[Chem. Pharm. Bull. 23(6)1315—1322(1975)]

UDC 547.466.1.04 : 542.98 : 615.36.033

Studies on Biotransformation of Lysozyme. II.[1] Tissue Distribution of ^{131}I-Labeled Lysozyme and Degradation in Kidney after Intravenous Injection in Rats

Teruaki Yuzuriha, Kouichi Katayama and Takeshi Fujita

Department of Drug Metabolism, Section of Experimental Therapeutics Research, Research & Development Division, Eisai Co., Ltd.[2]

(Received November 21, 1974)

The tissue distribution of TCA-precipitable ^{131}I following intravenous injection of 0.4 mg lysozyme labeled with ^{131}I per kg in rats was investigated. The renal concentration of TCA-precipitable ^{131}I was the highest in all the tissues tested, and the level in the kidney reached the maximum of 26.5% of the dose 30 min after injection. Sephadex G-50 gel filtration profiles of radioactivity solubilized by Triton X-100 demonstrated that the radioactivity in kidney was mainly in the form of ^{131}I-lysozyme.

The intracellular distribution of TCA-precipitable ^{131}I in the kidney was studied by the differential centrifugation of kidney homogenates during the first 3 hr after intravenous injection of ^{131}I-lysozyme in rats. The specific radioactivity in the $19600 \times g$ (20 min) fraction of kidney was found to be the highest among another fractions. The protein-bound radioactivity detected in the $19600 \times g$ fraction of kidney 30 min after injection was suggested to be present in phagolysosomes by sucrose density gradient centrifugation and solubilization with Triton X-100.

The degradation of injected ^{131}I-lysozyme by the $19600 \times g$ fraction or phagolysosomes was examined, in succession. The pH-degradation profile of the $19600 \times g$ fraction was a monopeak with the optimum at pH 5.0. Based on the observation of the activation by cysteine and the inhibition by iodoacetamide, it was indicated that the proteolytic enzyme with the optimal pH of 5.0 in phagolysosomes is cathepsin B_1. The main degradation product of injected ^{131}I-lysozyme in the phagolysosomes was identified as ^{131}I-diiodotyrosine by paper chromatography. In contrast, the radioactivity excreted in urine was mainly inorganic $^{131}I^-$.

Our previous reports[1,3] on the intestinal absorption of ^{131}I-labeled lysozyme (2 mg/kg) in rats have shown that approximately 2% of the dose is absorbed from the intestinal tract, and the main route in intestinal absorption of the enzyme is the portal vein, not lymphatics.

Perri, *et al.*[4] showed that exogenous or endogenous lysozyme was accumulated within the kidney. Sussman, *et al.*[5a] studying the intrarenal distribution of lysozyme in normal rats, found higher concentrations in the cortex than in the medulla. Maack, *et al.*[5b] studied the intrarenal distribution of injected ^{125}I-labeled egg white lysozyme in mice using autoradiography and analyses of renal subcellular fractions. Hansen, *et al.* have studied lysozyme turnover and renal uptake in the rat using ^{125}I-labeled rat lysozyme,[6a] and plasma lysozyme turnover in normal man and patients with diseases known to increase plasma lysozyme using ^{125}I-labeled human lysozyme.[6b] Royce[7a] and Maack, *et al.*[7b] found the formation of cyto-

1) Part I: T. Yuzuriha, K. Katayama and T. Fujita, *Chem. Pharm. Bull.* (Tokyo), **23**, 1309 (1975).
2) Location: *Koishikawa, Bunkyo-ku, Tokyo.*
3) T. Yuzuriha, K. Katayama and T. Fujita, *Chem. Pharm. Bull.* (Tokyo), **21**, 2807 (1973).
4) G.C. Perri, M. Faulk, E. Shapiro and W.L. Money, *Proc. Soc. Exptl. Biol. Med.*, **115**, 189 (1964).
5) *a*) M. Sussman, A.W. Asscher and J.A.S. Jenkins, *Invest Urol.*, **6**, 148 (1968); *b*) T. Maack, D.D.S. Mackensie and W.B. Kinter, *Am. J. Physiol.*, **221**, 1609 (1971).
6) *a*) N.E. Hansen, H. Karle and V. Andersen, *J. Clin. Invest.*, **50**, 1473 (1971); *b*) N.E. Hansen, H. Karle, V. Andersen and K. ϕlgaard, *ibid.*, **51**, 1146 (1972).
7) *a*) P.C. Royce, *Am. J. Physiol.*, **212**, 924 (1967); *b*) T. Maack, R. Brentani and M. Rabinovitgh, *Nature*, **186**, 158 (1960); *c*) S. Shibko and A.L. Tappel, *Biochem. J.*, **95**, 731 (1965).

plasmic droplets in proximal tubular cells of rat following intraperitoneal injection of egg white lysozyme. Shibko and Tappel,[7c] in studies of subcellular fractions of normal rat kidney, have primarily found lysozyme in the lysosomal fraction.

