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Accumulation of a Nondegradable Mannose Ligand within Rabbit Alveolar Macrophages. Receptor Reutilization Is Independent of Ligand Degradation†

Craig A. Hoppe and Yuan Chuan Lee*

ABSTRACT: Synthetic neoglycoproteins were made by reacting 5-(azidocarbonyl)pentyl 1-thio-α-D-mannopyranoside with poly(L-lysine) and poly(D-lysine). The ^{125}I-Man_{90}-poly-D-Lys and ^{125}I-Man_{104}-poly-L-Lys were tightly bound at 2 °C by the mannose receptor of the rabbit lung macrophage (K_d = 0.66 ± 0.18 and 0.59 ± 0.26 nM, respectively). Under saturating conditions in the cold, the macrophage bound 98 200 ± 7000 and 84 200 ± 10 500 ligand molecules per cell for the D- and L-polylysine derivatives, respectively. The cell-surface-bound ligands were dissociable by ethylenediaminetetraacetic acid and mannose at 2 °C. At 37 °C, the macrophages internalized both ^{125}I-Man_{90}-poly-D-Lys and ^{125}I-Man_{104}-poly-L-Lys efficiently. Although the internalized ^{125}I-Man_{104}-poly-L-Lys was degraded quickly by the macrophage to small radiolabeled peptide, the internalized ^{125}I-Man_{90}-H-poly-D-Lys apparently could not be degraded or exocytosed. The amount of ^{125}I-Man_{90}-poly-D-Lys which accumulated within the cell was 7-fold higher than the combined amount of surface and intracellular mannose receptors, strongly indicating reutilization of the receptors independent of degradation of the ligand.

Mammalian lung macrophages bind, internalize, and degrade macromolecular ligands which have terminal mannose[1] residues (Stahl et al., 1980). These steps occur quickly for ^{125}I-Man_{43}-AI-BSA[2] with $t_{1/2}$ = 0.6 min for surface-bound ligand to be internalized and $t_{1/2}$ = 10 min for cell-associated ligand to be released as degradation products into the medium (Hoppe & Lee, 1983). The surface receptors appear to be reutilized, since cell-surface binding activity recovers very quickly at 37 °C after greater than 70% of the surface binding activity is destroyed by trypsin treatment at 4 °C; however, when trypsin treatment is carried out at 37 °C, the cell's overall ability to take up mannose ligand is decreased by greater than 70% (Stahl et al., 1980). Furthermore, cycloheximide was found to have no effect on the recovery of receptor activity in cells treated with trypsin in the cold. Similarly, the mammalian hepatic Gal/GalNAc receptor is apparently reutilized since in the absence of protein synthesis the hepatocyte's ability to metabolize asialoorosomucoid far exceeds its capacity to bind ligand (Steer & Ashwell, 1980).

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[1] All sugars are of the D configuration in pyranoside form unless otherwise indicated.

[2] Abbreviations: BSA, bovine serum albumin; Man_{43}-AI-BSA, neoglycoproteins of BSA to which 43 mol of 1-thio-α-D-mannopyranoside has been attached by amidination using 2-imino-2-methoxyethyl 1-thio-α-D-mannopyranoside (Lee et al., 1976); EDTA, ethylenediaminetetraacetic acid; $Glyc_n$-H-poly-D(L)-Lys, neoglycoproteins of polylysine to which n moles of 1-thioglycosides has been attached by using the 5-(hydrazinocarbonyl)pentyl 1-thioglycopyranoside (Kawaguchi et al., 1980); Cbz, benzyloxycarbonyl; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Gal, galactose; GalNAc, *N*-acetylgalactosamine.

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It would be useful to have a ligand which could be internalized by the lung macrophage but could not be degraded and to compare its behavior during endocytosis with that of its counterpart, which is degradable. Poly(D-lysine) and poly(L-lysine) are ideal compounds which can be modified with mannose derivatives to form such a pair of ligands. We have therefore used previously developed techniques for covalently attaching sugars to side-chain amino groups of polypeptides (Stowell & Lee, 1980; Lee & Lee, 1982) to do such a modification of these polylysines. In this study, 5-(azidocarbonyl)pentyl 1-thio-α-D-mannopyranoside was used to modify poly(D-lysine) and poly(L-lysine) of comparable size. Here we present evidence that ^{125}I-Man_{90}-H-poly-D-Lys is specifically recognized by the mannose receptor of the lung macrophage, is internalized by the cell, accumulates in undegraded form within the cell, and is unable to exit the cell after its internalization.

Experimental Procedures

Materials. The following materials were obtained from the indicated sources: piperazine-*N*,*N*′-bis(2-ethanesulfonate) (as 1.5 sodium salt, monohydrate) and *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, Research Organics Inc.; male New Zealand white rabbits (1–1.5 kg), Bunnyville, Littletown, PA; carrier-free Na^{125}I, Amersham Corp.; silicone oil (DC 550), Accumetric, Elizabethtown, KY; mineral oil, *N*-hydroxysuccinimidyl 3-(*p*-hydroxyphenyl)propionate, poly(L-lysine) hydrobromide (DP 270), poly(D-lysine) hydrobromide (DP 250), and Triton X-100, Sigma Chemical Co.; 3-(*p*-hydroxyphenyl)propionic acid, Aldrich Chemical Co., Inc.; BSA (Pentex, fraction V), Miles Laboratories, Inc. The Man_{43}-AI-BSA was prepared by a method reported earlier (Lee et al., 1976). All other chemicals used in this work were of the highest purity available commercially and were used without further purification.

5-(Hydrazinocarbonyl)pentyl 1-thio-β-D-galactopyranoside and 5-(hydrazinocarbonyl)pentyl 1-thio-α-D-mannopyranoside were both prepared by a method reported earlier for the galactose analogue (Kawaguchi et al., 1981). Man_{104}-H-poly-L-Lys, Man_{90}-H-poly-D-Lys, and Gal_{116}-H-poly-L-Lys were prepared by the acyl azide method (Kawaguchi et al., 1980) with minor modifications. Polylysines (2.5 μmol) were reacted with 1.3 mmol of the acyl azide derived from 5-(hydrazinocarbonyl)pentyl 1-thio-$\alpha(\beta)$-D-glycopyranoside.

To allow radioiodination, the Glyc-H-polylysine derivatives were modified as follows: *N*-Succinimidyl 3-(*p*-hydroxyphenyl)propanoate (13.7 μmol) was stirred with each of the Glyc-H-polylysines (0.27 μmol) in a total volume of 5 mL of 0.2 M sodium borate, pH 8.5, at 4 °C for 12 h. The reaction mixture was purified by dialyzing against water for 2–3 days to remove salts and byproducts (30–50% of the polylysine is lost through the dialysis membrane during this step).

