Intracellular Metabolism of Indium-111-DTPA-Labeled Receptor Targeted Proteins

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The mechanisms of hepatic and renal accumulation and retention of ^{111}In-labeled proteins has been the subject of many investigations. Because the lysosome is a common intracellular destination for a variety of agents including antibodies and polypeptide hormones, we studied the in vitro and in vivo metabolism of ^{111}In-DTPA-labeled polypeptides using a series of glycoproteins that were concentrated within the lysosome by receptor mediated endocytosis. Indium-111-DTPA-labeled glycoproteins targeted to the mannose, asialoglycoprotein and mannose 6-phosphate receptors were studied in vitro using cell lines known to express these receptors and in vivo using Sprague-Dawley rats. Once internalized, the ^{111}In label was released slowly with 60%–90% (depending on the cell type) of the activity remaining cell associated at 24 hr. Subcellular fractionation using Percoll gradients indicated that the activity remained within the lysosome. Following internalization of the ^{111}In-DTPA-labeled glycoproteins, the label was rapidly converted to a low molecular weight species (estimated molecular weight ≤1000 daltons). This conversion was not seen with ^{111}In-DTPA-α-galactosidase. As a lysosomal enzyme, α-galactosidase is relatively resistant to proteolysis within the lysosome. These results suggest that following internalization, ^{111}In-DTPA-polypeptides are delivered to the lysosome where the polypeptide backbone can be degraded to yield ^{111}In-DTPA-amino acid(s). These metabolites remain within the lysosome and are only slowly released from the cell. The model systems used in these studies can also be used to evaluate the intracellular metabolism of polypeptides labeled by other techniques.

J Nucl Med 1993; 34:1728–1738

Radiolabeled polypeptides such as antibodies against tumor-associated antigens are being studied as potential therapeutic and diagnostic agents (*1*). The activity of these agents at any particular anatomic site represents a dynamic competition between the rate of accumulation and the rate of loss. While numerous methods of labeling polypeptides exist, one can best measure the rate of accumulation by choosing a strategy that provides a low rate of loss. Loss rates are affected not only by the stability of the radiolabel-polypeptide bond but also by events subsequent to tissue localization. These events include intracellular metabolism.

When considering intracellular metabolism, much evidence indicates that internalization and lysosomal degradation play important roles. Antibodies bound to cell surface antigens are often internalized and delivered to the lysosome for degradation (*2–5*). In addition, molecules such as low density lipoprotein (LDL) are rapidly delivered to the lysosome by receptor mediated endocytosis (*6,7*). The LDL and other receptor mediated endocytosis systems utilize specific high affinity cell surface receptors to capture their ligands from the extracellular milieu. The receptor-ligand complex is then internalized via invagination of the plasma membrane. The resulting intracellular vesicles, termed endosomes, rapidly acidify, and in this low pH environment, the ligand dissociates from the receptor. The receptor recycles back to the plasma membrane while the ligand is delivered to the lysosome. Because it recycles with a cycle time of approximately 15 min (*8*), a single receptor can deliver numerous ligand molecules to the lysosome. Thus, to study intracellular metabolism, receptor mediated endocytosis systems offer a means of delivering large amounts of radiolabeled polypeptides to the lysosome.

Although numerous receptor-mediated endocytosis systems exist, we have chosen to study systems whose receptors recognize the oligosaccharide moiety of glycoproteins. The mannose, mannose 6-phosphate and asialoglycoprotein receptor systems are well characterized examples (*7,9–12*) and they demonstrate several important points. First, almost any polypeptide can be targeted to the lysosome in these systems by attachment of the appropriate carbohydrate residues or modification of the existing oligosaccharide (*13–15*). Second, since the underlying polypeptide does not participate in binding, conditions or modifications which can denature proteins do not interfere with the oligosaccharide-receptor interaction (*16*). Third, receptor binding is easily blocked by adding a large excess of a simple carbohydrate. These attributes make them an ideal choice for studying the intracellular metabolism of radiolabeled polypeptides.

For these studies we have synthesized ligands to these receptors and labeled them with radiometals through bifunctional chelates. Several groups have studied the metabolism of proteins labeled in the same or similar ways

Received Apr. 2, 1993; revision accepted June 17, 1993.

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(*17–26*). These studies have suggested that intracellular metabolism yields a low molecular weight compound. Additionally, other studies suggest that the bifunctional chelate-radiometal complex remains intact (*17,20,26*). The exact structure of the radiolabeled metabolite, the intracellular location of metabolism and the reasons why the metabolite remains cell associated have not yet been determined. To answer these questions, we developed methods for analyzing the intracellular location and kinetics of radiolabeled polypeptide metabolism. We have used these systems to study the kinetics of intracellular metabolism and radiolabel release. Our approach uses immortalized cell lines as an in vitro model system and draws on the extensive background of knowledge concerning how these cells metabolize labeled polypeptides. The approach was validated by studying the metabolism of these same molecules in vivo.

MATERIALS AND METHODS

Materials

All reagents were of analytical grade. Indium-111-Cl_3 (4.19×10^5 Ci/g) was provided by Mallinckrodt Inc. (St. Louis, MO). Sodium-^{125}I (15.2 mCi/μg) was from Amersham (Arlington Heights, IL). Superose 12 HR 5/30 and PD-10 gel filtration columns were from Pharmacia (Alameda, CA). AG 50X2 (H^+ form), Chelex-100, Bio-Rad Protein Assay reagent and Biospin-6 columns were from Bio-Rad (Richmond, CA). Mannose 6-phosphate, aprotinin, antipain, leupeptin, chymostatin, pepstatin, human serum albumin, Percoll, thymidine 5′-monophosphate p-nitrophenylester, p-nitrophenyl N-acetyl-β-D-glucosaminide, p-nitrophenyl α-D-galactopyranoside, NADH, DTPA cyclic anhydride, yeast mannan, fetuin and asialofetuin were from Sigma Chemical Co. (St. Louis, MO). Mannose-bovine serum albumin and galactose-bovine serum albumin were from E-Y Labs (San Mateo, CA). The 2,4,6-trinitrobenzene sulfonic acid was from Pierce (Rockville, IL). Centricon-10 and -30 ultrafiltration units and PM-10 ultrafiltration membranes were from Amicon (Beverly, MA). Ultrafree-MC filter units and Millex-GS syringe filters were from Millipore (Bedford, MA). Fetal calf serum was from Intergen (Purchase, NY). Mature male (~250 gm) Sprague-Dawley rats were from Sasco (Omaha, NE). All animal experiments were were performed in compliance with guidelines specified by the Washington University Animal Care Committee. *Pichia holstii* o-phosphomannan was generously provided by M.E. Slodki (USDA, Peoria, IL). Recombinant human lysosomal α-galactosidase A was from Debra Barngrover, Genzyme Corporation (Framingham, MA).