In order to obtain more information about the biotransformation of ^{131}I-labeled lysozyme, the present study deals with the tissue and intracellular distribution of TCA-precipitable ^{131}I after intravenous injection of ^{131}I-labeled lysozyme in rats. Furthermore, the degradative activity of injected ^{131}I-labeled lysozyme in kidney were studied.

Experimental

Materials——Hen egg white lysozyme-hydrochloride (abbreviated as lysozyme) recrystallized five times was used for ^{131}I-labelling. ^{131}I-labeled lysozyme (abbreviated as ^{131}I-lysozyme) with the specific radioactivity of 100—113 μCi/mg was prepared by the method of McConahey and Dixon[8a] and Greenwood, *et al.*,[8b] as described previously.[1] Triton X-100 (Wako Pure Chemical Industries), bovine albumin (Armour Pharmaceutical Co.), glucose-6-phosphoric acid (B.D.H. Chemicals), β-glycerophosphate (Merck), cytochrome c (Type VI, Sigma Chemical Co.), cysteine (Nippon Rikagakuyakuhin Co.), iodoacetamide (Tokyo Chemical Industry Co), 3-iodo-L-tyrosine (Sigma Chemical Co.) and 3,5-diiodo-L-tyrosine (Wako Pure Chemical Industries) were used.

Tissue Distribution of ^{131}I-Lysozyme——Male rats of Wistar strain (180—280 g) were allowed to drink 1 mM KI aqueous solution in place of water for 4 days *ad libitum* and fasted for 24 hr prior to experiment. A 0.15M NaCl solution of ^{131}I-lysozyme was injected into the femoral vein at a dose of 0.4 mg per kg.

Rats were sacrificed by decapitation 5 min, 15 min, 30 min, 1 hr, 3 hr, 6 hr and 12 hr after injection of ^{131}I-lysozyme. Tissues were removed, rinsed with 0.15M NaCl and blotted with filter paper. After the tissue was homogenized in 3 vol. (v/w) of 0.25M sucrose using a Potter-Elvehjem type homogenizer with a Teflon pestle at 2000—2500 rpm with 3 strokes, the radioactivity in each homogenate was assayed by TCA-precipitation in a final concentration of 5% trichloroacetic acid (TCA) as described previously.[9]

Sephadex G-50 Gel Filtration of Radioactivity in Kidney and Liver——The kidney and liver, 30 min after intravenous injection of ^{131}I-lysozyme, were perfused *in situ* with cold 0.15M NaCl and then removed. The tissue was homogenized at 10% (w/v) in 0.2M KCl at 1200 rpm with 4 strokes. The tissue homogenates were solubilized by final 1% (w/v) Triton X-100 and then centrifuged at $105000 \times g$ for 1 hr at 4°. Five ml of the supernatant, in which approximately 88% of total radioactivity in the homogenate were recovered, was concentrated by addition of appropriate amount of dry Sephadex G-25. The concentrated supernatant was layered on a 1.5×30 cm column of Sephadex G-50 and then eluted with 0.15M NaCl. One ml of each fraction collected was assayed for radioactivity, immunoprecipitable ^{131}I, lysozyme activity and optical density as described previously.[1]

Subcellular Fractionation of Kidney——The kidney, during the first 3 hr after intravenous injection of ^{131}I-lysozyme, was perfused *in situ* with cold 0.15M NaCl and then removed. Tissue was homogenized in 3 vol. (v/w) of 0.2M KCl at 1200 rpm with 4 strokes. The kidney homogenate was fractionated at 4° by differential centrifugation according to the method of Straus.[10] The homogenate was centrifuged at $56 \times g$ for 5 min and the resulting pellet was discarded. The $56 \times g$ supernatant was centrifuged at $814 \times g$ for 20 min and the resulting supernatant was centrifuged at $19600 \times g$ for 20 min, and then the $19600 \times g$ supernatant was centrifuged at $105400 \times g$ for 45 min.

After all pellets had been homogenized in 4 vol. (v/w) of 0.2M KCl at 800 rpm with 1 stroke, the radioactivity in each fraction was analyzed by TCA-precipitation and the protein was measured. The fraction sedimented at $19600 \times g$ was further centrifuged with sucrose density gradient and was treated with Triton X-100.

Determination of Protein and Enzymatic Activity——Protein was determined with bovine albumin as a standard by the method of Lowry, *et al.*[11] Acid phosphatase was measured with β-glycerophosphate in the presence of 0.1% Triton X-100 by the method of Wattiaux and De Duve,[12] cytochrome oxidase was according to the method reported by Cooperstein and Lazarow[13] and glucose-6-phosphatase was by the method of Harper.[14]

8) *a*) P.J. McConahey and F.J. Dixon, *Int. Arch. Allergy*, **29**, 185 (1966); *b*) F.C. Greenwood, W.M. Hunter and J.S. Glover, *Biochem. J.*, **89**, 114 (1963).
9) K. Katayama and T. Fujita, *Biochim. Biophys. Acta*, **336**, 178 (1974).
10) W. Straus, *J. Cell Biol.*, **12**, 231 (1962).
11) O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
12) R. Wattiaux and C.De Duve, *Biochem. J.*, **63**, 606 (1956).
13) S.J. Cooperstein and A. Lazarow, *J. Biol. Chem.*, **189**, 665 (1951).
14) A.E. Harper, "Methods of Enzymatic Analysis," ed. by Bergmeyer, Academic Press, New York and London, 1963, pp. 788—792.

