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Studies on Biotransformation of Lysozyme. I. Preparation of Labeled Lysozyme and Its Intestinal Absorption

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The preparation and stability of lysozyme labeled with ^{3}H or ^{131}I were studied. ^{131}I-lysozyme was found to be convenient for investigation on biotransformation of the enzyme.

The transport of ^{131}I-lysozyme across rat intestine *in vitro* was studied using rat everted jejunum. The transport of lysozyme from the mucosal to serosal side was confirmed by several methods such as TCA-precipitation, immunoprecipitation and enzymatic activity assay.

The intestinal absorption of ^{131}I-lysozyme *in vivo* was studied in rats paying attention to the lymphatics and the portal vein as the route of absorption. The absorption rate of immunoprecipitable ^{131}I *via* lymphatics was 0.07% of the dose during the first 6 hr after intraintestinal administration of 2 mg/kg of ^{131}I-lysozyme. The absorption rate of immunoprecipitable ^{131}I *via* portal vein was calculated as 1.99% of ^{131}I-lysozyme dosed, by pharmacokinetic analyses on the concentration of immunoprecipitable ^{131}I in serum. Absorption *via* lymphatics was 3.24% of the total immunoprecipitable ^{131}I absorbed *via* both routes. The main route in intestinal absorption of ^{131}I-lysozyme following intraintestinal administration was suggested to be the portal vein, not lymphatics.

Recently, many interesting investigations on the intestinal absorption of enzyme proteins have been demonstrated that a small amount of the enzyme protein administered orally is absorbed from the intestinal tract.[2,3] However, the metabolic fate and tissue distribution of enzyme proteins absorbed and also the pharmacological action of enzymes have not yet been clarified. Our previous reports[4] on the intestinal absorption of elastase labeled with ^{131}I have shown that a small amount of ^{131}I-labeled elastase administered intraintestinally is absorbed from the intestinal tract. Furthermore, the preceding papers[5] have shown the biotransformation of ^{131}I-labeled elastase administered intravenously in rats.

Lysozyme has been reported to be effective mainly for chronic sinusitis[6] and viral hepatitis.[7] However, whether or not lysozyme administered orally is absorbed from intestinal tract has not been fully known.

In order to gain an information on the biotransformation of lysozyme of which molecular weight was about 14300, the present paper deals with the investigations on the preparation

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and stability of lysozyme labeled with ^{3}H or ^{131}I and the intestinal absorption of ^{131}I-labeled lysozyme in rats *in vitro* or *in vivo*. In the intestinal absorption experiment, the attention to the lymphatics and the portal vein as the route of absorption was paid. A preliminary report of the intestinal absorption *in vivo* has been published.[8]

Experimental

Materials——Hen egg white lysozyme-hydrochloride (abbreviated as lysozyme) recrystallized five times was used. $Na^{131}I$ (Daiichi Radioisotope Lab.) and rabbit anti-guinea pig IgG serum (Miles Lab.) were used. Anti-lysozyme serum obtained from the guinea pig, to which 2.5 mg of lysozyme was intramuscularly administered for six days every other day.

Preparation of Radioactive Lysozyme——^{3}H-labeled lysozyme (abbreviated as ^{3}H-lysozyme) was prepared by the exposure of 200 mg of lysozyme with 5 Ci of ^{3}H gas at room temperature for three days according to Wilzbach method. One hundred fifty five mg of ^{3}H-lysozyme was purified by gel filtration on Sephadex G-50 and then on Sephadex G-25 column (1.5 × 100 cm) with 10 mM ammonium acetate (pH 6.8) as an effluent.

^{131}I-labeled lysozyme (abbreviated as ^{131}I-lysozyme) was prepared based on the modified method of McConahey and Dixon[9a] and Greenwood, *et al.*[9b] Fifty μl of chloramine-T (0.35 μmole) in 50 mM phosphate buffer (pH 7.5) was dropwise added to the solution of lysozyme (0.14 μmole) dissolved in 0.5 ml of aqueous solution of ^{131}I-NaI (0.14 μmole, 0.5 mCi) at room temperature. After stirring this solution for 1 min, 50 μl of sodium metabisulfite (1.26 μmoles) in 50 mM phosphate buffer (pH 7.5) was added to the reaction mixture. The mixture was charged on a Sephadex G-50 column (1.5 × 30 cm) in a cold chamber (4°) and then fractionated using 0.15M NaCl as effluent to obtain ^{131}I-lysozyme. The absorbance at 280 nm and radioactivity was measured in each eluted fraction.