Cells

The mouse fibroblast cell line, L-SS, was obtained from Stuart Kornfeld, Washington University (St. Louis, MO) and grown as a monolayer in α-MEM containing 10% fetal bovine serum (FCS) and supplemented with 2 m*M* glutamine. The human hepatoma cell line, HepG2, was obtained from Alan Schwartz, Washington University (St. Louis, MO) and grown as a monolayer in α-MEM containing 15% FCS and supplemented with 2 m*M* glutamine. The mouse macrophage cell line, J774E, was obtained from Phillip Stahl, Washington University (St. Louis, MO) and grown as both a monolayer and suspension culture in α-MEM containing 10% FCS and supplemented with 60 μ*M* thioguanine and 2 m*M* glutamine. Except where noted, the cells were used as they neared maximal density.

Preparation of Pentamannosylphosphate Human Serum Albumin

Pentamannosylphosphate was prepared using a modification of the procedure described by Bretthauer et al. (*27*). Briefly, 10 gm of o-phosphomannan was dissolved in 370 ml of water containing 3.7 gm KCl. The pH was adjusted to 2.4 with HCl and the mixture boiled for 4 hr with refluxing. A precipitate was removed by centrifugation at 11,000 *g* for 10 min and the supernatant adjusted to pH 6.9 with NaOH. Barium acetate (4 g) was added and the pH brought to 8.0 with NaOH. The mixture was cooled to 4°C and 0.1 volumes of ice-cold ethanol were added to precipitate the acid resistant core. This was removed by centrifugation at 11,000 *g* for 10 min. The pentamannosylphosphate in the supernatant was then precipitated by adding 2 volumes of ice-cold ethanol. The precipitate was collected by centrifugation, the supernatant decanted and the pellets dried overnight in a dessicator. The pentamannosylphosphate (barium salt) was dissolved in a minimal amount of water and converted to the free acid by passage through a AG 50X2 (H^+ form) column. The column eluate was converted to the sodium salt by adjusting the pH to 7.0 with NaOH and the product lyophilized.

Pentamannosylphosphate was coupled to human serum albumin by reductive amination as described by Schwartz and Gray (*28*). Briefly, 1.8 gm of pentamannosylphosphate (sodium salt) was mixed with 130 mg of human serum albumin in 8 ml of 50 m*M* Bicine, pH 9.0. The mixture was sterilized by passage through a 0.22-μm filter and 79.2 mg of sodium cyanoborohydride was added. After 48 hr at room temperature, the mixture was diluted into 50 ml of the Bicine buffer and the free pentamannosylphosphate and cyanoborohydride removed by diafiltration using an Amicon model 8050 stirred cell and PM-10 filter until the ultrafiltrate was free of reducing sugars. Following concentration with a Centricon-10, the protein and sugar content were assayed and the coupling ratio calculated to be 21:1 (pentamannosylphosphate-to-albumin).

Glycoprotein Labeling

DTPA was attached to proteins using a modification of the procedure described by Hnatowich et al. (*29*). For pentamannosylphosphate-albumin, galactose-albumin, albumin and mannose-albumin, 0.75 ml of a 10 mg/ml solution in 100 m*M* Bicine pH 9.0 was added to 3.0 mg of DTPA cyclic anhydride (approximately 100-fold molar excess of DTPA anhydride) and the solution was vortexed immediately. For fetuin and asialofetuin, 1.0 ml of a 25 mg/ml protein solution was added to 36 mg DTPA cyclic anhydride (200-fold molar excess). After a 1-hr incubation at room temperature, the remaining anhydride was quenched by adding ethanolamine (20 m*M* final). The free DTPA and ethanolamine were removed by gel filtration on a PD-10 column equilibrated with 20 m*M* Hepes, 150 m*M* NaCl, pH 7.4 (HBS). The fractions were pooled only after assaying for both protein and amines.

For α-galactosidase, 200 μl of 1 mg/ml solution in 100 m*M* Bicine, pH 9.0, was mixed with 4 μl of a 5 mg/ml solution of DTPA cyclic anhydride dissolved in dimethylsulfoxide (100-fold molar excess). After a 1-hr incubation at room temperature, the remaining anhydride was quenched by adding excess ethanolamine. The α-galactosidase-DTPA was purified by affinity chromatography using a cation independent mannose 6-phosphate receptor-Affigel 10 column equilibrated in 20 m*M* Hepes and 150

m*M* NaCl, pH 7.4 (*30*). After extensive washing to remove the free DTPA, the α-galactosidase-DTPA was specifically eluted with buffer containing 10 m*M* of mannose 6-phosphate which was removed by diafiltration in a Centricon-10. Enzymatic assays demonstrated no loss of activity with the addition of DTPA.

Five to fifty μg aliquots of DTPA-glycoproteins were diluted into 100 μl of 0.1 *M* NaOAc, 150 m*M* NaCl (pH 6.0) and labeled with between 50 and 1000 μCi $^{111}InCl_3$. The labeled molecules were separated from the unbound ^{111}In either by gel filtration using BioSpin 6 columns and 20 m*M* of Hepes, 150 m*M* NaCl, pH 7.4, as the eluant or by metal affinity chromatography with Chelex-100 resin. For affinity chromatography, the resin was equilibrated in 0.1 *M* NaOAc, 150 m*M* NaCl pH 6.0 and 100 μl (gel volume) was collected in the upper chamber of a Ultrafree-MC filter unit. The unit was centrifuged at 14,000 rpm for 30 sec to remove the excess buffer. The sample was then applied and allowed to equilibrate with the resin for 15 min before the protein sample, free of unbound radiometal was collected by centrifugation. Both methods typically produced radiochemical yields in excess of 80% and specific activities of up to 20 μCi/μg. PMP-albumin-DTPA was iodinated by the iodogen method (*31*).

Endocytosis Assays

For experiments lasting less than 24 hr, cells were grown to confluence in six well dishes. Longer experiments used cells which were subconfluent. In each case, the growth medium was removed, cultures washed once and the assay was initiated by adding 1.5 ml of complete medium containing the labeled glycoprotein. Routinely, each ml of medium contained 1–3 μCi of the labeled glycoprotein (specific activity 2–10 mCi/mg protein). Control dishes contained one of the appropriate blocking compounds: 10 m*M* mannose 6-phosphate, 0.1 mg/ml galactose-albumin or asialofetuin or 2 mg/ml of yeast mannan. At the indicated times, the cells were cooled to 4°C, washed five times with ice-cold HBS, scraped into 1.0 ml of 0.1 N NaOH with a rubber policeman and cell-associated radioactivity determined in a gamma counter. For cultures incubated with the ^{125}I-labeled ligand, the medium was applied to a Centricon-10 and centrifuged for 90 min to separate small molecular weight metabolites from the intact ^{125}I-PMP-albumin-DTPA.