Sucrose Density Gradient Centrifugation——The fraction sedimented at 19600×*g* was homogenized in 2 vol. (v/w) of 0.25M sucrose. One half ml of this homogenate was used on sucrose density gradient centrifugation according to the method as described previously.[9]

Solubilization of the 19600×*g* Fraction——After 0.5 ml of various concentrations of Triton X-100 in 0.2M KCl was added to 0.5 ml of the homogenate of the 19600×*g* fraction in 0.2M KCl, the mixture was left for 30 min in an ice-cold water bath. Then the mixture was centrifuged at 19600×*g* for 20 min and the supernatant was assayed.

Determination of Degradative Activity——The isolated particles of kidney 30 min after intravenous injection of ^{131}I-lysozyme were homogenized in 2 vol. (v/w) of 0.25M sucrose and then an equal volume of 0.1M buffer (see below) containing 0.25M sucrose was added to the homogenate. After incubation at 37° for 4 hr, an equal volume of the 10% (w/v) TCA solution was added to the homogenate and the mixture was centrifuged. The radioactivities of supernatant and precipitate were determined. The radioactivity recovered in the supernatant against the total radioactivity was expressed as TCA-soluble ^{131}I as a measure of the degradative for injected ^{131}I-lysozyme.

Effect of pH on Degradative Activity——One half ml of 0.1M buffer at various pH values were added to 0.5 ml of the homogenate of the 19600×*g* fraction. The mixture was incubated for 4 hr at 37°. The 0.1M buffers used were as follows; glycine-HCl (pH 2.2—3.2), citrate (pH 3.6—5.4), phosphate (pH 5.8—7.4) and Tris (pH 8—9). The pH values of the mixtures were measured.

Effect of Activator and Inhibitor on Degradative Activity——After cysteine or iodoacetamide was added to the 19600×*g* fraction, the degradative activity was assayed at pH 5.0.

Paper Chromatography——Paper chromatography was carried out on Toyo filter paper No. 50 with 95% ethanol–2M ammonia (9: 1, v/v) and *n*-butanol–acetic acid–H_2O (4: 1: 5, by vol., upper phase) as developing solvents. Na^{131}I, 3-iodo-L-tyrosine and 3,5-diiodo-L-tyrosine were used as references.

Results

Tissue Distribution of TCA-Precipitable ^{131}I following Intravenous Injection of ^{131}I-Lysozyme

After intravenous injection of 0.4 mg ^{131}I-lysozyme per kg, the tissue concentration of TCA-precipitable ^{131}I was represented as ^{131}I-lysozyme eq μg per g of wet tissue and is shown in Fig. 1.

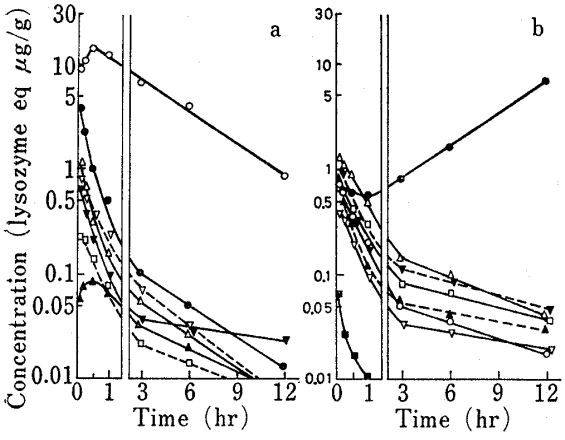

Fig. 1. Tissue Distribution of TCA-Precipitable ^{131}I after Intravenous Injection of 0.4 mg ^{131}I-Lysozyme per kg in Rats

Mean concentration of 3 experiments was represented as lysozyme eq μg/g of tissue. (a) —○— : kidney, —●— : serum, ···▽··· : heart, —△— : bone marrow, —▼—: pancreas, —▲—: testis, ···□···: muscle (b) —●—:thyroid gland, —△—:lung, ···▼···:stomach, —□— : spleen, —○— : liver, ···▲··· : adrenal gland, —▽—: intestine, —■—: brain