The radiochemical and chemical yields of purified ^{3}H-lysozyme were 8.7% and 47.1%, respectively. Those of purified ^{131}I-lysozyme were 34.4—44.2% and 70—91%, respectively. The specific radioactivity of the former and the later were 4.4 μCi/mg and 97—123 μCi/mg, respectively.

Stability of Labeled Lysozyme——5 ml of ^{3}H-lysozyme or ^{131}I-lysozyme (2 mg/ml) enclosed in a visking seamless cellulose tube (18/32″) was dialysed in 100 ml of Krebs-Ringer phosphate buffer under continuous stirring at 37°. During dialysis, 0.1 ml of the internal or external phase was taken out at predetermined intervals, and their radioactivity and enzyme activity were assayed.

***In Vitro* Transport of ^{131}I-Lysozyme**——The transportation of ^{131}I-lysozyme across the everted jejunum was performed based on Crane and Wilson's method.[10] Male rats of Wistar strain (240—300 g) were fasted for 24 hr and killed by decapitation. The everted jejunum was prepared according to previous report.[4a] The sac of rat jejunum, into which 1 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) containing 0.1% glucose was poured, was placed in a test tube containing 10 ml of 0.01% or 0.1% of ^{131}I-lysozyme in Krebs-Ringer solution. For the control experiments, Krebs-Ringer solution was added in mucosal side instead of ^{131}I-lysozyme. These test tubes were kept in an incubator at 37° for 2.5 hr, and a gas mixture consisting of O_2: CO_2 (95: 5, v/v) was bubbled into the mucosal fluid. In order to observe the time course of intestinal transport, 50 μl of serosal fluid was taken out every 30 min for 2.5 hr.

***In Vivo* Absorption of ^{131}I-Lysozyme**——Male rats of Wistar strain (230—290 g) were given 1 mM KI aqueous solution instead of water and fasted for 24 hr prior to experiments. The thoracic duct was cannulated as described previously.[4b] Following the injection of 2 mg of ^{131}I-lysozyme per kg into the lumen of upper jejunum of the rat, lymph was collected through the polyethylene tube at predetermined intervals during the first 6 hr and then blood was obtained by cardiac puncture. In order to determine the disappearance of lysozyme from blood and the transfer rate from blood to lymph, ^{131}I-lysozyme (0.4 mg/kg) was injected intravenously. The lymph and blood samples collected were centrifuged at 3000 rpm for 30 min and the supernatants were assayed.

Radioactivity Determinations——The counting of ^{3}H was done in a liquid scintillation counter (Aloka LSC-601) and that of ^{131}I was determined in a well-type scintillation counter (Aloka JDC-207).

Measurement of Enzyme Activity——Enzyme activity of lysozyme was determined with *Micrococcus Lysodeikticus* as a substrate based on Gorin's method.[11] The enzyme concentration in serum after injection of ^{131}I-lysozyme was calculated on the basis of the calibration curve which was obtained by the assay of various amounts of ^{131}I-lysozyme added to the constant volume of rat serum. In this method, the enzyme activity more than 0.5 μg of lysozyme per ml serum can be measured.

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11) G. Gorin, S.F. Wang and L. Papapavlou, *Anal. Biochem.*, **39**, 113 (1971).

Immunoprecipitation——Immunoprecipitable ^{131}I of serosal fluid, serum and lymph samples were assayed as follows. To conical centrifuge tube, the following solutions were added in succession; (1) 0.1 ml of serosal fluid, serum or lymph sample. (2) 0.1 ml of guinea pig anti-lysozyme serum diluted 1/100 with 10 mM phosphate buffer (pH 7.4) containing 0.15M NaCl and 0.5% bovine serum albumin. (3) 0.1 ml of rabbit anti-guinea pig IgG serum. After the tube was shaken and then kept at 4° for 24 hr, the precipitate separated by centrifugation was washed 3 times with 0.5 ml of 0.15M NaCl. The precipitate collected was dissolved in 0.5 ml of 0.1M NaOH and its radioactivity was counted. The recovery of radioactivity of ^{131}I-lysozyme added to rat serum or lymph was 95.7±0.5% (mean±S.E.) in the concentration range of 0.007—1.5550 μg ^{131}I-lysozyme per ml.