A reference compound, N^{ϵ}-[3-(*p*-hydroxyphenyl)-propanoyl]-L-lysine was synthesized by reacting 30 mg (107 μmol) N^{α}-Cbz-L-lysine with 30 mg (116 μmol) of *N*-succinimidyl 3-(*p*-hydroxyphenyl)propionate in 28 mL of dry dimethyl sulfoxide for 1 h at 23 °C. At this time, 85% of the total amino group was consumed as assayed by a fluorescamine assay (Udenfriend et al., 1972) using N^{α}-Cbz-L-lysine as the standard. The dimethyl sulfoxide was removed by flash evaporation at 40 °C and the residue brought up in 1.0 mL of glacial acetic acid. The Cbz group was removed by treating with 2.0 M HBr in glacial acetic acid for 15 min at 23 °C. The reaction mixture was neutralized with a concentrated solution of NaOH before being applied to a Sephadex G-25 column (1.5 × 73 cm) eluted with 1 M acetic acid. The desired product eluted as a peak with both absorbance at 290 nm and primary amino group measured by the fluorescamine assay.

Preparation of Macrophages. Lung macrophages were isolated from rabbits as reported earlier (Hoppe & Lee, 1982). The incubation medium used for preparing the cells and for assays was as follows: 0.12 M NaCl, 5 mM KCl, 2 mM $CaCl_2 \cdot 2H_2O$, 1 mM $MgSO_4 \cdot 7H_2O$, 5 mM glucose, 6×10^{-4}% (w/v) phenol red, 0.5% (w/v) BSA, 15 mM sodium piperazine-*N*,*N*′-bis(2-ethanesulfonate), and 15 mM *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid adjusted to pH 6.7 with NaOH. The cells used for experiments were routinely >95% viable by trypan blue exclusion and by the measurement of intracellular and extracellular lactate dehydrogenase activities (Berg et al., 1972).

Radioiodination of Proteins and Standards. Proteins, 3-(*p*-hydroxyphenyl)propanoylated polylysines, 3-(*p*-hydroxyphenyl)propionic acid, and N^{ϵ}-[3-(*p*-hydroxyphenyl)-propanoyl]-L-lysine were radioiodinated using Chloramine-T as reported previously (Krantz et al., 1976). The radioiodinated proteins and polylysines (approximately 5.0×10^7 cpm/μg) were used within 1–2 weeks. To ensure identity of the labeled and unlabeled ligands, labeled proteins were diluted with different amounts of unlabeled protein, and binding to macrophages was measured. The total number of ligand molecules bound per cell remained the same regardless of dilution. Therefore, for all binding experiments, radioiodinated derivatives were diluted with unlabeled carrier to the desired specific radioactivity.

Assay for Ligand Binding and Uptake by Cells. Ligand binding and uptake by cells were assayed in duplicate in incubation medium by using capped 12 × 75 mm polystyrene tubes (Falcon 2058), capped 17 × 100 mm polystyrene tubes (Falcon 2001), or 1.5-mL polypropylene tubes (no. 690, Sarstedt, Princeton, NJ) which were rotated end over end at 4 rpm during incubation. The 5.0 mM glucose in the incubation medium had no effect on ligand binding to cells. Assays were initiated by adding cells to an incubation mixture containing ^{125}I-labeled ligand. Both components were equilibrated to the assay temperature before the assay was initiated. Nonspecific binding and uptake were determined by adding unlabeled Man_{43}-AI-BSA to binding assays to a final concentration of 14 μM. The amount of ^{125}I-Man_{43}-AI-BSA bound in the presence of 14 μM unlabeled Man_{43}-AI-BSA was the same as the amount that was cell associated in the presence of 500 mM mannose or 10 mM EDTA. In most cases, cell incubations were done in 1.5-mL polypropylene tubes which were processed by adding 0.4 mL (4:1) of silicone oil–mineral oil directly to the incubation mixture followed by centrifugation in an Eppendorf microcentrifuge (Model 5412) for 60 s. In one experiment (Figure 2), 0.4-mL aliquots of incubation mixtures were pipetted on top of (4:1) silicone oil–mineral oil in 1.5-mL polypropylene tubes followed by centrifugation of the tubes for 60 s. After centrifugation, the polypropylene tube was cut at the middle of the oil layer with a dog toenail cutter, and the tip of the tube, containing the cell pellet, was counted in a Packard PRIAS auto-γ counter (Model PGD).

Assay of Ligand Degradation. Degradation products in the medium were routinely assayed by gel filtration in the presence of cold carriers. After centrifugation of cells through oil, a 75-μL sample of the supernatant was made 0.2 mM in iodotyrosine, 0.1 mM in diiodotyrosine, and 0.15 M in KI before application in a 150-μL volume to a column (0.8 × 24 cm) of Sephadex G-25 (medium). The column was eluted with 1.0 M acetic acid, and 1.05-mL fractions were collected. The

radioactive material eluting after the void volume was degraded ligand as determined by calibrating the elution volumes of monoiodotyrosine, diiodotyrosine, KI, and ^{125}I-Man$_{104}$-H-poly-L-Lys degradation product purified with Sephadex G-10. In experiments to characterize the ^{125}I-Man$_{104}$-poly-L-Lys degradation product, Sephadex G-10 columns (0.8 × 24 cm) eluted with 1.0 M acetic acid followed by 1.0 M ammonium acetate were used. Routinely, greater than 90% of the applied radioactivity could be recovered in the effluent of the Sephadex G-25 and G-10 columns.

Analysis of Binding and Dissociation Data. Binding and dissociation data were analyzed by using a nonlinear regression analysis program for two-parameter equations (Duggleby, 1981) implemented on a Heath H-89 microcomputer. The analysis of binding data using a one-site model (two-parameter equation) is valid since the binding of Man$_{43}$-AI-BSA, Man$_{90}$-H-poly-D-Lys, and Man$_{104}$-H-poly-L-Lys to macrophages was shown to fit best to a one-site binding model using SCAFIT (Munson & Rodbard, 1980).

Assay of Total Cellular Receptor. Macrophages (5.0 × 10^6 cells) were pelleted (200*g*, 5 min) in capped 12 × 75 mm polystyrene tubes (Falcon 2058), and the supernatant medium was removed. The cell pellet was then brought up in assay buffer [25 mM sodium piperazine-*N*,*N*′-bis(2-ethanesulfonate), pH 6.7, 1.0 M NaCl, 1% (v/v) Triton X-100, 0.6% (w/v) BSA, and 50 mM $CaCl_2 \cdot 2H_2O$] with either 10 nM ^{125}I-Man$_{90}$-H-poly-D-Lys or 10 nM ^{125}I-Man$_{43}$-AI-BSA. The tubes were incubated at 2 °C for 2 h with end over end rotation at 4 rpm. Nonspecific binding of ligands was determined by adding unlabeled Man$_{43}$-AI-BSA to binding assays to a final concentration of 14 μM.