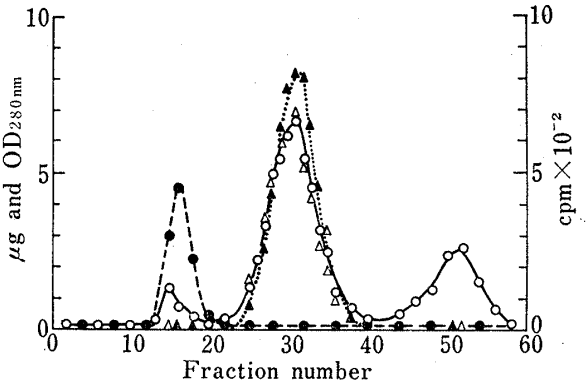

Fig. 2. Sephadex G-50 Gel Filtration Pattern of Radioactivity in Kidney 30 min following Intravenous Injection of 0.4 mg ^{131}I-Lysozyme per kg in Rat

Size of fraction eluted with 0.15 M NaCl was 1 ml and the void volume of the column was 15 ml. One tenth ml of each fraction was assayed.
···▲···: enzyme activity, ···●···: optical density, —○—: radioactivity, —△—: immunoprecipitable ^{131}I

The time course of tissue levels in kidney and testis attained the maximum 30 min after injection and then declined monoexponentially. In contrast, the concentrations of TCA-precipitable ^{131}I in serum and the other tissues except for thyroid gland reached the peaks

5 min or less after injection and decreased biexponentially. The pattern in thyroid gland, in which the level increased with time lapse, was similar to that following intravenous injection of K^{131}I. Therefore, the increase seems not to be in the form of ^{131}I-lysozyme.

The content of TCA-precipitable ^{131}I in kidney 30 min after injection was 26.5% of the dose, and the content in liver 5 min following injection was found to be 7.5% of the dose.

Nature of Radioactivity in Kidney

Fig. 2 shows a Sephadex G-50 gel filtration pattern of the radioactivity in kidney 30 min after intravenous injection of 0.4 mg ^{131}I-lysozyme per kg in rats.

As shown in Fig. 2, the elution pattern of radioactivity demonstrated three peaks. In the first peak eluted at the void volume, 6.2% of the total radioactivity was recovered, but both of the immunoprecipitable ^{131}I and lysozyme activity were not detected in the effluent of the first peak. The radioactivity in the second peak, in which hen egg white lysozyme was eluted, was 69.9% of the total and completely precipitated by anti-lysozyme antibody. Since the concentration (8.20 μg as lysozyme/0.1 ml) of enzymatic activity in the fraction No. 30 was approximately 100 times higher than that (0.08 μg as lysozyme/0.1 ml) of radioactivity, most of enzymatic activity in the second peak may originate from native lysozyme of rat kidney. Immunoprecipitation and lysozyme activity were not observed in the third peak of radioactivity corresponding to 23.9% of the total radioactivity applied. Accordingly, the radioactivity in the third peak seems to be the degradative products of injected ^{131}I-lysozyme.

The gel filtration of the radioactivity solubilized from the liver 30 min after the intravenous injection also showed a pattern quite similar to that of the kidney.

Intracellular Distribution of TCA-Precipitable ^{131}I in Rat Kidney after Intravenous Injection of ^{131}I-Lysozyme

In order to determine the predominant composition of the four fractions based on the Straus's method,[10] the relative specific activity of cytochrome oxidase as a mitochondrial marker, acid phosphatase as a lysosomal marker and glucose-6-phosphatase as a microsomal marker were measured in each of the 814×g, 19600×g, 105400×g and the final supernatant. Fig. 3 shows the renal intracellular distribution of those marker enzymes.

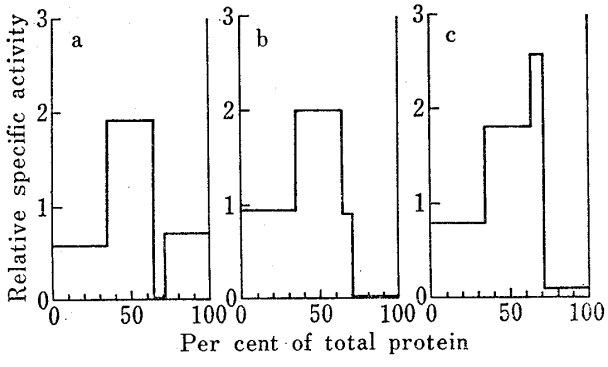

Fig. 3. Distribution Pattern of Acid Phosphatase (a), Cytochrome Oxidase (b) and Glucose-6-Phosphatase (c) in Rat Kidney Homogenate

Fraction are plotted in the order in which they were isolated, *i.e.* from left to right; 814×g, 19600×g, 105400×g, and the final supernatant. Each fraction is represented separately on the ordinate by relative specific activity. On the abscissa each fraction is represented by its percentage of the total protein in the homogenate. Data are expressed as the mean (n=3).