TCA-Precipitation——TCA-precipitable ^{131}I was determined in the final 5% solution of trichloroacetic acid (TCA) as described previously.[4a)]

Electrophoreses and Paper Chromatography——Electrophoresis was conducted in 70 mM sodium diethylbarbiturate buffer (pH 8.6) with a potential gradient of 0.6 mA/cm for 1 hr using a Fujiox model AS-D cellulose acetate apparatus.

Immunoelectrophoresis was carried out using the modified method of Grunbaum and Dong.[12)]

Paper chromatography was performed on Toyo filter (No. 50) using 95% ethanol–2M ammonia (9:1, v/v) as a solvent.

Results

Properties of Labeled Lysozyme

Both the purified lysozyme labeled with ^{3}H or ^{131}I were found to have the same enzyme activity with that of the starting lysozyme and to have a single radioactivity peak by electrophoresis, immunoelectrophoresis and paper chromatography. Based on these facts, the radiochemical and enzymatic purities were more than 99.8%, respectively.

Stability of Labeled Lysozyme

Fig. 1 demonstrates the radiochemical and enzymatic stability of labeled lysozymes during 8 hr dialysis at 37°.

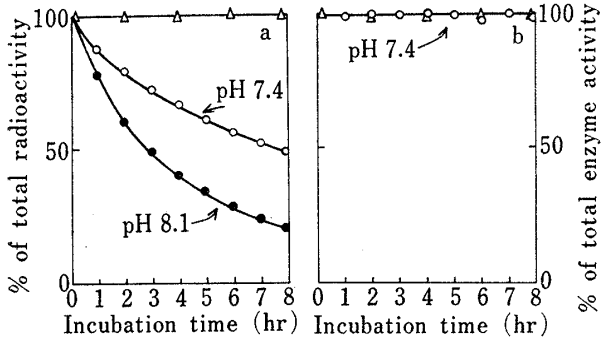

Fig. 1. Stability of Labeled Lysozyme during 8 hr Dialysis at 37°

Dialyses: seamless cellulose tubing, ϕ 14.3 m/m (18/32″); inner., 5 ml of (a) ^{3}H-lysozyme or (b) ^{131}I-lysozyme(2 mg/ml)in Krebs-Ringer phosphate buffer; outer., 100 ml of Krebs-Ringer phosphate buffer. —○—,—●—: radioactivity, —△—: enzyme activity

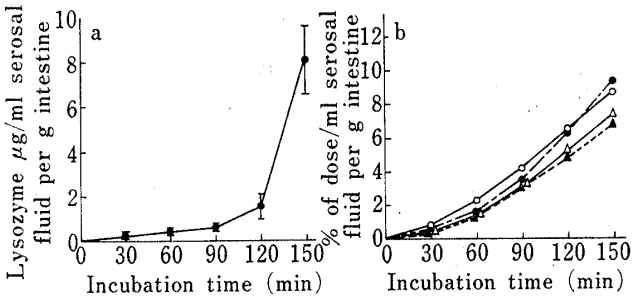

Fig. 2. *In Vitro* Transport of ^{131}I-Lysozyme across Everted Rat Intestine during the Incubation at 37°

(a) Time course of enzyme activity of native rat lysozyme appearing from intestine to serosal fluid. Data are represented as the mean with the S.E. of 3 experiments. (b) Time Course of transport rate of ^{131}I-lysozyme. Conditions: jejunum 7cm; mucosal fluid, 10ml of 0.1% ^{131}I-lysozyme in Krebs-Ringer bicarbonate (pH 7.4) containing 0.1 % glucose; serosal fluid, 1 ml of Krebs-Ringer solution.

—○—: radioactivity, —●—: enzyme activity, —△—: TCA-precipitable ^{131}I, —▲—: immunoprecipitable ^{131}I

The radioactivity of ^{3}H-lysozyme placed in the seamless cellulose tube during dialysis at pH 7.4 and 8.1 decreased with time lapse, respectively, although the enzyme activity was constant. The remaining radioactivity at pH 7.4 and 8.1 after dialysis for 8 hr was 49% and 21% of the initial radioactivity, respectively. In contrast, no decrease of both enzyme activity and radioactivity of ^{131}I-lysozyme in the inner fluid was observed in the same

12) B.W. Grunbaum and L. Dong, *Nature*, **194**, 185 (1962).

conditions. Accordingly, ^{131}I-lysozyme was used for the further investigations on the intestinal absorption of the enzyme.

