mp 179—182°) and unchanged material I (122 mg) were obtained after recrystallization from AcOEt, respectively.

(c) **Dimerization in Dioxane**——A mixture of I (460 mg) and dioxane (0.5 ml) was heated under the conditions described in Table I. The reaction mixture was separated as described for dimerization (a). The less polar fraction (246 mg) and the more polar fraction (97 mg) were recrystallized from AcOEt to afford III (220 mg, mp 178—181°) and unchanged material I (77 mg), respectively.

(d) **Dimerization in THF**——A mixture of I (480 mg) and THF (0.5 mg) was heated under the conditions described in Table I. The reaction product was separated as described above. The less polar fraction (247 mg) and the more polar fraction (73 mg) were recrystallized from AcOEt to give III (236 mg, mp 179—181°) and unchanged material I (58 mg), respectively.

II. Formation of Dimer IV from 16,17-Dehydrodigitoxigenin 3-Acetate (I) in Slightly Basic Medium. (a) **Dimerization in Toluene-AcONa**——A mixture of I (300 mg), anhydrous sodium acetate (10 mg), and toluene (0.5 ml) was heated at 115—120° for 144 hr in a closed test tube under nitrogen in an oil bath, and the reaction mixture was separated into three fractions by quintuply developed preparative TLC (SiO_2, benzene: Et_2O: $CHCl_3$=2:2:1). The less polar fraction (27 mg) was recrystallized from AcOEt to give III (20 mg) mp 178—181°. The more polar fraction (234 mg) was recrystallized from AcOEt to give IV (206 mg) as colorless needles, mp 296—298°, $[\alpha]_D^{24}$ −40.1° (c=0.50, $CHCl_3$). *Anal.* Calcd for $C_{50}H_{68}O_{10}\cdot 2H_2O$: C, 69.42; H, 8.39; Mol. Wt., 865.07. Found: C, 69.36; H, 8.48; Mol. Wt., 914.0. The most polar fraction (17 mg) was identified as unchanged material (I) by TLC.

(b) **Dimerization in DMF**——A mixture of I (458 mg) and redistilled DMF (0.5 ml) was heated under the conditions described in Table I, and the reaction mixture was separated by the method used in the case of dimerization (a). The more polar fraction (345 mg) was recrystallized from AcOEt to afford IV (283 mg) as colorless needles, mp 296—298°. The most polar fraction (23 mg) was identified as unchanged I by TLC.

Dianhydro derivative (V) from IV——A solution of IV (50 mg) in dry pyridine (1 ml) was cooled at −15° with ice-NaCl mixture and a solution of $SOCl_2$ (0.1 ml) in dry pyridine (0.5 ml) was added dropwise with stirring. After further stirring for 1 hr at the same temperature, excess $SOCl_2$ was decomposed by additng ice and the precipitate thus formed was collected by filtration and dried *in vacuo*. The crude product (43 mg) was recrystallized from $CHCl_3$–MeOH to give V (25 mg), mp 266—270°. *Anal.* Calcd for $C_{50}H_{64}O_8\cdot 2H_2O$: C, 72.44; H, 8.27. Found: C, 72.18; H, 8.12.

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Chemical Modification of Tryptophan and Histidine Residues in Lipoprotein Lipase from *Pseudomonas fluorenscens*[1]

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Lipoprotein lipase (glycerol ester hydrolase; EC 3.1.1.3) from *Pseudomonas fluorescens* was oxidized with N-bromosuccinimide at 37°. One mol of tryptophan residue per mol of enzyme was oxidized, but the enzymatic activity was unaffected.

The enzyme was inactivated by photooxidation in the presence of methylene blue, and the inactivation was pH-dependent. In addition, the decrease in the enzymatic activity was accompanied by the loss of a histidine residue. It appears that the histidine residue in involved in the catalytic activity of the enzyme.

Keywords——*Pseudomonas fluorescens*; chemical modification; tryptophan; histidine; lipoprotein lipase; methylene blue; photooxidation

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Introduction

Chemical modification is a very useful technique to investigate the active site of an enzyme. So far, little information is available concerning the active site of lipase, and a classification of lipase on the basis of the active site amino acid residue, has not been carried out, as it has in the case of proteases.[3]

In 1971, Semeriva *et al.*[4] reported that porcine pancreatic lipase has a histidine residue in the active site. In the case of lipases from microorganisms, Liu *et al.*[5] suggested that a tryptophan residue participated in the activity of lipase from *Humicola lanuginosa*. Isobe and Sugiura[6] demonstrated that one histidine residue was required for the enzyme activity of lipase B from *Chromobacterium viscosum*.

Previously, the present authors reported that lipoprotein lipase (LPL) from *Pseudomonas fluorescens* was strongly inhibited by iodine and N-bromosuccinimide.[7] It is well known that these reagents attack certain amino acid residues, including tyrosine, tryptophan and histidine residues in the enzyme molecule.[8] This study was undertaken to investigate the roles of tryptophan and histidine residues in LPL, in order to identify the active site of LPL.