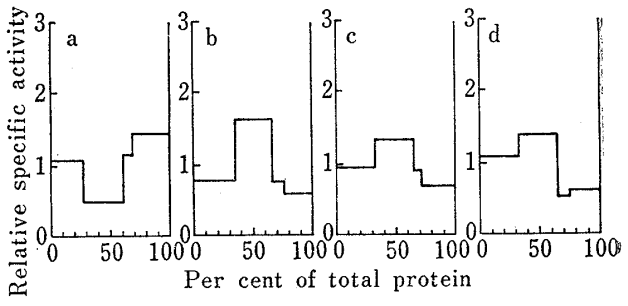

Fig. 4. Distribution Pattern of TCA-Precipitable ^{131}I in Kidney Homogenate after Intravenous Injection of 0.4 mg ^{131}I-Lysozyme per kg in Rat

(a) ^{131}I-lysozyme in 0.15M NaCl solution added to kidney homogenate of untreated rat. (b) 5 min, (c) 30 min and (d) 3 hr after the injection. Relative specific activity = % of radioactivity in each fraction per that of protein in each fraction. Data are expressed as the mean (n=3).

As indicated in Fig. 3, the distribution of acid phosphatase was the highest in the 19600×*g* fraction, in which approximately 59% of the total kidney homogenate were recovered. The distribution of cytochrome oxidase was also fairly concentrated in the 19600×*g* fraction. On the other hand, the relative specific activity of glucose-6-phosphatase in the 105400×*g* fraction was the highest among all the fractions.

Fig. 4 shows the renal intracellular distribution of TCA-precipitable ^{131}I 5 min, 30 min and 3 hr after the injection, in comparison with the case of the addition of approximately 14 μg ^{131}I-lysozyme to the homogenate of kidney (1 g) of untreated normal rat.

In Fig. 4a, 44.6% of the lysozyme added to the homogenate were recovered in the final supernatant, while the distribution of radioactivity in the 814×*g*, 19600×*g* and 105400×*g* fractions may be due to the adsorption of ^{131}I-lysozyme. In Fig. 4b, 4c, 4d, the intracellular distribution pattern of TCA-precipitable ^{131}I to renal particles during 3 hr after the injection was the highest in the 19600×*g* fraction and markedly differed from the distribution pattern in the case of addition of ^{131}I-lysozyme to homogenate.

Nature of the 19600×*g* Fraction containing TCA-Precipitable ^{131}I

As shown in Fig. 3 and 4, the distribution of radioactivity in the 19600×*g* fraction corresponding to the mitochondria-lysosome fraction was the highest in all the fractions.

Then, the 19600×*g* fraction of kidney 30 min after the injection was analyzed by density gradient centrifugation. Fig. 5 shows the distribution of TCA-precipitable ^{131}I and acid phosphatase activity in the 19600×*g* fraction after sucrose density gradient centrifugation.

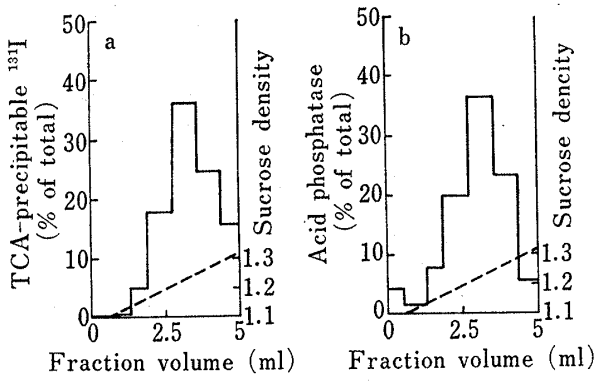

Fig. 5. Sucrose Density Gradient Centrifugation of the 19600×*g* Fraction

One half ml of the 19600×*g* fraction was layered on 4.5 ml of a linear sucrose (density 1.10—1.32) and centrifuged at 30000 rpm for 3 hr at 4°

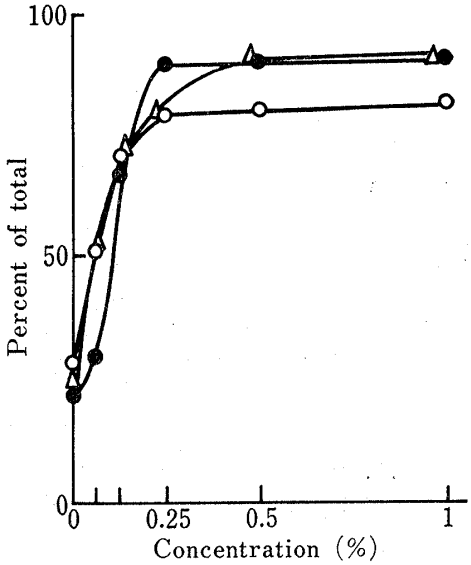

Fig. 6. Solubilization of Radioactivity from the 19600×*g* Fraction with Triton X-100

Ordinate represented the percentage of the solubilized amount to the total amount. —○—: TCA-precipitable ^{131}I, —●—: acid phosphatase, —△—: protein

As demonstrated in Fig. 5, both TCA-precipitable ^{131}I and acid phosphatase activity were distributed in the same fraction of density approximately 1.23.