After incubation, the assay suspension was centrifuged in a microfuge for 3 min. Greater than 98% of the total radioactivity was found in the supernatant after this procedure. The remaining radioactivity (2%) left in the pellet introduced a maximal error of 15% in the binding data reported. A 25-μL volume of each supernatant was brought up to 0.5 mL with assay buffer at 4 °C before the addition of 0.5 mL of a cold, saturated solution of ammonium sulfate which had been adjusted to pH 7.8 with solid Tris. After 10 min at 4 °C, the suspension was filtered on Whatman GF/C under reduced pressure, and the tube and filter were washed with three 3-mL portions of half-saturated ammonium sulfate, 10 mM $CaCl_2 \cdot 2H_2O$, and 0.1% (w/v) BSA, adjusted to pH 7.8 with solid Tris. Radioactivity retained on the filter disk was then determined. Nonspecific binding was <1% of specific binding for ^{125}I-Man$_{90}$-H-poly-D-Lys; however, 35% of the ligand was precipitated by ammonium sulfate in the absence of added cellular protein. For ^{125}I-Man$_{43}$-AI-BSA, 5% of the ligand was precipitated by ammonium sulfate in the absence of added cellular protein, and nonspecific binding was 50% of specific binding.

Analysis of Intracellular Radioactivity. After incubation with ^{125}I-labeled ligand, cells were washed twice with incubation medium and then solubilized in 3 M acetic acid, 0.1% (w/v) BSA, and 2% (v/v) Triton X-100 for 5 min at 4 °C with occasional mixing. After centrifugation of the Triton extract in a microfuge for 2 min, >99% of the radioactivity was found in the supernatant. The supernatant was fractionated on a Sephadex G-50 (fine) column (1.5 × 120 cm), eluting with 3 M acetic acid–0.1% (w/v) BSA.

Results

Equilibrium Binding of ^{125}I-Man-polylysine Molecules to Macrophages. Equilibrium binding analysis indicated that ^{125}I-Man$_{90}$-H-poly-D-Lys and ^{125}I-Man$_{104}$-H-poly-L-Lys were bound to macrophages at 2 °C in a similar fashion and their binding closely resembled that of ^{125}I-Man$_{43}$-AI-BSA (Figure 1). The binding parameters are compiled in Table I. All three classes of molecules had a similar number of cell-surface binding sites, ranging from 71 400 to 98 200 sites/cell; however, the affinity of ^{125}I-Man$_{90}$-H-poly-D-Lys and ^{125}I-Man$_{104}$-H-poly-L-Lys (with K_d = 0.66 ± 0.18 and 0.59 ± 0.26 nM, respectively) for the macrophage was higher than that of ^{125}I-Man$_{43}$-AI-BSA (K_d = 1.17 ± 0.17 nM). Using SCAFIT (Munson & Rodbard, 1980), the data fit best to a one-site binding model and gave similar results.

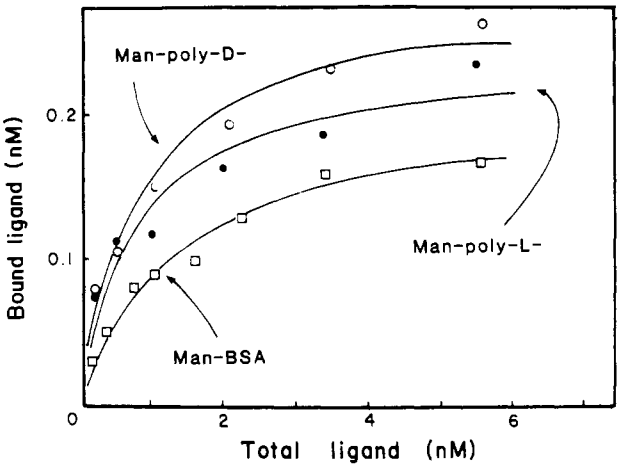

FIGURE 1: Equilibrium binding of ^{125}I-Man$_{90}$-H-poly-D-Lys and ^{125}I-Man$_{104}$-H-poly-L-Lys to macrophages. The macrophages (1.7 × 10^6 cells/mL) were incubated with various concentrations of ^{125}I-Man$_{90}$-H-poly-D-Lys (○), ^{125}I-Man$_{104}$-H-poly-L-Lys (●), and ^{125}I-Man$_{43}$-AI-BSA (□) at 2 °C in 1.5-mL polypropylene tubes. After 6 h, specifically bound ^{125}I-labeled ligand was determined, and the data were fitted by using a nonlinear regression program for two-parameter equations (Duggleby, 1981). Both the fitted curves (solid lines) and the experimental points (○, ●, and □) are shown. The best-fit binding parameters for each analysis are given in Table I.

Table I: Binding Parameters of Man-H-polylysine Derivatives to Macrophages[a]

ligand	K_d[b] (nM)	R[c] (sites/cell)
Man$_{90}$-H-poly-D-Lys	0.66 ± 0.18	98 200 ± 7 000
Man$_{104}$-H-poly-L-Lys	0.59 ± 0.26	84 200 ± 10 500
Man$_{43}$-AI-BSA	1.17 ± 0.17	71 400 ± 3 400

[a] The data of Figure 1 were analyzed by using a nonlinear regression program for two-parameter equations (Duggleby, 1981). [b] Dissociation constant for the ligand-receptor complex. [c] Receptor concentration.

Determination of Total Cellular Receptor. Total cellular receptor (i.e., binding sites) was determined by an ammonium sulfate precipitation assay (see Experimental Procedures). Triton X-100 solubilized cells had 236 000 binding sites/cell for ^{125}I-Man$_{90}$-H-poly-D-Lys and 212 000 binding sites/cell for ^{125}I-Man$_{43}$-AI-BSA.

Dissociation of the Cell-Surface-Bound ^{125}I-Man-polylysine Derivatives. Figure 2 shows that cell-surface-bound ^{125}I-Man$_{90}$-H-poly-D-Lys and ^{125}I-Man$_{104}$-H-poly-L-Lys had very similar dissociation kinetics when EDTA was used but differed somewhat in their dissociation when mannose was used. EDTA and mannose dissociated both ^{125}I-Man-H-polylysines to the same extent as ^{125}I-Man$_{43}$-AI-BSA (>90%); however, the rate of dissociation was slower for the polylysine derivatives. By 3 min, greater than 75% of the cell-surface-bound Man-H-polylysines were dissociated by EDTA treatment, but a slower dissociating component was seen after this point (Figure 2A). Therefore, the dissociation rate constants in EDTA for