Materials and Methods

Materials——LPL from *Pseudomonas fluorescens* was purified as described previously.[7] N-Bromosuccinimide (NBS) was purchased from Tokyo Kasei Kogyo Co., Ltd. Methylene blue was obtained from Wako Pure Pharm. Co., Ltd. All other chemicals were of reagent grade quality.

Assay of LPL Activity——LPL activity was determined according to the previous paper.[7]

N-Bromosuccinimide Oxidation——The oxidation of LPL by NBS was carried out in a 10 mm cuvette at 37°. Two ml of 1.5×10^{-4} M enzyme dissolved in 0.1 M succinate buffer (pH 5.5) was incubated with an equal volume of various concentrations of NBS dissolved in the same buffer. After 15 min, the decrease in absorbance at 280 nm was measured and the extent of the tryptophan oxidation was calculated by Spande's method.[9]

Photooxidation——LPL from *Ps. fluorescens* was photooxidized in the presence of methylene blue according to the method of Weil and Seibles.[10] For this experiment, 0.5 ml of 0.0018—0.018% methylene blue dissolved in distilled water was added to a test tube containing 1 ml of 0.018—0.18% LPL and 0.5 ml of buffers with various pH values (pH 4—6, 0.5 M citrate buffer; pH 6—8, 0.5 M phosphate buffer; pH 8—9, 0.5 M Tris-HCl buffer) previously incubated at 37° for 5 min. Subsequently, the reaction mixture was irradiated with a 150-watt incandescent lamp at a distance of 15 cm at 37°. Aliquots were withdrawn from the reaction mixture at 2 hr intervals to determine the enzymatic activity and/or amino acid composition. The control experiment was carried out without methylene blue, and in complete darkness.

Amino Acid Analysis——Samples from the photooxidation experiment were gel-filtered to remove methylene blue on Sephadex G-25 (0.9 × 30 cm) previously equilibrated with 10 mM citrate buffer (pH 4.5) and protected from the light. The protein fraction obtained was dialyzed against distilled water, lyophilized, and weighed.

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The lyophilized samples were hydrolyzed for 24 hr at 110° in evacucated sealed tubes with 6 N HCl, and then hydrolysates were analyzed with a Nihondenshi amino acid analyzer, model JLC-6AH. The half-cystine content was determined by the method of Robyt *et al.*[11] The tryptophan content was determined by the method of Dalby and Tsai.[12] Methionine sulfoxide was determined according to the method of Neumann.[13] The lyophilized samples were hydrolyzed for 16 hr at 110° with 15% NaOH, and the hydrolysates were analyzed with the amino acid analyzer.

Results and Discussion

NBS Oxidation

LPL from *Ps. fluorescens* was previously shown to be strongly inhibited by NBS, at a molar ratio of NBS to LPL of 530.[7] Thus, the enzyme was oxidized with various concentrations of NBS in order to examine its behavior in further detail. The relationship between the remaining activity and the amount of oxidized tryptophan residue is shown in Fig. 1. At a molar ratio of NBS to LPL of 6, 50% oxidation of the tryptophan residues occurred. In the previous paper,[14] it was demonstrated that one mole of LPL contained two tryptophan residues. Thus, it was concluded that one mole of tryptophan residues was oxidized. However, the enzyme was not inactivated by NBS at the concentration used here. The present and previous data suggested that inactivation of LPL by NBS was not due to the oxidation of tryptophan, but another amino acid residue.

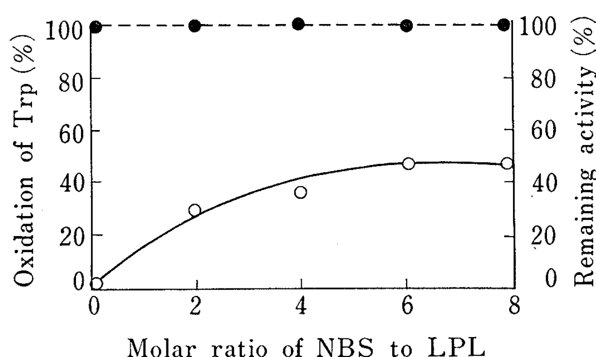

Fig. 1. The Relationship between the Oxidation of Tryptophan Residues and the Remaining Activity of Lipoprotein Lipase from *Pseudomonas fluorescens*

For details, see the text.
---●---: remaining activity, —○—: oxidation of Trp.