In order to confirm that the radioactivity in the 19600×*g* fraction exists in lysosomes, the 19600×*g* fraction was treated with Triton X-100. Fig. 6 shows the solubilization patterns of radioactivity, acid phosphatase and protein from the 19600×*g* fraction.

Eighty three point one % of the total radioactivity were solubilized by 0.25% Triton X-100 and the solubilization pattern of radioactivity in various concentrations of Triton X-100 was observed to be in good agreement with that of acid phosphatase.

Effect of pH, Activator and Inhibitor on the Degradation of Injected ^{131}I-Lysozyme in the 19600×*g* Fraction

The degradative activity of freshly prepared 19600×*g* fraction of rat kidney 30 min after intravenous injection of ^{131}I-lysozyme was determined within the range from pH 2.2 to 9.0, and is presented in Fig. 7a.

As indicated in Fig. 7a, the TCA-soluble ^{131}I formed by the degradation of ^{131}I-lysozyme was found to have a peak at pH 5.0.

It is suggested that the optimal pH value of 5.0, in the degradation of injected ^{131}I-lysozyme in the 19600×*g* fraction, corresponded to that of cathepsin B_1.[15] Accordingly, the effects of cysteine as activator and iodoacetamide as inhibitor for cathepsin B_1 on degradative activity were examined using the 19600×*g* fraction of kidney 30 min after injection.

Fig. 7b shows the effect of 20 mM cysteine or 5 mM iodoacetamide on the degradation at pH 5.0. As indicated in Fig. 7b, the degradative activity was activated a little by 20 mM cysteine and was almost completely inhibited by 5 mM iodoacetamide.

Degradative Activity of Particulate Fractions

The optimal pH of the degradation in the 19600×*g* fraction was recognized to be 5.0, and the activity in the 19600×*g* fraction was compared with that in the 814×*g* or 105400×*g* fraction at this pH value. Fig. 8 shows the time courses of the degradation of injected ^{131}I-lysozyme in particulate fractions of kidney 30 min after intravenous injection of ^{131}I-lysozyme.

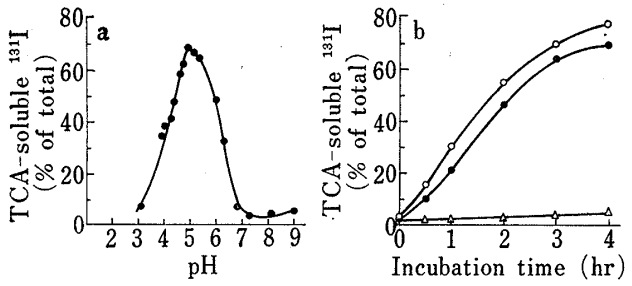

Fig. 7. Effect of pH and Reagents on Degradative Activity of Injected ^{131}I-Lysozyme by the 19600×*g* Fraction

(a) Effect of pH on degradative activity; incubation time at 37° was 4 hr in 0.05M buffer containing 0.25M sucrose.
(b) Effect of cysteine and iodoacetamide on the release of TCA-soluble ^{131}I; the fraction was incubated at pH 5.0 in 0.05M buffer containing 0.25 M sucrose.
—○—: 20 mM cysteine, —●—: 0.25M sucrose, —△—: 5 mM iodoacetamide

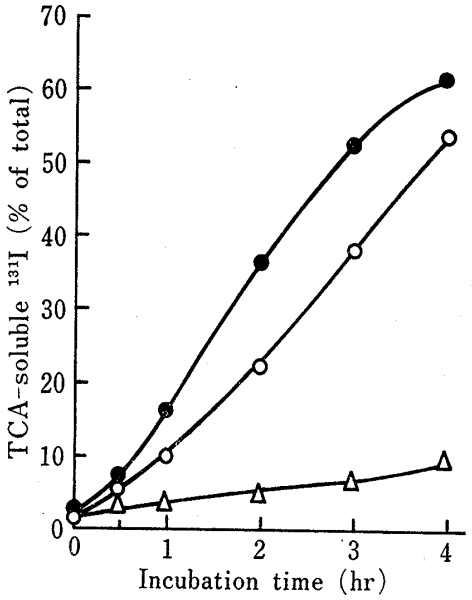

Fig. 8. Degradation of Injected ^{131}I-Lysozyme in Particulate Fraction during the Incubation at 37°

The particles sedimented at 814×*g* (○), 19600×*g* (●) and 105400×*g* (△) were incubated at pH 5.0.

As shown in Fig. 8, the 19600×*g* fraction was found to have the highest degradative activity among the particulate fractions after incubation for 4 hr at pH 5.0, followed by the 814×*g* and 105400×*g* fractions.

15) *a*) A.J. Barrett, *Anal. Biochem.*, **47**, 280 (1972); *idem*, *Biochem. J.*, **131**, 809 (1973); *b*) S.G. Franklin and R.M. Metrione, *Biochem. J.*, **127**, 207 (1972).