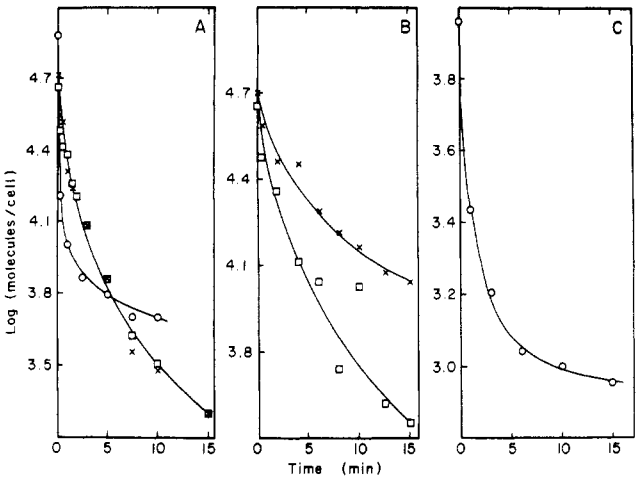

FIGURE 2: Dissociation of cell-surface ^{125}I-Man-H-polylysine derivatives by EDTA and mannose. (A) The macrophages (1.2 × 10^7 cells/mL for Man-H-polylysines, 9.1 × 10^6 cells/mL for Man_{43}-AI-BSA) were incubated at 2 °C in 12 × 75 mm polystyrene tubes in 2 nM ^{125}I-Man_{90}-H-poly-D-Lys (×), 2 nM ^{125}I-Man_{104}-H-poly-L-Lys (□), and 47 nM ^{125}I-Man_{43}-AI-BSA (○) in a total volume of 3 mL. After 4 h, the number of molecules bound per cell for each ligand was as follows: ^{125}I-Man_{90}-H-poly-D-Lys, 5.1 × 10^4; ^{125}I-Man_{104}-H-poly-L-Lys, 4.6 × 10^4; ^{125}I-Man_{43}-AI-BSA, 7.6 × 10^4. Aliquots (0.2 mL) of the incubation mixture were then diluted at 2 °C in separate tubes for each time point to one-fifth their original concentration in incubation medium devoid of Ca, Mg, and BSA but containing sufficient EDTA to reach a final concentration of 10 mM. These tubes were incubated at 2 °C, and at various times, duplicate samples (0.4 mL) were taken to determine cell-bound ^{125}I-Man-ligand. (B) The macrophages (2.6 × 10^6 cells/mL) were incubated in 17 × 100 mm polystyrene tubes at 2 °C in medium containing 2 nM ^{125}I-Man_{90}-H-poly-D-Lys (×) and 2 nM ^{125}I-Man_{104}-H-poly-L-Lys (□) in a total volume of 6 mL. After 4 h, the number of molecules bound per cell was as follows: ^{125}I-Man_{90}-H-poly-D-Lys, 5.6 × 10^4; ^{125}I-Man_{104}-H-poly-L-Lys, 5.6 × 10^4. Aliquots (0.2 mL) of cells were then diluted at 2 °C to half their original concentration in incubation medium containing sufficient mannose to reach a final concentration of 0.5 M. These tubes were incubated at 2 °C, and at various times, tubes were processed by adding 0.6 mL of medium to dilute mannose to 0.2 M and then removing duplicate 0.4-mL samples to determine cell-bound ^{125}I-labeled ligand. The dilution of the mannose solution was necessary because 0.5 M mannose is too dense to allow medium separation from cells by the oil centrifugation technique. (C) The macrophages (1.0 × 10^7 cells/mL) were incubated in 0.7 nM ^{125}I-Man_{43}-AI-BSA under the same conditions given in (B). Subsequently, the time course of dissociation was determined by the same procedure used in (B). Under the conditions of the first incubation, 8.7 × 10^3 molecules of ^{125}I-Man_{43}-AI-BSA were bound per cell.

the Man-H-polylysines [analyzed as first-order kinetics by nonlinear regression (Duggleby, 1981)] were determined for those data points up to and including 3 min. The EDTA dissociation rate constants for ^{125}I-Man_{90}-H-poly-D-Lys and ^{125}I-Man_{104}-H-poly-L-Lys were 0.40 ± 0.03 and 0.33 ± 0.02 min^{-1}, respectively. These were 15-fold slower than that for ^{125}I-Man_{43}-AI-BSA which was 6.15 ± 1.60 min^{-1}.

In the case of mannose-mediated dissociation, greater than 80% of both cell-surface-bound Man-H-polylysines were dissociated by mannose treatment by 17.5 min. The rate constants were therefore determined for the Man-H-polylysines by using those data up to and including 17.5 min. A slower dissociating component became apparent after this point. In the presence of mannose, ^{125}I-Man_{43}-AI-BSA dissociated 6 times faster (k_{dis} = 1.08 ± 0.31 min^{-1}) than ^{125}I-Man_{104}-H-poly-L-Lys (k_{dis} = 0.17 ± 0.02 min^{-1}) and 12 times faster than ^{125}I-Man_{90}-H-poly-D-Lys (k_{dis} = 0.09 ± 0.008 min^{-1}). Dissociation of cell-surface ligand in 0.5 M mannose was slower than in 10 mM EDTA for all three ligands. ^{125}I-Man_{43}-AI-BSA dissociated 5-fold faster in the presence of EDTA,

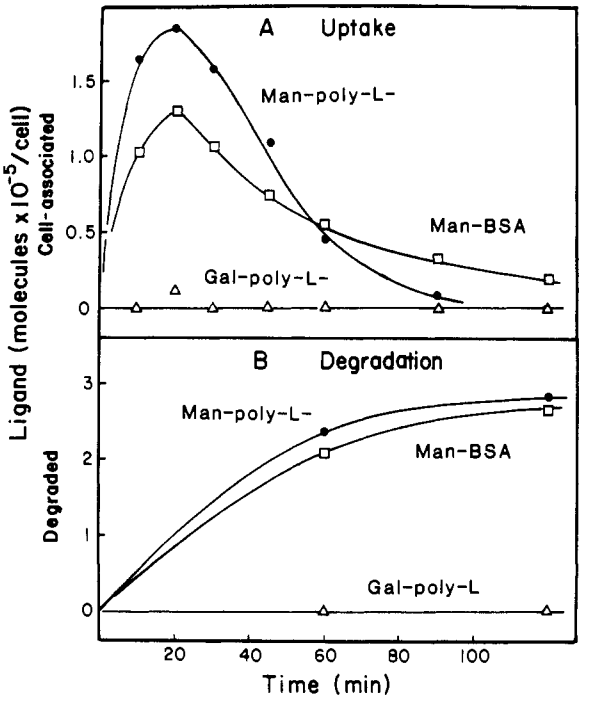

FIGURE 3: Uptake and degradation of ^{125}I-Man_{104}-H-poly-L-Lys by macrophages. Macrophages (7.4 × 10^6 cells/mL) were incubated in 5.2 nM ^{125}I-Man_{104}-H-poly-L-Lys (●), 5.7 nM ^{125}I-Gal_{116}-H-poly-L-Lys (△), and 5.5 nM ^{125}I-Man_{43}-AI-BSA (□) at 37 °C in 1.5-mL polypropylene tubes. (A) Time course of uptake. At various times, uptake was determined as described under Experimental Procedures. (B) Time course of degradation. At various times, ligand degradation was determined as described under Experimental Procedures. At 120 min, approximately 60% of the total doses of ^{125}I-Man_{104}-H-poly-L-Lys and ^{125}I-Man_{43}-AI-BSA were present in the medium as degradation product.