Cellular Release Assay

L-SS and HepG2 cells were grown in 60-mm dishes. J774E cells were grown in suspension. After a single wash, uptake was initiated by adding 3 ml of medium containing 3.0 μCi of the appropriate ligand. Control plates contained either mannose 6-phosphate, asialofetuin or mannan as above. All components were sterilized by passage through a 0.22-μm filter prior to use. At the indicated times, the chase was initiated by removing the medium, washing the cultures three times, and adding 5 ml of chase medium which contained either 5 m*M* mannose 6-phosphate, 0.05 mg/ml asialofetuin or 1 mg/ml mannan to inhibit release and receptor mediated recapture of radiolabeled molecules. At the indicated times, the medium was removed, the monolayer washed four times with ice-cold HBS, the cells lysed in 1 ml of 0.1 N-NaOH and cell associated radioactivity determined. The radioactivity in the cell lysate and medium was measured. Aliquots of the medium were fractionated using Centricon-10 or -30 ultrafiltration devices.

Subcellular Fractionation

HepG2 cells were grown to confluence in 75-mm^2 T-flasks. The monolayer was washed once, and endocytosis initiated by adding either 1.0 μCi galactose-albumin-DTPA-^{111}In or 5.0 μCi PMP-albumin-DTPA-^{111}In in 25 ml of growth medium. After a 4-hr incubation, the medium was removed, the monolayers washed three times and 20 ml of fresh medium added. After a 1-hr (PMP-albumin) or 2-hr (galactose-albumin) chase, the cells were washed three times with ice-cold HBS before adding 2.5 ml of 0.25 *M* sucrose, 10 m*M* Tris, pH 7.5, containing a cocktail of protease inhibitors (20 μg/ml final each of antipain, pepstatin, chymostatin and leupeptid, 25 IU/ml final concentration of aprotinin). The cells were collected by scraping with a rubber policeman and then allowed to swell for 10 min on ice. The cells were then disrupted by 10 passes through a 22-gauge needle and either 20 or 50 strokes in a Dounce homogenizer (Kontes). Intact cells and nuclei were pelleted by centrifugation at 400 g for 10 min. The postnuclear supernatant contained between 33% (20 strokes in Dounce) and 70% (50 strokes in Dounce) of the total ^{111}In activity. Two milliliters of the postnuclear supernatant was carefully added to tubes containing 6 ml of a 6.75% Percoll solution containing 0.25 *M* sucrose, 10 m*M* Tris (pH 7.5) and protease inhibitors which had been layered over 1.2 ml of 2.5 *M* sucrose. The gradients were centrifuged for 30 min at 28,000 rpm in a Ti70 rotor with the brake off. Fractions (0.5 ml) were collected from the top of the gradient using a Buchler Auto Densi-Flow II connected to a peristaltic pump and fraction collector. Radioactivity was determined using a gamma counter. Aliquots were assayed for marker enzymes as described below.

Degradation Assay

L-SS cells were grown to confluence in 60-mm dishes. The monolayers were washed once before adding 5.0 ml of growth medium containing either 3.2 μCi PMP-albumin-DTPA-^{111}In or 0.1 μCi α-galactosidase-DTPA-^{111}In. After a 30-min uptake, cells were washed three times before adding 5.0 ml of growth medium containing 5 m*M* mannose 6-phosphate to initiate the chase. At the indicated times, cells were washed four times with HBS, scraped into 1.0 ml of HBS containing protease inhibitors and disrupted by sonication (Branson sonicator model 183 at power level 3 for 5 sec). The lysate was frozen overnight, thawed and sonicated a second time. The samples were passed through a 0.22-μm filter prior to gel filtration on a Superose 12 HR 5/30 column using a fast protein liquid chromatography system (FPLC, Pharmacia). The column was eluted with HBS at a flow rate of 0.4 ml/min and 1-min fractions were collected and assayed for radioactivity. Since the cultures which contained mannose 6-phosphate during the original uptake endocytosed little radioactivity (1%–2% compared to companion cultures), fresh ^{111}In-labeled glycoprotein was added to these lysates as a means of assessing whether proteolysis occurred after cell lysis.

Biodistribution

Biodistribution experiments were performed as previously described (*32*). Routinely, rats received 5–7.5 μCi of ^{111}In-labeled glycoproteins (specific activity 5–10 mCi/mg protein). Animals were sacrificed at 1, 4, 24 and 48 hr after injection and tissue samples removed and weighed. Radioactivity content was determined using a well type gamma counter.

Assays

Protein concentration was determined using the Bio-Rad Protein Assay reagent and bovine serum albumin as a standard. Reducing sugars were assayed by the phenol sulfuric method (*33*) using mannose as a standard. Amines were detected using 2,4,6-trinitrobenzene sulfonic acid. β-Hexosaminidase and α-galactosi-

TABLE 1
Glycoprotein Receptors and Suitable Ligands

Receptor	Cells expressing receptor	Suitable ligands	References
Asialoglycoprotein	HepG2 (human hepatoma cell line) hepatocytes	galactose-albumin asialofetuin	9,13,14,36
Mannose 6-phosphate (cation independent)	L-SS (mouse fibroblast cell line) HepG2 most cell types with lysosomes	α-galactosidase PMP-albumin	9,11,36–39
Mannose	J774E (mouse macrophage cell line) macrophages Kupffer cells hepatic endothelial cells splenic sinusoidal cells	mannose-albumin	9,13,14,40–43

dase were measured using the substrates, p-nitrophenyl N-acetyl-β-D-glucosaminide and p-nitrophenyl α-D-galactopyranoside respectively (*34*). Lactate dehydrogenase was assayed by measuring the conversion of NADH to NAD^+ after the addition of pyruvate. Alkaline phosphodiesterase I was measured using thymidine 5′-monophosphate p-nitrophenylester as a substrate (*35*). Following subcellular fractionation, enzyme activity was normalized to the amount measured in the most active fraction.

RESULTS

Preparation of Radiolabeled Glycoproteins

To study the metabolism of radiolabeled compounds in a variety of cell types, five different agents targeted towards three different glycoprotein receptors were prepared. These agents and their corresponding glycoprotein receptors are summarized in Table 1. The ligands fall into two broad groups. Fetuin and α-galactosidase are members of the first group consisting of proteins glycosylated during their biosynthesis and modified during post-translational processing. The lysosomal enzyme, α-galactosidase, binds to mannose 6-phosphate receptors via the phosphomannosyl residues in its N-linked oligosaccharides (*39*). In fetuin, the terminal sialic acid residues were removed by the supplier to generate asialofetuin, a molecule which can bind to the asialoglycoprotein receptor via its exposed terminal galactose residues. Pentamannosylphosphate-albumin (PMP-albumin), galactose-albumin and mannose-albumin constitute the second class where the appropriate carbohydrate moiety was added to a nonglycosylated protein. Pentamannosylphosphate was coupled to albumin via reductive amination to produce a semisynthetic ligand for the mannose 6-phosphate receptor. Ligands for the mannose receptor and the asialoglycoprotein receptor, mannose-albumin and galactose-albumin respectively, were obtained from commercial sources.