In Vitro Transport of ^{131}I-Lysozyme

Fig. 2a shows the time course of enzyme activity concentration in serosal fluid in the control experiment.

The result indicates that the endogenous rat lysozyme was released from intestinal wall to the serosal fluid. In order to know the true transport of ^{131}I-lysozyme, the apparent concentration of enzyme activity in the serosal fluid, therefore, was corrected by the value originated from endogenous lysozyme at each time interval in Fig. 2a during the incubation with ^{131}I-lysozyme.

The data on the time course of the concentration determined by immunoassay and the other analyses are presented in Fig. 2b. The results obtained by these methods were approximately in agreement with each other. These concentrations in the serosal fluid were already observed 30 min after the initiation of incubation at 37° and thereafter the concentrations in serosal fluid increased with time. The transfer rate of TCA-precipitable ^{131}I and immunoprecipitable ^{131}I in the incubation for 2 hr was approximately 5%, respectively. This was the same rate with both 0.1% and 0.01% ^{131}I-lysozyme.

In Vivo Absorption of ^{131}I-Lysozyme

Fig. 3 shows the concentration of TCA-precipitable and immunoprecipitable ^{131}I in serum and lymph as lysozyme eq μg per ml after intraintestinal administration of ^{131}I-lysozyme (2 mg/kg).

As shown in Fig. 3, the levels of TCA-precipitable and immunoprecipitable ^{131}I in both serum and lymph were observed to be approximately in accordance with each other. The

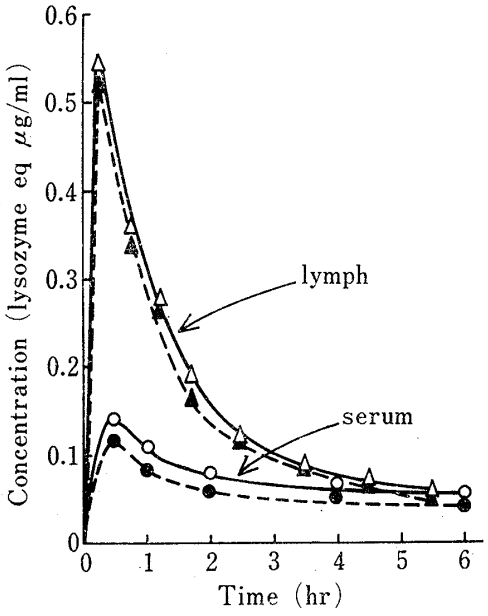

Fig. 3. *In Vivo* Intestinal Absorption following Intraintestinal Administration of 2 mg ^{131}I-Lysozyme per kg in Rats

The mean concentrations of 4 experiments in serum and lymph levels of immunoprecipitable ^{131}I (▲, ●) or TCA-precipitable ^{131}I (△, ○) were represented as lysozyme eq μg per ml.

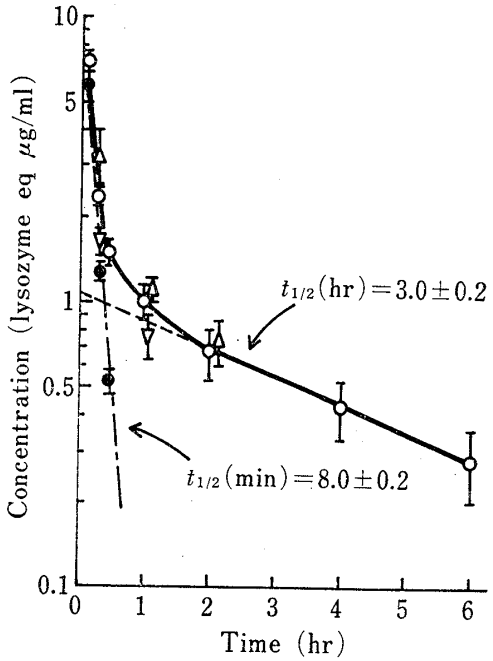

Fig. 4. Serum Level of Immunoprecipitable ^{131}I (o) after Intravenous Injection of 0.4 mg ^{131}I-Lysozyme per kg in Rats

The serum level is contrasted also with TCA-precipitable ^{131}I (△) and enzyme activity (▽) assayed at same time. Data are represented as the mean with the S.E. of 3 experiments.

serum and lymph levels increased rapidly, reached the maximum levels at 30 min and thereafter decreased. The maximum level observed in lymph, 0.529±0.106 μg/ml, was approximately 5 times higher than that in serum, 0.112±0.016 μg/ml.