^{125}I-Man_{104}-H-poly-L-Lys 2-fold faster, and ^{125}I-Man_{90}-H-poly-D-Lys 4-fold faster.

Cell remained >95% viable in 0.5 M mannose and 10 mM EDTA after 30 min at 2 °C as assayed by trypan blue exclusion and the measurement of intracellular and extracellular lactate dehydrogenase activities (Berg et al., 1972).

Uptake and Degradation of ^{125}I-Man-H-polylysine Derivatives by Macrophages. Both ^{125}I-Man_{104}-H-poly-L-Lys and ^{125}I-Man_{90}-H-poly-D-Lys were taken up by macrophages at 37 °C at a faster initial rate than ^{125}I-Man_{43}-AI-BSA (Figures 3 and 4). For ^{125}I-Man_{104}-H-poly-L-Lys and ^{125}I-Man_{43}-AI-BSA, degradation of the internalized ligand was very efficient, with >60% of the starting dose present in the medium as degradation products by 120 min of incubation at 37 °C (Figure 3). In contrast, ^{125}I-Man_{90}-H-poly-D-Lys was taken up by the cells, but no degradation products appeared in the medium during 37 °C incubation for 120 min (Figure 4). The treatment of these incubated cells with 10 mM EDTA to dissociate cell-surface ligand (Figure 2) resulted in a decrease of only 5% of the cell-associated radioactivity. Under optimal experimental conditions where the uptake of ^{125}I-Man_{90}-H-poly-D-Lys was maximized, macrophages accumulated 1.7 × 10^6 molecules/cell of the nondegradable ligand by 120 min of incubation at 37 °C (2.5-fold higher than that shown in Figure 4). Thus, the macrophages were capable of internalizing 7-fold greater ^{125}I-Man_{90}-H-poly-D-Lys molecules than the total mannose receptor sites present in the cell. As expected, ^{125}I-Gal_{116}-H-poly-L-Lys was not taken up by the macrophages (Figure 3). After 2 h at 37 °C in the presence of Man-H-polylysine concentrations up to 50 nM, the viability of the macrophages remained >90% by the trypan blue and lactate dehydrogenase assays.

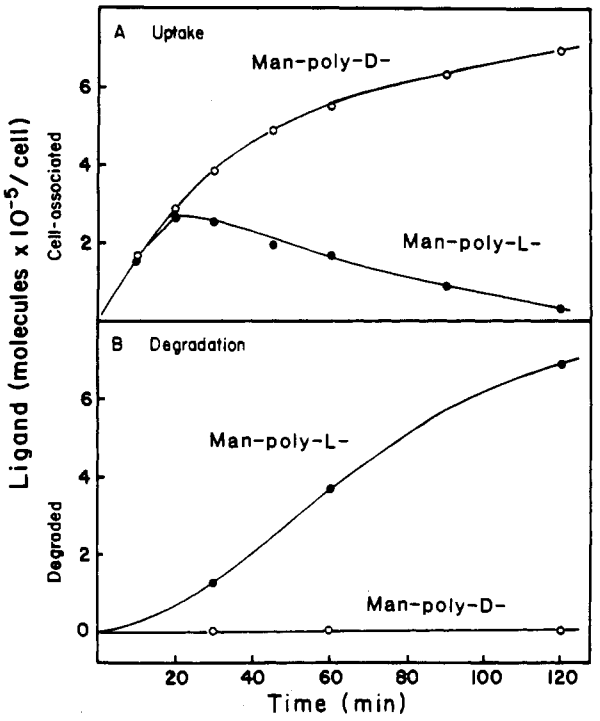

FIGURE 4: Absence of ^{125}I-Man$_{90}$-H-poly-D-Lys degradation after uptake by macrophages. Macrophages (2.8 × 10^6 cells/mL) were incubated in 5.3 nM ^{125}I-Man$_{90}$-H-poly-D-Lys (○) and 5.4 nM ^{125}I-Man$_{104}$-H-poly-L-Lys (●) at 37 °C in 1.5-mL polypropylene tubes. (A) Time course of uptake. At various times, uptake was determined as described under Experimental Procedures. At 120 min, 60% of the ^{125}I-Man$_{90}$-H-poly-D-Lys was cell associated. (B) Time course of degradation. At various time, ligand degradation was determined as described under Experimental Procedures. At 120 min, 60% of the ^{125}I-Man$_{104}$-H-poly-L-Lys dose was present in the medium as degradation products.

Characterization of the ^{125}I-Man$_{104}$-H-poly-L-Lys Degradation Product. Unlike ^{125}I-Man-AI-BSA, which was degraded to iodotyrosine (Hoppe & Lee, 1983), the ^{125}I-Man$_{104}$-H-poly-L-Lys degradation product reproducibly chromatographed slightly ahead of monoidotyrosine on Sephadex G-10 and at a different position from ^{125}I-N^{ϵ}-[3-(*p*-hydroxyphenyl)propanoyl]-L-lysine (Figure 5). Exhaustive carboxypeptidase Y treatment of the degradation product (Kuhn et al., 1974) did not change its chromatgraphic behavior on Sephadex G-10. However, hydrolysis of the product in 2 M NaOH at 100 °C for 7.5 h did give a radiolabeled compound which coincided with authentic monoiodo-3-(*p*-hydroxyphenyl)propionic acid on Sephadex G-10 columns (Figure 6).

Characterization of ^{125}I-Man$_{90}$-H-poly-D-Lys Accumulated in the Macrophage. Triton X-100 was used to solubilize the radioactivity which accumulated in cells incubated with 5.6 nM ^{125}I-Man$_{90}$-H-poly-D-Lys for 2 h at 37 °C. After centrifugation of the Triton extract in a microfuge for 2 min, >99% of the radioactivity was found in the supernatant. Upon chromatography of the supernatant on a Sephadex G-50 column, the intracellular radioactivity comigrated with standard ^{125}I-Man$_{90}$-H-poly-D-Lys, indicating that very little if any size modification of the ligand occurred after its internalization by the cell (data not shown). In contrast, when cells were incubated with 5 nM ^{125}I-Man$_{104}$-H-poly-L-Lys for 30 min at 37 °C followed by Triton extraction of the intracellular radioactivity, a smaller fragment (30% of the total radioactivity) was seen by Sephadex G-50 chromatography. The slower moving peak coincided on the Sephadex G-10 column with the ^{125}I-Man$_{104}$-H-poly-L-Lys degradation product