The bifunctional chelate, DTPA cyclic anhydride, was added to each of these glycoproteins in preparation for labeling with radiometals. Since DTPA cyclic anhydride reacts with nucleophilic sites in the polypeptide backbone and leaves the oligosaccharide unaffected, it should not alter binding to glycoprotein receptors. This was verified by comparing the binding of PMP-albumin and PMP-albumin-DTPA to purified cation independent mannose 6-phosphate receptor (CI-M6PR). No difference was found in a competitive binding assay (data not shown). At the coupling ratios used, DTPA derivatization of α-galactosidase did not affect its enzymatic activity (results not shown). The number of DTPA moieties per protein was determined for asialofetuin-DTPA and albumin-DTPA using a radiometal of known specific activity. Asialofetuin-DTPA and albumin-DTPA bound 8 and 12 moles of metal per mole of protein respectively (data not shown).

Receptor-Mediated Endocytosis of Glycoproteins

The endocytosis and subsequent metabolism of PMP-albumin-DTPA-^{111}In was compared with PMP-albumin-DTPA which was labeled with ^{125}I by direct iodination. As shown in Figure 1, mouse fibroblasts which possess mannose 6-phosphate receptors rapidly accumulated both compounds. When mannose 6-phosphate was added to the medium, this accumulation was inhibited by greater than 90% indicating that the uptake was mediated by mannose 6-phosphate receptors. In these experiments, no attempt

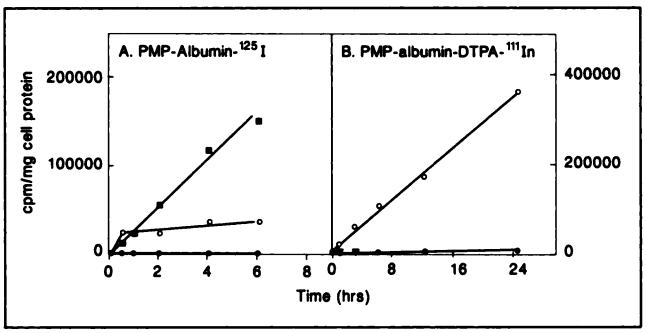

FIGURE 1. Receptor mediated endocytosis of pentamannosylphosphate-albumin-DTPA (PMP-albumin-DTPA). Mouse fibroblasts (L-SS) were allowed to endocytose either ^{125}I-labeled (A) or ^{111}In-labeled (B) PMP-albumin-DTPA as described in Materials and Methods. The open symbols (○) represent cell associated radioactivity from cultures without inhibitors while the filled symbols (●, ▲) are data from cultures which included 10 m*M* of mannose 6-phosphate (●), or 100 μ*M* of chloroquine (▲) to inhibit receptor mediated endocytosis. In Panel A, the small molecular weight metabolites (■) were separated from intact ^{125}I-PMP-albumin-DTPA using a Centricon-10 ultrafiltration device.

was made to distinguish between uptake mediated by either the cation-independent mannose 6-phosphate receptor (CD-M6PR) and the cation-independent mannose 6-phosphate receptor (CI-M6PR). However, previous studies under similar conditions with this same (*44*) or similar cell lines (*45*) indicate that the CD-M6PR contributes little, if any, to total uptake. The uptake had other attributes of receptor mediated endocytosis: specificity for ligands containing mannose 6-phosphate, linear correlation with cell number and temperature dependence (results not shown). In addition, the uptake was inhibited by the diprotic amine chloroquine (Fig. 1B). Chloroquine is known to accumulate within acidic vesicles and disrupt receptor mediated endocytosis by increasing the intravesicular pH (*46–49*).

After 30 min, marked differences in radiolabel behavior were seen. The cell associated ^{125}I activity plateaued while the ^{111}In activity steadily increased. In addition, a low molecular weight ^{125}I-labeled metabolite accumulated in the culture medium (Fig. 1A). This compound did not accumulate in cultures where receptor mediated endocytosis was inhibited. These results suggest that the low molecular weight compound results from the intracellular degradation of the PMP-albumin-DTPA and cellular release of the ^{125}I-labeled catabolic product. Several studies have demonstrated similar results with other iodinated molecules targeted to the lysosome (*13,14,50*).

The above results suggest that in contrast to ^{125}I, ^{111}In exits the cell slowly. To test whether ^{111}In retention represents a generalized phenomena, two other glycoproteins, galactose-albumin-DTPA-^{111}In and mannose-albumin-DTPA-^{111}In were tested in two other cell lines. As indicated in Table 1, the human hepatoma cell line, HepG2, expresses large amounts of the asialoglycoprotein receptor, and the mouse macrophage cell line, J774E, expresses the mannose receptor. As shown in Figure 2, both cell lines accumulated the ^{111}In-labeled ligand in a time dependent fashion that was markedly inhibited by excess unlabeled ligand. The HepG2 cell line also expresses the cation independent mannose 6-phosphate receptor and thus readily accumulated both PMP-albumin-DTPA-^{111}In and α-galactosidase-DTPA-^{111}In (Fig. 4 and unpublished results).

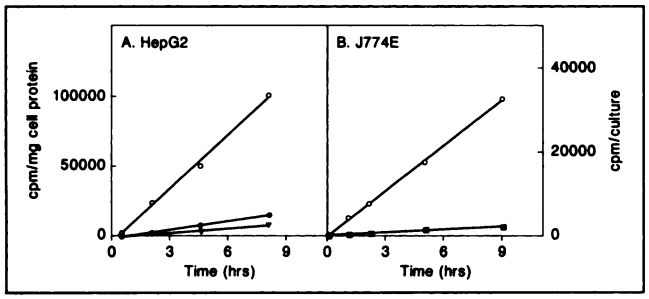

FIGURE 2. Receptor mediated endocytosis of mannose-albumin-DTPA-^{111}In and galactose-albumin-DTPA-^{111}In. (A) Cells from the human hepatoma cell line, Hep G2, or (B) mouse macrophage cell line, J774E were grown to confluence and allowed to endocytose ^{111}In labeled galactose-albumin-DTPA (A) and mannose-albumin-DTPA (B). Open symbols (○) represent the cell associated activity from cultures without inhibitors while filled symbols (●, ▼, ■) represent cultures which included 100 μg/ml asialofetuin (▼), 100 μg/ml galactose-albumin (●), or 2 mg/ml mannan (■) to inhibit receptor mediated endocytosis.