Fig. 4 shows the semilogarithmic plots of immunoprecipitable ^{131}I in serum *vs.* time after intravenous injection of ^{131}I-lysozyme (0.4 mg/kg).

As shown in Fig. 4, the serum concentration of immunoprecipitable ^{131}I, which was in good accordance with that of TCA-precipitable ^{131}I or enzyme activity, declined biexponentially with two different half lives of 8.0 min and 3.0 hr.

Fig. 5 presents the transfer rate and cumulative amounts of immunoprecipitable and TCA-precipitable ^{131}I in thoracic duct lymph after intraintestinal (2 mg/kg, Fig. 5a) and intravenous (0.4 mg/kg, Fig. 5b) administration of ^{131}I-lysozyme.

As shown in Fig. 5b, the cumulative amount of immunoprecipitable ^{131}I in lymph after intravenous injection of ^{131}I-lysozyme was only 1% of the dose during the first 6 hr. This finding demonstrates that the transfer of ^{131}I-lysozyme from blood to lymph is negligible. Accordingly, TCA-precipitable and immunoprecipitable ^{131}I in lymph following intraintestinal administration might be mainly due to a direct absorption *via* lymphatics.

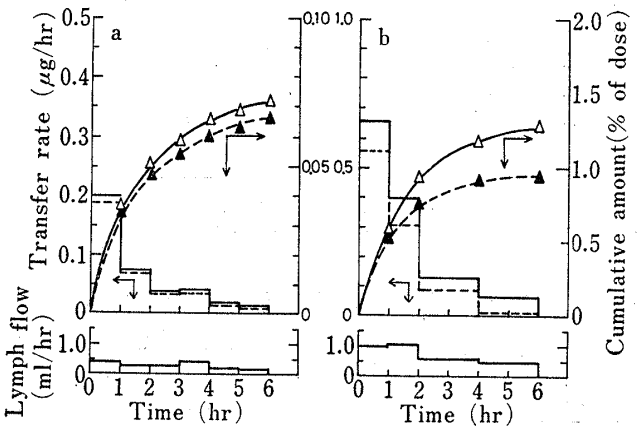

Fig. 5. Transfer Rate and Cumulative Amount of Immunoprecipitable ^{131}I (▲) and TCA-precipitable ^{131}I (△) in Thoracic Duct Lymph after Administration of ^{131}I-Lysozyme

(a) 2 mg/kg administered intraintestinally, (b) 0.4 mg/kg injected intravenously. Each value is represented as the mean with the S.E. of 4 experiments.

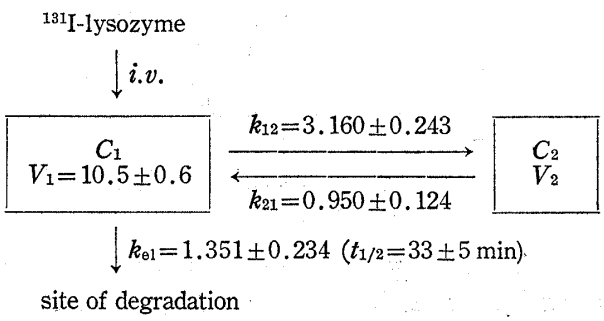

Fig. 6. Two-Compartment Model postulated for Kinetic Analyses of Serum Level of Immunoprecipitable ^{131}I in Fig. 4

Compartment 1 is the central compartment of which blood is considered to be a part and Compartment 2 is the peripheral compartment. k_{12}, k_{21} and k_{e1} are first-order rate constants for the designated processes, and V is the volume of distribution. unit of k, hr^{-1}; unit of V, ml. mean±S.E. ($n=3$).