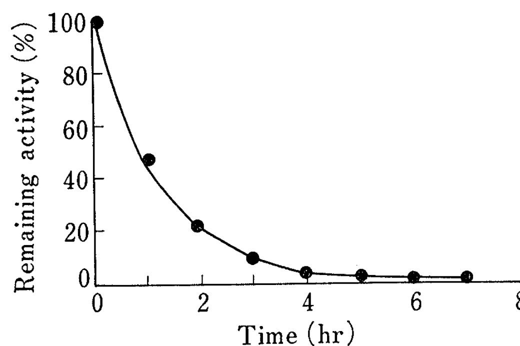

Fig. 2. The Inactivation of the Lipoprotein Lipase from *Pseudomonas fluorescens* by Photooxidation in the Presence of Methylene Blue

Photooxidation was performed at 37° with 0.172% protein and 0.0086% methylene blue in 0.125 M Tris-HCl buffer (pH 8.5).

Photooxidation

LPL was photooxidized in the presence of methylene blue. Aliquots of the reaction mixture were removed at various times to determine the remaining activity. As shown in Fig. 2, the enzyme was largely inactivated within 4 hr. The effect of pH on the inactivation of the enzyme by photooxidation was examined. As shown in Fig. 3, the inactivation depended on pH, and the apparent pK_a value of the inactivation curve was around 7, indicating that the amino acid residue involved had a pK_a of 7.0. Figure 4 shows the relationship of the number of histidine and tryptophan residues photooxidized to the extent of inactivation. About one histidine residue out of 5 in the enzyme was lost when 90% inactivation occured, and complete inhibition was observed when 1.9 mol of histidine residues and 0.7 mol of tryptophan residues had been lost. Thus, the photooxidation rate of tryptophan was slower than the inactivation rate of LPL, indicating that this residue is not significantly involved

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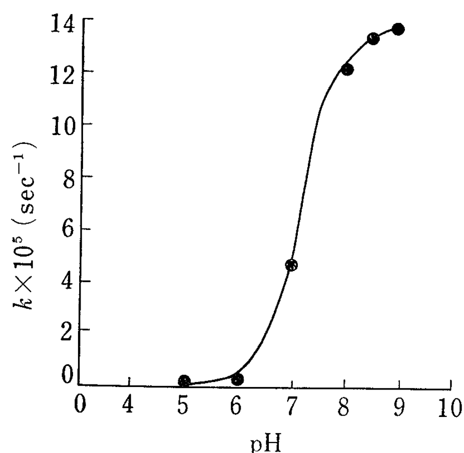

Fig. 3. pH Dependence of the Inactivation of Lipoprotein Lipase from *Pseudomonas fluorescens* by Methylene Blue-catalyzed Photooxidation

Photooxidation was performed at 37° for 2 hr with 0.063% protein and 0.0032% methylene blue.
k: the pseudo-first order rate constant for the inactivation of lipoprotein lipase.

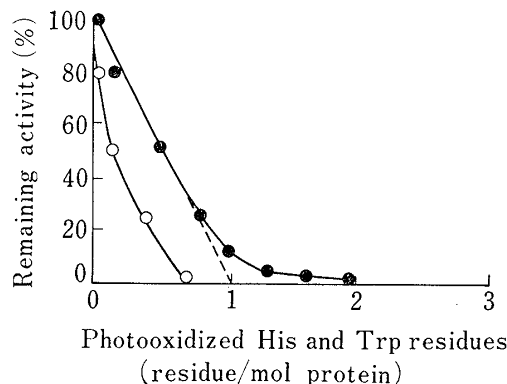

Fig. 4. The Relationship between the Lipoprotein Lipase Activity and the Amount of Photooxidized Histidine and Tryptophan Residues in the Lipoprotein Lipase from *Pseudomonas fluorescens*

For details, see the text.
—●—: His residue, —○—: Trp residue.

in the enzyme activity. In addition, no disapperance of other amino acid residues such as tyrosine was observed, and methionine sulfoxide formation was not detected. Thus, it appears that one histidine residue is specifically involved in the catalytic activity of LPL.

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Enhancement of Pulmonary Absorption of Fluorescein Isothiocyanate-Labelled Dextran (FITC-dextran) by Sodium Glycocholate in Rats

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The effects of a sulfonic acid dye and a surfactant on the pulmonary absorption of fluorescein isothiocyanate-labelled dextran (FITC-dextran), a model macromolecular compound (MW=150000) which is not absorbed after pulmonary administration, were studied. Bromphenol blue, a sulfonic acid dye, did not show an enhancing effect. However, sodium glycocholate (SGC) considerably enhanced the pulmonary absorption of FITC-dextran. Therefore, SGC may be a useful additive to increase the systemic availability of macromolecular compounds administered as aerosol dosage forms.

Keywords——absorption; intrabronchial administration; macromolecular compound; sodium glycocholate; rat

Recently, much attention has been paid to the feasibility of intrabronchial administration of drugs such as heparin,[2)] salbutamol,[3)] disodium cromoglycate,[4)] cyanocobalamin,[5)]

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