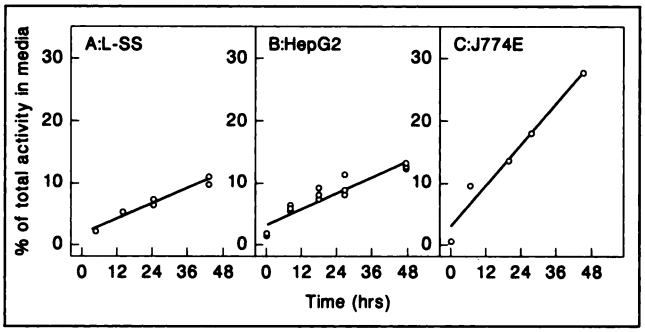

FIGURE 3. Pulse chase release study of ^{111}In-DTPA-glycoproteins in three different cell lines. (A) L-SS, (B) G2 Hep or (C) J774E cells were pulse chase labeled with either PMP-albumin-DTPA-^{111}In (A), galactose-albumin-DTPA-^{111}In (B) or mannose-albumin-DTPA-^{111}In (C) as described in Materials and Methods. After the indicated chase times, radioactivity in both the media and cells was measured in a gamma counter and the fraction in the media calculated.

Measuring the Cellular Release of Indium-111

A pulse chase type experiment was performed to more accurately determine the rate of release. Cells were allowed to internalize the labeled glycoproteins for a short period of time (pulse). The medium containing the radiolabeled ligand was removed and replaced with a medium containing excess unlabeled ligand to initiate the chase. After varying chase lengths, the amount of radioactivity released into the culture medium was compared to the amount remaining cell associated. As shown in Figure 3, the majority of the activity remains cell associated despite chase lengths up to 48 hr. Initially, only a small fraction of the total activity was recovered in the medium but this fraction increased steadily with time. In a similar experiment, Naruki et al. used an anti-CD5 monoclonal antibody antibody, three different T-cell lines and peripheral blood mononuclear cells and demonstrated that these cells also retained the ^{111}In label but rapidly released the ^{125}I label (*25*).

The activity recovered in the culture medium was fractionated by ultrafiltration to determine whether it represented small molecular weight metabolite(s) or protein bound activity. For macrophages, greater than 70% of the activity from each time point could pass through a 30,000-dalton filter. For hepatoma cells, initially 72% of the activity passed through a 30,000-dalton filter but this fell to 50% by 48 hr. This data suggests that most of the extracellular activity does not represent transchelation to the transferrin present in the culture media.

Indium-111 Is Retained in the Lysosome

The intracellular location where the ^{111}In activity was sequestered was determined next. The lysosome was considered a likely possibility since receptor mediated endocytosis delivers ligands to the lysosome and studies with ^{125}I residualizing labels indicated the labeled metabolite remained within the lysosome (*5*). This hypothesis was

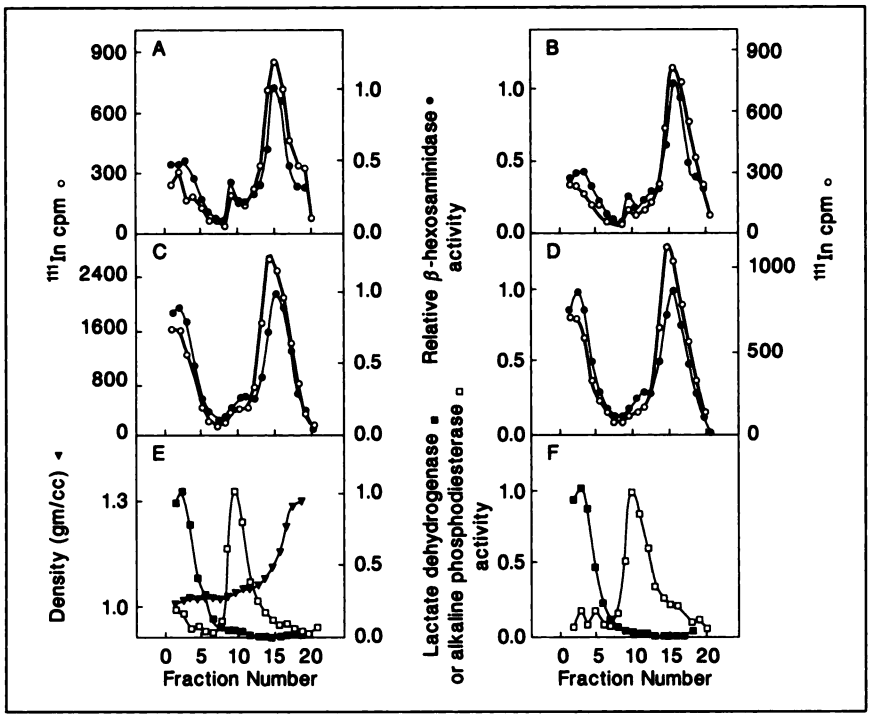

FIGURE 4. Percoll gradient analysis of ^{111}In-DTPA glycoproteins following endocytosis. Hep G2 cells were pulse chase labeled with either PMP-albumin-DTPA-^{111}In (A,C,E) or galactose-albumin-DTPA-^{111}In (B,D,F) as described in Materials and Methods. After either a 1-hr (A,C,E) or 2-hr (B,D,F) chase, the cells were collected, lysed and centrifuged on 6.75% Percoll gradients to separate subcellular components. Fractions were assayed from radioactivity (○), a lysosomal enzyme, β-hexosaminidase (●); a cytosolic enzyme, lactate dehydrogenase (■); a plasma membrane marker, alkaline phosphodiesterase I (□) or density (▼). In Panels A and B, cells were lysed with 20 strokes in a Dounce homogenizer. In Panels C–F, cells were lysed with 50 strokes in a Dounce homogenizer.

tested by labeling HepG2 cells with ^{111}In-DTPA-labeled galactose-albumin or PMP-albumin, chasing a short period, homogenizing the cells and separating lysosomes from other intracellular organelles and the cytosol on Percoll gradients.

As shown in Figure 4 (panels A–D), the radioactivity distribution was nearly identical for cells labeled with either PMP-albumin or galactose-albumin. In addition, the distribution of ^{111}In activity closely paralleled the distribution of the lysosomal marker, β-hexosaminidase. The majority of both the radioactivity and β-hexosaminidase were recovered in the later fractions which had densities characteristic of lysosomes. A smaller fraction of the radioactivity was found in the uppermost (less dense) fractions which contained the cytosolic marker, lactate dehydrogenase. However, the ^{111}In activity was accompanied by a nearly equal fraction of the β-hexosaminidase suggesting that lysosomes had been broken during the homogenization procedure. This hypothesis is supported by the results seen with two different homogenization protocols. Fewer strokes in the Dounce homogenizer resulted in much less radioactivity or β-hexosaminidase in the cytosolic fractions (compare Fig. 4 panels A and B with panels C and D).