Identification of Degradation Product

TCA-soluble ^{131}I in a 4 hr incubation of the 19600×*g* fraction at pH 5.0 was subjected to paper chromatography, and the results are indicated in Fig. 9.

As shown in Fig. 9, the *Rf* of the peak of radioactivity was observed to be identical with that of diiodotyrosine on paper chromatograms.

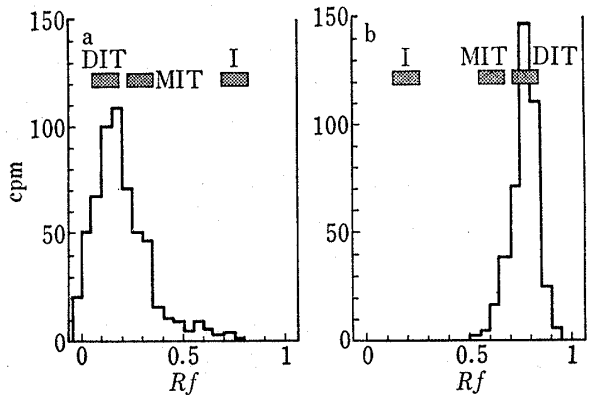

Fig. 9. Paper Chromatograms of TCA-Soluble ^{131}I from 19600×*g* Fraction after a 4-hr Incubation at pH 5.0 in Fig. 8

(a) 95% ethanol-2M ammonia (9: 1, v/v); (b) *n*-butanol-acetic acid-water (4: 1: 5, by vol., upper phase). DIT, 3,5-diiodo-L-tyrosine; MIT, 3-iodo-L-tyrosine; I, $Na^{131}I$

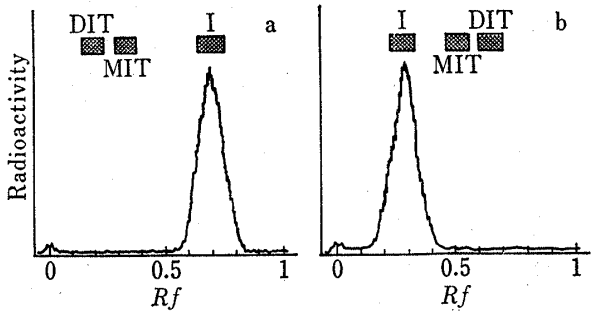

Fig. 10. Paper Chromatograms of Urine after Intravenous Injection of ^{131}I-Lysozyme

(a) 95% ethanol–2M ammonia (9: 1, v/v). (b) *n*-butanol–acetic acid–water (4: 1: 5, by vol., upper phase). DIT, 3,5-diiodo-L-tyrosine; MIT, 3-iodo-L-tyrosine; I, $Na^{131}I$. Radioscanning, 3 kcpm; speed, 5 cm/min; time const., 10 sec

The urinary and fecal excretion of radioactivity after intravenous injection of 0.4 mg ^{131}I-lysozyme per kg reached 93.6% and 1.5% of the dose during the first 3 days, respectively. The radioactivity excreted in urine within 1 day after injection was 80% of the dose and then it was subjected to paper chromatography.

As indicated in Fig. 10, 96.5% of the radioactivity excreted in urine was found to be inorganic $^{131}I^-$.

Discussion

In the present study, the tissue distribution, intracellular distribution and degradation of injected ^{131}I-lysozyme in rats were investigated. As shown in Fig. 1, the uptake of TCA-precipitable ^{131}I in kidney was found to be remarkably high during the first 12 hr after intravenous injection of ^{131}I-lysozyme. The fact that some considerable amount of egg white lysozyme after intraperitoneal administration is taken up by kidney, has been reported in rats by Perri, *et al.*,[4] Miller, *et al.*,[16a] Hansen, *et al.*[6a] and in mice by Maack.[16b] These reports on renal uptake of lysozyme agreed with the results in Fig. 1.

In Fig. 2, it was found that ^{131}I-lysozyme after injection was transported in intact form from serum to kidney and liver. Perri, *et al.*,[4] in IRC-50 column chromatography, indicated that extracts of the rat kidney showed two distinct peaks of enzyme activity corresponding to the positions expected for rat kidney lysozyme and administered egg white lysozyme, respectively. Maack, *et al.*,[5b] in fractionation of mice kidney extracts on Sephadex G-75 column after intraperitoneal injection of 35 mg ^{125}I-labeled egg white lysozyme, demonstrated that the peak of radioactivity coincided exactly with the peak of lysozyme activity, and the result was associated with the intact lysozyme molecules. Steiner[17] suggested that lysozyme

16) *a*) T.E. Miller, C.M. Cameron and J.D.K. North, *Proc. Soc. Exptl. Biol. Med.*, **128**, 749 (1968); *b*) T. Maack, *J. Cell Biol.*, **35**, 268 (1967).