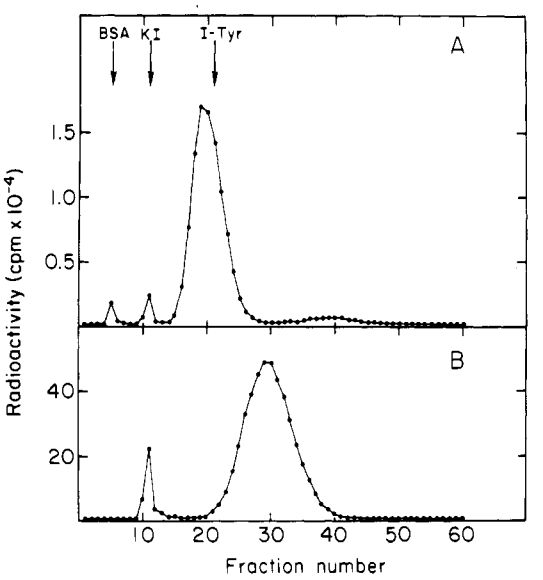

FIGURE 5: Gel chromatography of the ^{125}I-Man$_{104}$-H-poly-L-Lys degradation product. (A) Macrophages (4.5 × 10^6 cells/mL) were incubated in 5.4 nM ^{125}I-Man$_{104}$-H-poly-L-Lys for 2 h at 37 °C in 1.5-mL polypropylene tubes. The cells were separated from the medium through silicone oil–mineral oil by centrifugation. A 75-μL sample of the supernatant, mixed with monoiodotyrosine (0.2 mM), diiodotyrosine (0.1 mM), and KI (0.15 M), was fractionated on a Sephadex G-10 column (0.8 × 23 cm) eluted with 1.0 M acetic acid (1.05-mL fractions were collected). The total radioactivity recovered in the column effluent was 93% of that which was applied. The elution positions for BSA, KI, and monoiodotyrosine standards are indicated. Monoiodo-3-(*p*-hydroxyphenyl)propionic acid did not elute from this column until the acetic acid concentration of the eluent was increased to 6 M. (B) Standard ^{125}I-N^{ϵ}-[3-(*p*-hydroxyphenyl)propanoyl]-L-lysine was chromatographed as in (A).

Table II: Behavior of Cell-Associated ^{125}I-Man$_{90}$-H-poly-D-Lys[a]

incubation time (min)	cell-associated ^{125}I-Man$_{90}$-H-poly-D-Lys (molecules × 10^{-5}/cell)	medium radioactivity (molecules × 10^{-5}/cell)
0	4.41	0.04
5	4.36	0.05
10	4.25	0.06
15	4.07	0.08
20	4.13	0.10
30	4.17	0.13
40	4.13	0.15
50	3.88	0.16
60	3.96	0.16

[a] Macrophages (3 × 10^6 cells/mL) were incubated in 5.6 nM ^{125}I-Man$_{90}$-H-poly-D-Lys in a 17 × 100 mm polystyrene tube at 37 °C for 20 min in a total volume of 12 mL. The cells were cooled at 4 °C, centrifuged at 200*g* for 5 min at 4 °C, and then washed twice in incubation medium at 4 °C. The cells were then suspended in 6 mL of incubation medium at 4 °C. Aliquots of cells (0.3 mL) were added to 0.3 mL of incubation medium at 37 °C in 1.5-mL polypropylene tubes to give a final cell concentration of 3 × 10^6 cells/mL. The tubes were further incubated at 37 °C and assayed at various times for cell-associated ligand and radioactivity in the medium. At 60 min, 93% of the initial radioactivity was recovered.

found extracellularly. Interestingly, no other radioactive peaks were seen between the intact ligand and degradative end-product peaks.

Fate of Cell-Associated ^{125}I-Man$_{90}$-H-poly-D-Lys. Cell-associated ^{125}I-Man$_{90}$-H-poly-D-Lys was slowly released at 37 °C from cells which had been allowed to take up the ligand in a preincubation at 37 °C (Table II). After a 60-min incubation at 37 °C, about 5% of the preloaded ligand was

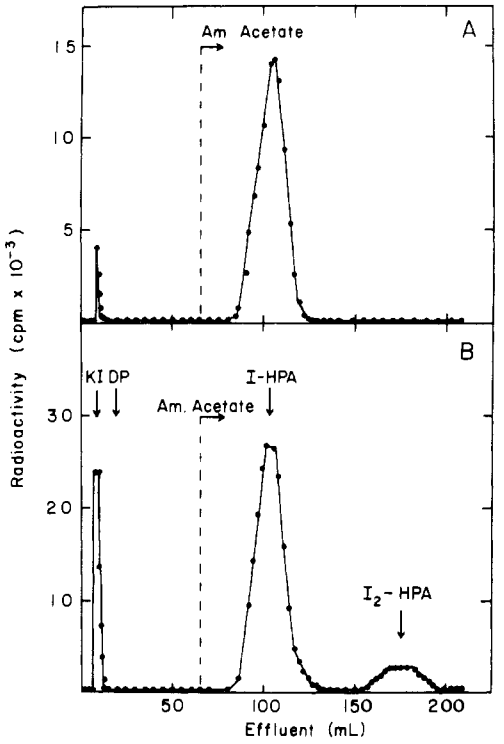

FIGURE 6: Analysis of the products from base hydrolysis of the ^{125}I-Man$_{104}$-H-poly-L-Lys degradation product. (A) Fractions 16–25 of Figure 5A were pooled, lyophilized, and brought up in 2 M NaOH and 85 μM unlabeled mono- and diiodo-3-(*p*-hydroxyphenyl)propanoic acid in a total volume of 0.5 mL. This solution was incubated at 100 °C for 7.5 h. A 100-μL volume of hydrolysate was applied to a Sephadex G-10 column (0.8 × 23 cm) and eluted with 67 mL of 1.0 M acetic acid, conditions which elute iodide and unhydrolyzed degradation product (see Figure 5A), followed by elution with 1.0 M ammonium acetate. (B) Standard [^{125}I]iodo-3-(*p*-hydroxyphenyl)-propionic acid and [^{125}I]diiodo-3-(*p*-hydroxyphenyl)propionic acid were treated exactly like fractions 16–25 in (A). Greater than 98% of the starting radioactivity was recovered in the effluent of both (A) and (B). DP, I-HPA, and I_2-HPA are ^{125}I-Man$_{104}$-H-poly-L-Lys degradation product, monoiodo-3-(*p*-hydroxyphenyl)propionic acid, and diiodo-3-(*p*-hydroxyphenyl)propionic acid standards, respectively.

released into the medium as radioactivity which coincided on Sephadex G-10 columns with the ^{125}I-Man$_{104}$-H-poly-L-Lys-degradation product.

Discussion

Table I shows that the binding affinities of the ^{125}I-Man-H-polylysines were somewhat higher (i.e., lower K_d) than that for ^{125}I-Man$_{43}$-AI-BSA. This may be caused by the greater density of mannose residues on the polylysine derivatives or the nonglobular nature of the lysine polymers. Interestingly, the Man-H-polylysine derivatives and Man$_{43}$-AI-BSA had similar numbers of cell-surface binding sites on the macrophages. We have previously observed that Man$_{43}$-AI-BSA and Man$_{24}$-AI-BSA have similar numbers of binding sites per macrophage but have approximately half the number of sites per cell compared to Man$_{13}$-AI-BSA and Man$_5$-AI-BSA (Hoppe & Lee, 1983). This phenomenon was interpreted to mean that there is a finite number of available subsites for ligand attachment on the cell surface and that binding of higher affinity ligands requires more subsites for attachment than lower affinity ligands. If this view is correct, then the Man-poly-Lys derivatives are bound to the cells by approximately the same number of attachment subsites as the Man$_{43}$-AI-BSA derivative.