Neoglycoproteins Are Rapidly Degraded in the Lysosome

Since earlier results with ^{125}I-labeled PMP-albumin-DTPA suggested that the polypeptide backbone was rapidly degraded (Fig. 1A), we studied the fate of PMP-albumin-DTPA-^{111}In. For these experiments, L-SS fibroblasts were pulse labeled, the culture medium exchanged and mannose 6-phosphate added to initiate the chase. Cells were lysed in buffer containing protease inhibitors to prevent further degradation and the molecular size of the radiolabeled moiety determined by gel filtration chromatography. As shown in Figure 5, the radiolabel from PMP-albumin-DTPA-^{111}In is completely converted to a small molecular weight form within 60 min. Similar experiments with the HepG2 and J774E cell lines indicated that these cells also rapidly degraded PMP-albumin and mannose-albumin respectively (results not shown). In these experiments, the conversion could result from release of the ^{111}In from DTPA, degradation of the chelate itself, hydrolysis of the DTPA-polypeptide bond or degradation of the polypeptide backbone. These possibilities were evaluated using α-galactosidase-DTPA-^{111}In.

Lysosomal enzymes such as α-galactosidase are only slowly hydolyzed in the catabolic environment of the lysosome and thus α-galactosidase-DTPA-^{111}In was used to test the contribution polypeptide backbone degradation makes to the observed conversion. Previous studies have shown that α-galactosidase is efficiently delivered to the lysosome by mannose 6-phosphate receptors (*39*). α-Galactosidase-DTPA-^{111}In was prepared and shown to enter L-SS cells by mannose 6-phosphate receptor mediated endocytosis (results not shown). The protocol used in Figure 5 was repeated with α-galactosidase and the results are presented in Figure 6. In striking contrast to PMP-albumin-DTPA-^{111}In, the majority of the α-galactosidase-DTPA-^{111}In retains its original molecular size even after a 6-hr chase (compare Fig. 5D with Fig. 6E). This result suggested that the molecular size conversion predominantly reflects degradation of the polypeptide backbone. Still some conversion is seen with α-galactosidase-DTPA-^{111}In and this could reflect the possibilities discussed above or a finite, albeit low (*39*), rate of α-galactosidase proteolysis.

In Vivo Studies

The above in vitro studies were performed in cell lines known to express the relevant cell surface receptor. These studies suggested that each of the ^{111}In-labeled glycoproteins was targeted to a common intracellular location, the lysosome. The final set of experiments were designed to analyze the metabolism of ^{111}In-DTPA-labeled proteins in vivo. In vivo, these compounds should distribute among different tissues according to that tissue's level of functioning cell surface receptors. These receptors then deliver the compounds to the lysosome. Thus, while the amount of activity in individual tissues or organs should differ between compounds, the subsequent intracellular metabolism should be similar. Other investigators have examined the clearance and biodistribution of similar polypeptides but these studies have either utilized different radiolabeling strategies or lacked the later time points needed to best measure metabolism and release (*13,14,51–54*).

As shown in Figure 7, the liver accumulated greater than 90% of the receptor ligands, galactose-albumin-DTPA-^{111}In, asialofetuin-DTPA-^{111}In or mannose-albumin-DTPA-^{111}In. This accumulation was rapid, with very little activity remaining in the blood after 1 hr. The biodistribution of molecules targeted to the asialoglycoprotein receptor (galactose-albumin-DTPA-^{111}In and asialofetuin-albumin-DTPA-^{111}In) were expected, given the results of Vera and Stadalnik with ^{99m}Tc-galactosyl-neoglycoalbumin (*51,53*) and Hubbard et al. with ^{125}I-asialofetuin (*13,14*). The results with mannose-albumin-DTPA-^{111}In are consistent with prior studies which utilized ^{125}I-labeled ligands for the mannose receptor (*13,14*). The rapid blood clearance most likely reflects receptor mediated endocytosis since the control compounds, albumin-DTPA-^{111}In and fetuin-DTPA-^{111}In were cleared much more slowly (Fig. 7, Panel K). The biodistribution of ligands for the mannose 6-phosphate receptor was examined and will be reported elsewhere.

Following hepatic deposition, the ^{111}In activity was slowly cleared from this organ (Fig. 7, panels B and G). By 48 hr, approximately 40%–60% of the activity delivered to the liver had been cleared. This rate of loss was higher than expected from experiments with the HepG2 hepatoma cell line where 12% of activity was recovered in the culture medium at 48 hr (Fig. 3B) but nearly equivalent to the rate of loss seen with macrophages in culture, 27% in medium at 40 hr (Fig. 3C). Given the number of alterations between cells in vivo and their immortalized tissue culture counterparts, the observed differences in metabolism and release are considered minor. When compared to other studies, the rate of ^{111}In loss is also much less than that observed with ^{99m}Tc-galactosylneoglycoalbumin where 40%–60% of

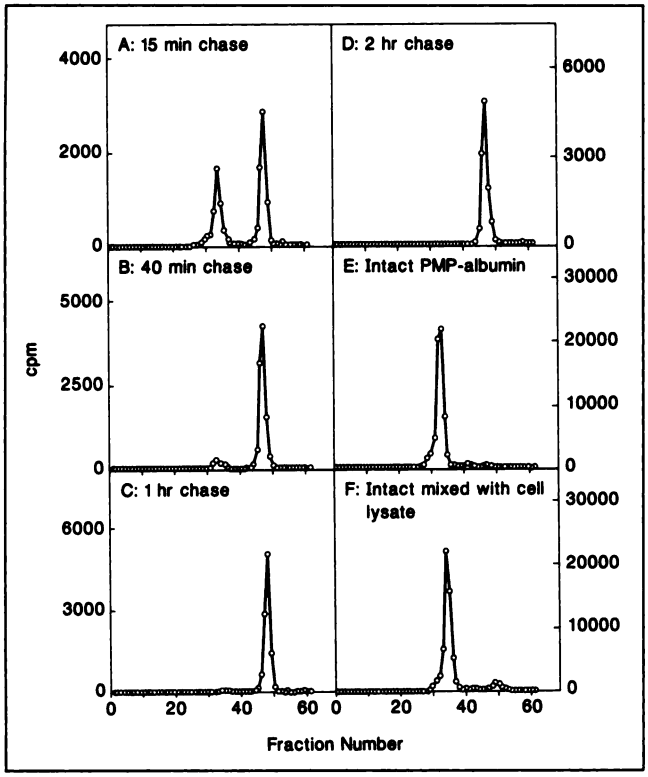

FIGURE 5. Intracellular degradation of PMP-albumin-DTPA-^{111}In. L-SS cells were pulse chase labeled with PMP-albumin-DTPA-^{111}In as described in Materials and Methods. Panels A–D: after the indicated chase times, the cells were lysed in a buffer containing protease inhibitors and the cell associated radioactivity analyzed by gel filtration. Panel E: gel filtration profile for PMP-albumin-DTPA-^{111}In. Panel F: gel filtration profile of PMP-albumin-DTPA-^{111}In mixed with cell lysate. In Panels A–F, the material eluting in fractions 45–49 corresponds to the column's included volume (estimated molecular weight 1000 daltons or less).