17) R.F. Steiner, *Arch. Biochem. Biophys.*, **47**, 56 (1953).

formed an electrostatic complex with plasma albumin at pH of blood. In our preliminary experiment, the mixture of ^{131}I-lysozyme and rat serum was analysed by Sephadex G-50 gel filtration. As a result, it was found that more than 90% of the radioactivity was recovered at the same elution volume as that of lysozyme. These facts including the result shown in Fig. 2 suggest that an interaction of ^{131}I-lysozyme with plasma albumin or other proteins *in vivo* is very weak.

Renal cell uptake of protein filtered at glomeruli has been widely studied by cytochemical means. From these studies, it is indicated that, subsequently to appearance in tubular fluid, protein is taken up into the cell particles rich in hydrolytic (lysosomal) enzymes.[5b,18] As seen in Fig. 4, the radioactivity of injected ^{131}I-lysozyme was the highest in the 19600×***g*** fraction. The radioactivity in the 19600×***g*** fraction showed a pattern similar to a lysosomal marker enzyme or acid phosphatase as shown in Fig. 5 and Fig. 6. Accordingly, these results and the fact (Fig. 8) that the 19600×***g*** fraction has an ability to degrade injected ^{131}I-lysozyme, suggest that the protein-bound radioactivity in the 19600×***g*** fraction may be present in phagolysosomes reported by Straus.[18a]

The fate of lysozyme reabsorbed in the proximal tubules remains to be clarified. The pH-degradation profile of the 19600×***g*** fraction (Fig. 7a) was shown a single peak at pH 5.0. The optimal pH observed at pH 5.0 was in agreement with that of cathepsin B_1.[15a] The complete inhibition by iodoacetamide or the slight activation by cysteine on the release of TCA-soluble ^{131}I (Fig. 7b) coincided with the results of Franklin and Metrione[15b] or Mego and McQueen[19] and the findings reported previously.[20] These facts indicate that the enzyme with the optimum pH of 5.0 is cathepsin B_1. In Fig. 8, the degradation activity of injected ^{131}I-lysozyme was found to be the highest in the 19600×***g***, followed by the 814×***g*** and 105400×***g*** fraction. It is suggested that the 814×***g*** fraction, which is referred to as large phagolysosomes by Straus[18a] has ability to degrade injected ^{131}I-lysozyme, while the 105400×***g*** fraction as phagosomes has not. The main degraded product of injected ^{131}I-lysozyme in the phagolysosomes at pH 5.0 was ^{131}I-diiodotyrosine, as shown in Fig. 9. In contrast, the urinary product was almost exclusively inorganic $^{131}I^-$ (Fig. 10). Maunsbach,[21] in studies on degradation of ^{125}I-labeled homologous albumin reabsorbed by proximal tubule cells in rat kidney, indicated that albumin in isolated kidney lysosomes may be degraded by cathepsin B with an optimal pH of about pH 5.0. In addition, he reported that the radioactive degradation product of ^{125}I-labeled albumin was identified as monoiodotyrosine by paper chromatography. Our observations on degradation of ^{131}I-lysozyme were in good agreement with that of ^{125}I-labeled albumin, but the fact that the radioactive degradation product is either monoiodotyrosine or diiodotyrosine, may result from the labeling difference of proteins.

From the results reported here, it is likely that ^{131}I-lysozyme injected intravenously was cleared from serum by the kidney, then degraded into ^{131}I-diiodotyrosine by cathepsin B_1 in the renal phagolysosomes and ^{131}I-diiodotyrosine was deiodinated by deiodinase,[22] followed by excretion in urine as inorganic $^{131}I^-$.

Acknowledgement The authors thank Dr. S. Ohtake, Director, Section of Experimental Therapeutics Research, for his valuable advice and Mr. T. Naito, Vice president of Eisai Co., for his support of our research.

18) *a*) W. Straus, *J. Cell Biol.*, **20**, 497 (1964); *idem*, *J. Histochem. Cytochem.*, **15**, 381 (1967); *b*) F. Miller and G.E. Palade, *J. Cell Biol.*, **23**, 519 (1964); A.B. Maunsbach, *J. Ultrastruct. Res.*, **15**, 197 (1966); J.E. Bourdeau, E.R.Y. Chen and F.A. Carone, *Am. J. Physiol.*, **225**, 1399 (1973).
19) J.L. Mego and J.D. McQueen, *Biochim. Biophys. Acta*, **100**, 136 (1965).
20) K. Katayama and T. Fujita, *Biochim. Biophys. Acta*, **336**, 191 (1974).
21) A.B. Maunsbach, "Lysosomes in Biology and Pathology," Vol. 1, eds. by J.T. Dingle and H.B. Fell, North-Holland Publishing Company, Amsterdam and London, 1969, pp. 115—154.
22) J.B. Stanburt, *J. Biol. Chem.*, **288**, 801 (1957); S. Matsuzaki and M. Suzuki, *J. Biochem.*, **62**, 746 (1967).