Despite the difference in the stereochemistry of the isomers, the binding parameters of ^{125}I-Man$_{90}$-H-poly-D-Lys and ^{125}I-Man$_{104}$-H-poly-L-Lys are quite similar. Since both derivatives are probably in a random-coiled form under the conditions of the binding assay (Applequist & Doty, 1962) and there is a considerable amount of freedom of the D-mannose orientation through the aliphatic side chains of the lysine, the D and L isomers may assume an equally favorable orientation of mannose residues for binding to the receptor.

The ability of EDTA and mannose to dissociate the Man-H-polylysine derivatives from the macrophage surface (Figure 2) was strong evidence that the polylysine compounds were bound to the cell surface by the mannose receptor. This conclusion is further supported by the inability of the macrophage to take up ^{125}I-Gal$_{116}$-H-poly-L-Lys (Figure 3), thus eliminating the possibility that the binding of the ^{125}I-Man-H-polylysines was due to nonspecific cellular binding of the polylysine or the aglycon group which linked sugar to polylysine.

Robbins et al. (1981) have shown that a trimannosyldilysine binds to the mannose receptor of the rat alveolar macrophage at 0 °C with a dissociation constant of 2 μM and is specifically internalized when the temperature is raised to 21 °C. Similar to our results, the galactose analogue of this derivative is not recognized by the mannose receptor.

The lower initial rate constants of dissociation by EDTA and mannose for the ^{125}I-Man-polylysines (when compared with ^{125}I-Man$_{43}$-AI-BSA) (Figure 2) were probably a reflection of the different binding affinities of the polylysine compounds and Man$_{43}$-AI-BSA. The Man-H-polylysine derivatives have higher affinities for the macrophage and thus may dissociate slower than the Man$_{43}$-AI-BSA, if the association rates of the Man-H-polylysines and Man$_{43}$-AI-BSA are similar.

The Man-H-polylysines exhibited at least biphasic dissociation kinetics in mannose and EDTA (shown in Figure 2). Greater than 75% of the surface-bound ligands were dissociated considerably faster than another minor population of molecules. This dissociation heterogeneity could be caused by size heterogeneity in the lysine polymers. It is not known why the Man-polylysines exhibited multiphase dissociation in the presence of mannose and EDTA while their equilibrium binding data fit best to a one-site binding model (see Results). It is possible that two or more binding sites may exist for each of the Man-polylysine molecules. The dissociation of prebound ligands (Figure 2) may be a more sensitive assay than equilibrium binding for detecting populations of the ligand with different binding properties. It is also possible that either some of the polylysine derivatives or the receptors have undergone changes during the process of prebinding ligand and dissociation in the presence of mannose and EDTA.

The slower dissociation of cell-surface ligand in 0.5 M mannose vs. 10 mM EDTA (Figure 2) was probably caused by the different mechanisms by which the two agents caused dissociation. While EDTA chelates calcium which is necessary for receptor binding, mannose acts as a competitive inhibitor of ligand binding at the sugar binding site of the receptor. EDTA has a binding constant of $K_d = 2 \times 10^{-11}$ M for calcium whereas the Gal/GalNAc receptor, which is calcium dependent for binding, has a K_d for 10^{-5} M for calcium (Blomhoff et al., 1982). If the mannose receptor's affinity for calcium is similar to that of the Gal/GalNAc receptor, there is 6 orders of magnitude difference between receptor and EDTA affinity for calcium. One would expect 10 mM EDTA (the conditions of the EDTA dissociation experiment of Figure 2) to quickly sequester the calcium away from the binding protein, assuming both receptor and EDTA had equal access to the available calcium. It was necessary to use greater than 10 mM (EDTA concentration used for dissociation) mannose to effect disso-

ciation of surface-bound ligand. Mannose at 10 mM is an ineffective dissociating agent for two reasons. First, this concentration of mannose causes enhanced binding of carboxypeptidase Y and Man_{43}-AI-BSA to macrophages (Hoppe & Lee, 1982) and would presumably also cause enhanced binding of the Man-polylysine derivatives. Second, the K_d of mannose binding to macrophages is probably on the order of 10 mM [i.e., one-thirtieth the mannose concentration at which complete inhibition of Man_{43}-AI-BSA binding to macrophages occurs (Hoppe & Lee, 1982)]. Thus, this concentration of mannose would not be adequte to effect complete dissociation of the Man-polylysines.

Since both Man-H-polylysines bound tightly to the mannose receptor of the macrophage, their internalization and degradation by the macrophage were tested. Two main points can be made from the data of Figures 3 and 4: (1) ^{125}I-Man_{90}-H-poly-D-Lys and ^{125}I-Man_{104}-H-poly-L-Lys were initially taken up by the cells approximately 1.5-fold faster than ^{125}I-Man_{43}-AI-BSA and (2) the macrophages did not release degradation products into the medium after ^{125}I-Man_{90}-H-poly-D-Lys uptake, unlike ^{125}I-Man_{43}-AI-BSA and ^{125}I-Man_{104}-H-poly-L-Lys which were degraded at comparable rates by the macrophage. The ^{125}I-Man-H-polylysines were initially taken up faster than ^{125}I-Man_{43}-AI-BSA by the macrophage probably because of their higher affinity for the mannose receptor (Table I). A similar observation was made for increasingly higher mannose-coupled Man-AI-BSA derivatives (Hoppe & Lee, 1983). At comparable concentrations, the higher affinity ligands (higher mannose coupled) were taken up faster by macrophages than ligands of lower affinity (lower mannose coupled).

The product of ^{125}I-Man_{104}-H-poly-L-Lys degradation might be expected to be ^{125}I-N^{ϵ}-[3-(*p*-hydroxyphenyl)propanoyl]-L-lysine. However, the degradation product did not coincide with standard ^{125}I-N^{ϵ}-[3-(*p*-hydroxyphenyl)propanoyl]-L-lysine on Sephadex G-10 chromatography (Figure 5). We have tentatively concluded that the degradation product is a short peptide of lysine [with an ^{125}I-3-(*p*-hydroxyphenyl)propionyl group attached] which is resistant to carboxypeptidase Y digestion. The action of carboxypeptidase Y is influenced dramatically by the side chains of carboxyl-terminal and neighboring amino acids (Kuhn et al., 1974). The presence of the ^{125}I-3-(*p*-hydroxyphenyl)propionyl group (Figure 6) and perhaps a sugar derivative on the peptide could dramatically hinder the carboxypeptidase activity of the enzyme. The inability of carboxypeptidase Y to digest the ^{125}I-Man_{104}-H-poly-L-Lys degradation product is not surprising given that the macrophage, itself a very potent degradative machine, is unable to degrade this putative peptide to smaller end products.