Discussion

From the experiment of *in vitro* transport of ^{131}I-lysozyme, it is suggested that this enzyme is transported across the intestinal wall and the concentration as lysozyme eq in the serosal fluid which was detected by several analytical methods might be the protein originated from ^{131}I-lysozyme added in the mucosal side before incubation. In this experiments, there were no monitoring of the viability of everted sac over the period of incubation. However, for *in vitro* transport of elastase,[4a] the viability of isolated intestine has been ascertained and similar result has been reported by Kimura.[2b] Since Fig. 2a shows a marked discharge of rat native lysozyme into the serosal fluid after 2 hr, the viability of everted sac seems to be assured until incubation 2 hr.

The findings of TCA-precipitable and immunoprecipitable ^{131}I in both lymph and serum after intraintestinal administration of ^{131}I-lysozyme indicate the possibility of intestinal absorption of lysozyme *in vivo*. Since the serum and lymph levels attained the maxima at 30 min after administration and thereafter decreased (Fig. 3), it is suggested that the absorp-

tion rate of ^{131}I-lysozyme is rapid. This finding is in agreement with the report of Ishihara and Murachi[13] using the *in situ* mesentric perfusion method.

In order to calculate the amount of absorption, parameters obtained from the data of serum concentration of immunoprecipitable ^{131}I after intravenous injection of ^{131}I-lysozyme. The serum levels (Cp) shown in Fig. 4 were fitted to the equation, $Cp=A\exp(-\alpha t)+B\exp(-\beta t)$, by the least-square method. The parameters of A, B, α and β were 6.57±0.32, 1.10±0.22 μg per ml, 5.225±0.138 and 0.236±0.017 hr^{-1}, respectively. Fig. 6 is a two-compartment model postulated for kinetic analyses of the present data.

The amount of immunoprecipitable ^{131}I absorbed *via* the portal vein was calculated with the parameters according to the method of Loo and Riegelman.[14] The amounts of absorption *via* lymphatics and portal vein, and the percentages of absorption *via* lymphatics to the total immunoprecipitable ^{131}I absorbed are shown in Table I.

Table I. Absorption of Immunoprecipitable ^{131}I *via* Portal Vein and Lymphatics after Intraintestinal Administration of 2 mg ^{131}I-Lysozyme per kg in Rats

Absorption	Lysozyme eq μg	% of the dose
Amount *via* portal vein (A)	10.61±2.40	1.99±0.41
Amount *via* lymphatics (B)	0.37±0.18	0.07±0.04
Amount *via* both routes ($A+B$)	10.98±2.45	2.06±0.43
Percentage of lymphatics $[100\times(B)/(A+B)]$	3.24±1.69	

Absorption amounts of immunoprecipitable ^{131}I by both portal vein and lymphatic route were calculated with the data in Fig. 3 as lysozyme eq. Each value is represented as the mean with the S.E. of 4 experiments.

The average absorption amounts *via* the portal vein and lymphatics during the first 6 hr after intraintestinal administration of 2 mg/kg of ^{131}I-lysozyme were 10.61 μg and 0.37 μg as lysozyme eq, respectively. Therefore, the total amount absorbed *via* both routes was 10.98 μg, which corresponded to 2.06% of the dose. The absorption rate of ^{131}I-lysozyme was found to be relatively higher than that of ^{131}I-elastase (1 mg/rat), 0.15% of the dose.[4b]

The percentage of absorption *via* lymphatics to the total absorbed immunoprecipitable ^{131}I was found to be 3.24%. The result indicates that the main route in intestinal absorption of ^{131}I-lysozyme is the portal vein, not lymphatics. The result seems to be in accordance with the facts that heparin[3a] and insulin[3b] administered orally passed directly into the blood. However, as compared with the results that 36% of the total amount of elastase[4b] absorbed and 40.6—56.5% of the total amount of ^{3}H-labeled bovine serum albumin[3c] absorbed were *via* lymphatics, the percentage of lymphatic absorption of ^{131}I-lysozyme is extremely small. Thus, further studies on the relationship between the physicochemical properties of macromolecules and the absorption rate *via* lymphatics will be necessary. In addition, whether or not the immunoprecipitable and protein-bound ^{131}I in serum and lymph originates from intact ^{131}I-lysozyme administered remains to be elucidated.

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14) J.C.K. Loo and S. Riegelman, *J. Pharm. Sci.*, **57**, 918 (1968).