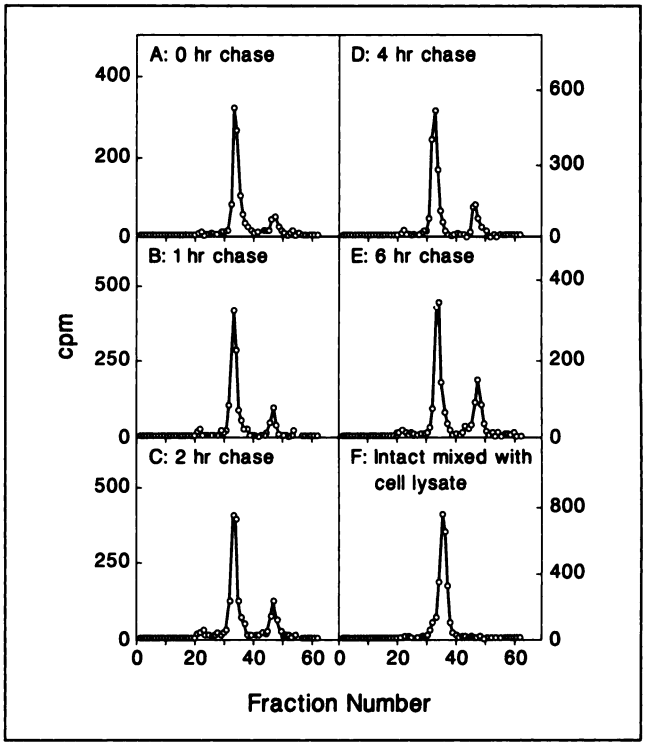

FIGURE 6. Intracellular Stability of ^{111}In-DTPA-α-galactosidase. L-SS cells were pulse chase labeled with ^{111}In-DTPA-α-galactosidase. Panels A–E: after the indicated chase times, cells were lysed in a buffer containing protease inhibitors and the cell associated radioactivity analyzed by gel filtration. Panel F: gel filtration profile of ^{111}In-DTPA-α-galactosidase.

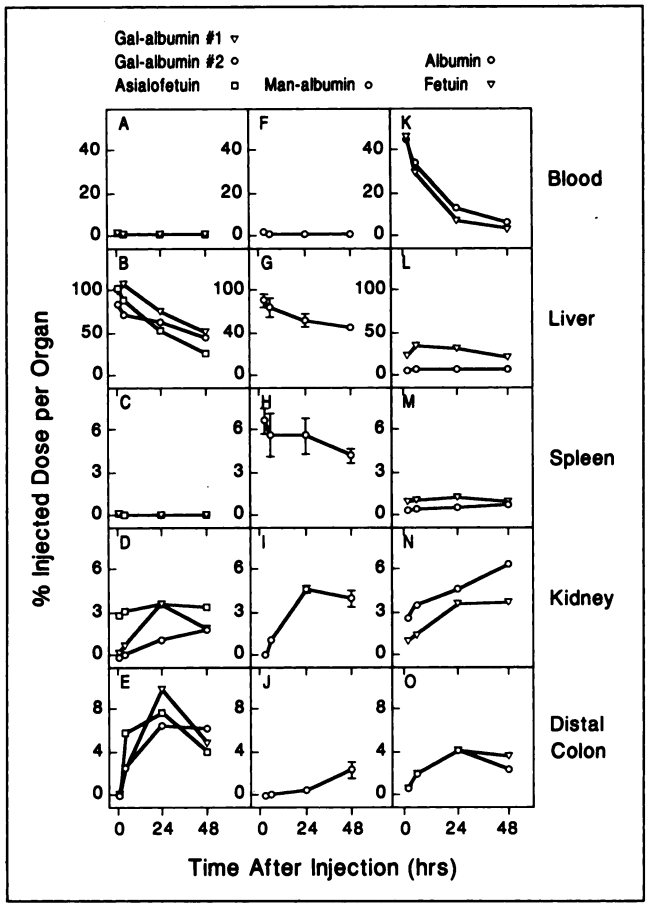

FIGURE 7. In vivo metabolism of ^{111}In-DTPA labeled proteins. The biodistributions of ^{111}In-labeled proteins in mature Sprague-Dawley rats was performed as described in Materials and Methods. The results of two separate experiments are combined. Experiment 1: galactose-albumin-DTPA-^{111}In (#1-▽), mannose-albumin-DTPA-^{111}In (○), and albumin-DTPA-^{111}In (○); Experiment 2: galactose-albumin-DTPA-^{111}In (#2-○), asialofetuin-DTPA-^{111}In (□) and fetuin-DTPA-^{111}In (▽). Symbols represent the averaged values for either four (experiment one) or three (experiment two) animals. Error bars in Panels A–E and K–O were omitted to improve clarity.

the activity had left the liver by 1 hr (*53,54*). Gore et al. found 40% of the activity was secreted into the bile within 1 hr and postulated this may result from active transport of the TcO_4-anion (*54*).

A small but significant fraction of the mannose-albumin-DTPA-^{111}In radioactivity was recovered in the spleen (Fig. 7H). This result is in agreement with prior data using ^{125}I-labeled ligands which indicated that splenic cells express a functional mannose receptor (*40–42*). The 38% decrease in splenic activity over 48 hr was nearly equal to the 39% decrease seen in the liver.

Renal activity increased with time for all animals except those given asialofetuin-DTPA-^{111}In (Fig. 7D, I, N). The increase was most conspicuous for animals given galactose- or mannose-albumin-DTPA-^{111}In because their initial renal activity was essentially zero. This increase most likely reflects hepatic metabolism, release and renal uptake. It is unlikely that the renal activity results from simple filtration since the activity in the blood remained low. Instead, the kidneys appear to actively accumulate and retain the radioactive compound released from the liver. The apparent lack of renal accumulation in animals given asialofetuin-DTPA-^{111}In may indicate that this molecule is metabolized differently within the liver than the neoglycoproteins. The structure of the radiolabeled compound(s) in the kidney and the mechanism of their accumulation are currently under investigation. In the two experiments with galactose-albumin-DTPA-^{111}In, different amounts of activity were found in the kidneys at 24 hr (Fig. 7D). While this could result from normal variation, animals in the first experiment were fasted for 12 hr prior to injection and animals in the second experiment were allowed free access to food, thus fasting versus nonfasting states may have influenced the results.