After macrophages were incubated with ^{125}I-Man_{90}-H-poly-D-Lys for 2 h, no Sephadex G-10 included radioactive materials could be detected in the medium (Figure 4). In contrast, degradation products appeared in the medium after 7.5 min of incubation at 37 °C for ^{125}I-Man_{43}-AI-BSA.[3] To test whether cells released intact ligand back into the medium after its uptake, cells were preincubated at 37 °C for 20 min in ^{125}I-Man_{90}-H-poly-D-Lys, medium radioactivity was removed, and incubation was continued at 37 °C. Cells released radioactivity very slowly on continued incubation at 37 °C (Table II). To determine whether this cell-associated ^{125}I-Man_{90}-H-poly-D-Lys remained intact, Man_{90}-H-poly-D-Lys which had accumulated intracellularly was analyzed on Sephadex G-50 columns. The intracellular radioactivity coincided with the original ^{125}I-Man_{90}-H-poly-D-Lys, indicating that no detectable difference in size existed between the radiolabeled molecules. Thus, intact ^{125}I-Man_{90}-H-poly-D-Lys could not exit the macrophage after its internalization. This is analogous to the case of the internalized β-glucuronidase (Shepherd et al., 1983) which is poorly degraded and accumulates in secondary lysosomes in bone marrow macrophages over a 24-h incubation.

In contrast, a similar analysis of the intracellular radioactivity using the degradable ^{125}I-Man_{104}-H-poly-L-Lys yielded two peaks by Sephadex G-50 chromatography. Peaks eluted with intact ^{125}I-Man_{104}-H-poly-L-Lys and medium degradation products (Figure 5). There are three possible explanations for the absence of any intermediate-sized radiolabeled compounds: (1) ^{125}I-Man_{104}-H-poly-L-Lys may be labeled near the termini, requiring very few hydrolytic steps to liberate the end product: (2) if the ligand is labeled at the inner lysyl residues, the entire degradation process may be very fast and may not allow the detectable accumulation of any intermediates: (3) lysosomal enzymes may attack the polypeptide chain at the point where the iodinated 3-(*p*-hydroxyphenyl)-propionyl group is attached.

Our results reveal some information about the permeability of the lysosomal membrane. When the poly(L-lysine) derivative was used, the degradation product from lysosomes was rapidly transported outside by some unknown mechanism. This mechanism obviously does not apply to the undegraded poly(D-lysine) derivative which accumulated in the macrophage. Similarly, Furuno et al. (1983) have shown that rat hepatocyte lysosomes are unable to degrade pepstatin after it is internalized as a pepstatin/asialofetuin conjugate. However, the pepstatin is eventually secreted into the bile by the hepatocyte.

We wanted to establish whether mannose receptors were being reutilized during the accumulation of the Man_{90}-H-poly-D-Lys ligand within the macrophage. Thus, the total number of functional mannose receptors associated with the macrophage was determined by using Triton X-100 in a soluble binding assay. Approximately 200 000 total functional receptors (as defined by the number of ligand molecules bound) were found by direct binding asssy of solubilized macrophages. Assuming no alteration of receptor binding activity by detergent, the total receptor activity in the soluble assay can be compared with ligand binding to intact cells. Accordingly, 30–40% of the total mannose receptor of the macrophage is located on the cell surface (see Table I). The 20–30% of total receptor found at the cell surface of the rat alveolar macrophage estimated by using trypsin treatment of cells at 4 and 37 °C (Stahl et al., 1980) is comparable to this value. The total ^{125}I-Man_{90}-poly-D-Lys which accumulated in macrophages (1.7×10^6 molecules/cell) was 7-fold greater than the total receptor present in the macrophage (236 000 sites/cell as determined with Man-poly-D-Lys), supporting the notion that the receptor used for internalization of ligand can be reutilized even in the absence of ligand degradation. An analogous situation apparently exists for the mammalian Gal/GalNAc receptor (Steer & Ashwell, 1980; Baenziger & Fiete, 1982; Bridges et al., 1982).

Acknowledgments

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Registry No. N^{ϵ}-[3-(*p*-hydroxyphenyl)propanoyl]-L-lysine, 88916-49-8; N^{α}-Cbz-L-lysine, 2212-75-1; *N*-succinimidyl 3-(*p*-

[3] C. A. Hoppe and Y. C. Lee, unpublished results.

hydroxyphenyl)propionate, 34071-95-9.

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Anticoagulantly Active Heparin-like Molecules from Vascular Tissue†

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ABSTRACT: Mucopolysaccharides were isolated from calf cerebral microvasculature and calf aorta. The only complex carbohydrates that exhibited anticoagulant activity were heparin-like components. The biologic potencies of calf cerebral and aortic heparin-like species were 2.92 units/mg of anti-factor Xa activity and 2.85 units/mg of anti-factor IIa activity, as well as 0.56 unit/mg of anti-factor Xa activity and 0.19 unit/mg of anti-factor IIa activity, respectively. Additional experiments revealed that the anticoagulantly active aortic components were significantly present only within the intima. The above populations of heparin-like species were affinity fractionated with antithrombin. The highly active component obtained from calf cerebral microvasculature exhibited an anti-factor Xa activity of 40.7 units/mg as well as an anti-factor IIa activity of 36.8 units/mg, constituted about 4.2% of the initial mass of the starting material, and represented about 75% of the biologic potency of the starting material. The highly active component derived from calf aorta exhibited an anti-factor Xa activity of 55.4 units/mg as well as an anti-factor IIa activity of 11.3 units/mg, constituted about 0.3% of the initial mass of the starting material, and represented about 60% of the biologic potency of the starting material. The highly active cerebral microvascular species possessed a molecular weight and charge density similar to that of heparan sulfate whereas the highly active aortic species displayed a molecular weight and charge density equivalent to that of a hexadecasaccharide fragment of heparin.

Heparin is known to be synthesized by mast cells and is capable of accelerating the neutralization of hemostatic enzymes by antithrombin (Metcalfe et al., 1979; Damus et al., 1973; Stead et al., 1976; Rosenberg et al., 1975b). Heparan sulfate, a heparin-like substance, is found on various cell surfaces as well as in basement membranes and is known to exhibit small amounts of anticoagulant activity (Kanwar & Farquhar, 1979; Kraemer, 1971; Buonassisi & Root, 1975; Teien et al., 1976; Thomas et al., 1979; Radhakrishnamurthy et al., 1977; Oegema et al., 1979). The present investigation was undertaken to determine whether heparan sulfate associated with vascular tissue contains a small population of anticoagulantly active mucopolysaccharide chains that function in a biochemically identical manner with heparin.

To this end, heparin-like molecules were isolated from calf cerebral microvessels and aortas. The ability of these components to enhance the rate of inactivation of thrombin or factor Xa by antithrombin was established by standard chromogenic assays. Affinity fractionation of the above preparations with protease inhibitor revealed that only a small portion of these products could bind to antithrombin. Fur-

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