin and 8.0 × 10⁻⁵ M I.*

The approximate k_3 value at pH 7.4 and 25°, however, was estimated using Eq. 3. The value $7.0 \times 10^2\ M^{-1}\ sec^{-1}$ was obtained and compares with the literature value of $5.25 \times 10^3\ M^{-1}\ sec^{-1}$ at pH 8.0 and 25.4° for trinitrophenyl β-alanine (22). In addition, the k_{-3} value at pH 7.4 and 25° was calculated as $9.5 \times 10^{-3}\ sec^{-1}$ from the values for K_P and k_3.

Localization of Reactive Site on Albumin for I—In a series of studies on the effects of drug binding on the esterase-like activity of albumin (5, 18), it was found that albumin has some specific drug binding sites, and one of them is located near the tryptophan-214 (Trp-214) residue (23). When a drug binds to a site near Trp-214, the fluorescence intensity of Trp-214 is reduced when excited at 300 nm. To localize the reactive site for I, the fluorescence spectra of the reaction mixture of albumin with I and of each reactant solution were measured (Fig. 4). The intensity of the emission spectrum for the mixture (spectrum 3) is lower than that for the albumin solution (spectrum 1), indicating that the reactive site for I is located near the Trp-214 residue.

It has been reported that VII and VIII acetylate the ϵ-amino group of the lysine-199 (Lys-199) residue located near Trp-214 (5, 24). To examine further the reactive site for I, the effect of albumin acetylation by VII and VIII on the trinitrophenylation rate was investigated. The results are shown in Fig. 5. In this figure, $\log\{(A_\infty - A_t)/(A_\infty - A_0)\}$ is plotted against time, where A_∞, A_t, and A_0 are the absorbances at 360 nm at the completion of the reaction, at times t and zero, respectively. It is obvious that the trinitrophenylation of acetylalbumins with I (curves 2–4) is slower than that of the control albumin (curve 1). For acetylalbumins the trinitrophenylation is not a single process, but consists of an initial phase and a slower second phase. The slopes of the second phase are approximately equal to each other. Furthermore, these slopes (in curves 2–4) are almost identical with the slope (in curve 5) of the second phase obtained from the plot for the trinitrophenylation with excess I. These identities suggest that the second phase for acetylalbumins is due to the secondary reactive site(s) of albumin for I, of which the location(s) is(are) unknown at present.

For trinitrophenylation with excess I (curve 5) it is evident that a value (~0.63) on the ordinate obtained by extrapolating the line of the second phase to time zero indicates the fraction of I consumed by the secondary reactive site(s) of albumin $[(8.0 \times 10^{-5} - 3.3 \times 10^{-5})/8.0 \times 10^{-5} \approx 0.59]$. The slight discrepancy between 0.63 and 0.59 may be due to experimental error. For acetylalbumins (curves 2–4) the fractions were estimated by similar extrapolations (as expressed by dotted lines). The values obtained are ~0.22, 0.67, and 0.77 for mono-, di-, and triacetylalbumin, respectively. These values imply that $7.8 \times 10^{-6}\ M$ ($= 1.0 \times 10^{-5}\ M - 2.2 \times 10^{-6}\ M$), $3.3 \times 10^{-6}\ M$, and $2.3 \times 10^{-6}\ M$ of I were consumed by the primary reactive site, respectively. However, $5.0 \times 10^{-5}\ M$ of each acetylalbumin (fivefold excess of albumin over I) was employed for the trinitrophenylation reactions. With respect to the albumin concentration, therefore, only about 16 $[\approx(7.8 \times 10^{-6}/5.0 \times 10^{-5}) \times 100]$, 7, and 5% of the primary reactive site are unmodified (unacetylated) with VIII for mono-, di-, and triacetylalbumin, respectively. In other words, about 84, 93, and 95% of the primary reactive site for I are blocked by VIII. Since 5-nitroaspirin (VIII) primarily acetylates the ϵ-amino group of the Lys-199 residue (5, 24), these results on the effects of the acetylation suggest that the primary reactive group for I is the Lys-199 residue. The results from the experi-

ments using the albumin acetylated with aspirin (VII) also led to a similar conclusion concerning the reactive site for I.

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Pharmaceutical Approach to Subcutaneous Dosage Forms of Insulin

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Abstract □ The present studies were undertaken to describe the dynamic nature of the degradation and absorption of insulin in the subcutaneous injection site and to develop agents which would stabilize this dosage form. [^{125}I]Insulin with 0.2-U/kg of unlabeled insulin in 10 μl of aqueous solution was injected subcutaneously in rats under the depilated skin of the back. At various times, radioactive skin tissue was extracted and assayed for insulin and/or its metabolites by gel filtration. Using these data, absorption and degradation rate constants of these substances were estimated according to a one-compartment model. Absorption rate constants of insulin and its metabolite of low molecular weight (monoiodotyrosine) were 0.021 min^{-1} and 0.107 min^{-1}, respectively, while the degradation rate constant for insulin to monoiodotyrosine was 0.013 min^{-1}. Thus, the bioavailability of insulin injected subcutaneously was lower than expected, suggesting the necessity of stabilizing methods. The protection of insulin from degradation at the site of injection was examined by the addition of various peptides. It was found that benzyloxycarbonyl-Gly-Pro-Leu-Gly was a good stabilizing agent, and remarkably inhibited insulin degradation. This inhibition was confirmed by the increase of immunoreactive insulin level and the decrease of the blood glucose level. We postulated that this peptide protects the injected insulin from degradation by inhibiting the peptidase present in subcutaneous tissue.

Keyphrases □ Insulin—absorption and degradation at the subcutaneous injection site, stabilizing agents for the subcutaneous dosage form □ Stabilizing agent—for insulin, subcutaneous dosage forms, use of the peptide benzyloxycarbonyl-Gly-Pro-Leu-Gly □ Subcutaneous injection—absorption and degradation of insulin, use of benzyloxycarbonyl-Gly-Pro-Leu-Gly peptide for stabilization

Endogenous peptides having specific biological effects have attracted attention as new drugs; however, effective dosage forms of these peptides for clinical usage have been difficult to assess in any but the parenteral dosage forms. Insulin, administered subcutaneously, has been the primary treatment for severe diabetes for several years, even though effective methods have been described for oral (1–3), nasal (4), aerosol (5, 6), and rectal (7, 8) administration. In previous attempts to assess the absorption of insulin from its subcutaneous depot, radioiodinated insulin preparations were injected and the disappearance of the radioactivity from the injection site was measured by an external gamma-counter (9, 10). The stability and bioavailability of insulin administered subcutaneously are not well understood since the metabolites of insulin could not be measured. The kinetics of insulin in the blood after subcutaneous injection are the result of many factors including absorption and degradation in the injection site (11).

Recently, Berger reported that aprotinin increased the absorption rate of subcutaneously injected insulin and amplified its biological effect by inhibiting the local degradation of exogenous insulin at the injection site (12). Little else has been reported about the degradation and absorption of insulin at the injection site, although the absorption characteristics of insulin and other drugs from intramuscular injection sites were investigated (13, 14). The present studies were undertaken to investigate the dynamic nature of the degradation and the absorption of insulin in the subcutaneous injection site and to develop new stabilizing agents for this dosage form.

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