Radioactivity levels in the distal colon also varied considerably (Fig. 7, Panels E, J and O). The largest increases were observed in animals given compounds targeted to hepatocyte asialoglycoprotein receptors. A possible explanation is that hepatocytes can release radiolabeled metabolites into both the bile and the bloodstream. Exocytosis of lysosomal contents into the biliary system has been observed (*55,56*) and would manifest itself here as increased intestinal activity. This hypothesis is supported by the results with ^{99m}Tc-galactosyl-neoglycoalbumin (*53,54*) and two other observations. First, after 48 hr, 17% and 31% of the initial dose was recovered in the feces of animals given indium-labeled asialofetuin or galactose-albumin, respectively. Second, little activity was found in the distal colon of animals given mannose-albumin-DTPA-^{111}In. This difference likely results from the different cellular distribution of these two receptors in the liver. The mannose-albumin compound is targeted to Kupffer and hepatic endothelial cells and these cells cannot release metabolites directly into the biliary system.

DISCUSSION

These results indicate that molecules coupled to the cyclic anhydride of DTPA and labeled with ^{111}In are endocytosed by their appropriate receptors and delivered to the lysosome. After reaching the lysosome, the molecules can be hydrolyzed to small molecular weight compounds by the resident lysosomal enzymes. These metabolites are retained by the lysosome and slowly released from the cell.

Studies with antibodies directed against cell surface antigens have suggested a similar scheme. Geissler et al. have studied the fate of such antibodies labeled with ^{125}I coupled through a residualizing label or directly linked to the tyrosine residues in the polypeptide (*4,5*). Their data indicate that following cell surface binding, the labeled molecules are internalized and degraded. Autoradiography and subcellular fractionation confirmed that these molecules were delivered to the lysosome. While degradation may begin in endosomes via the action of hydrolases such as cathepsin D (*57*), the majority of degradation likely occurred in the lysosome. Further, different methods of ^{125}I labeling

caused marked differences in the stability of cell associated radioactivity. The ^{125}I linked to an aryl carbohydrate moiety remained cell associated while the the ^{125}I linked to the polypeptide's tyrosines was rapidly released from the cell mainly as ^{125}I-monoiodotyrosine (*5*).

The rapid release of activity from ^{125}I-tyrosine-labeled antibodies is similar to that of other ^{125}I-labeled molecules which are degraded in the lysosome. LaBadie et al. studied the metabolism of asialofetuin labeled with ^{125}I through tyrosine residues (*50*). After delivery to hepatocytes, the molecule was rapidly degraded to mono- and diiodotyrosine in the lysosome. These metabolites rapidly left the lysosome and could be converted to iodide by a cytoplasmic enzyme. Both monoiodotyrosine and iodide were then found in the blood. Our current results indicate that the fate of asialofetuin-DTPA-^{111}In is much different. While the ^{125}I activity was rapidly released, the ^{111}In activity was only slowly released (Fig. 7B). This difference was not confined to glycoproteins targeted to hepatocyte receptors since the ^{125}I label of molecules targeted to mannose receptors found on Kupffer and endothelial cells was also rapidly released (*13,14*) while the ^{111}In label was only slowly released (Fig. 7, Panels G and H). These differences likely reflect the different biochemical pathways available to iodotyrosine derivatives versus ^{111}In-DTPA-labeled metabolites. With this in mind, the rapid intracellular degradation and transmembrane transport of iodinated compounds is not surprising since thyroid cells efficiently degrade thyroglobulin within lysosomes and release the iodinated products (*58*).

These data suggest that certain radiolabeled molecules can utilize existing biochemical pathways to leave cells. If no such pathway exists, or if the pathway is blocked, release is slowed. Lysosomal enzymes labeled with ^{125}I through tyrosine residues are an example of the latter. These proteins are only slowly degraded in the lysosome and thus the ^{125}I release is greatly slowed (*13,14*). Thus, an understanding of a metabolite's structure and the available biochemical pathways can be used to predict which radiolabeled molecules will be retained by the cell.

The exact structure of the radiolabeled metabolites generated from molecules conjugated to various bifunctional chelates are unknown. Sands et al. studied antibodies conjugated to the cyclic anhydride of DTPA and found that these ^{111}In-DTPA-antibodies are degraded to small molecular weight metabolites in the liver (*26*). Using ion exchange chromatography, they identified ^{111}In-DTPA as a minor product and argue that the major product likely represents ^{111}In-DTPA-amino acid(s). In similar experiments with an ^{111}In-DTPA-antibody, Paik et al. found that the radiolabeled compound in the liver was metabolized to a low molecular weight compound that had nearly the same molecular size and ionic character as ^{111}In-DTPA (*17*). Deshpande et al. studied the metabolism of antibodies coupled to SCN-Benzyl-EDTA and found that the metal chelate bond remains intact (*20*). Our data is entirely consistent with these results. With one exception, each of the ^{111}In-DTPA-polypeptides generated using the cyclic anhydride of DTPA was rapidly converted to small molecular compounds. The exception, α-galactosidase-DTPA-^{111}In, suggests that the amino acid backbone is more readily hydrolysed than the DTPA-polypeptide linkage. Together these results suggest that the catabolic product is ^{111}In-DTPA-amino acid(s). This hypothesis is further supported by the recent work of Franssen et al. (*59*). They synthesized a large series of low molecular weight compounds linked to proteins or peptides through an amide linkage. These were exposed to cellular or lysosomal homogenates and the catabolic products analyzed. In every case, the amide bonds between amino acids was hydrolysed while the amide bond between the amino acid and the small molecular weight compound remained intact.

Our results indicate that these labeled low molecular weight metabolites are retained within the lysosome. Others have found the indium-labeled metabolites in what they termed cytosolic fractions (*26*) or nonsedimentable fractions (*19–21*). This apparent discrepancy likely results from differences in subcellular fractionation techniques. Lysosomes can be disrupted when lysing cells (Fig. 4). Prior to centrifugation, other groups have used detergents or sonication and freeze thaw cycling to disrupt cells. These steps are known to disrupt lysosomes and are often employed to release the lysosomal contents prior to further analysis (Figs. 5 and 6). From this, we predict that lysosomes retain the ^{111}In-labeled metabolites. While we did not specifically evaluate the rate at which metabolites leave the lysosome, it is slow compared to the rates of proteolysis and ^{125}I-labeled metabolite release.

In answering the question why ^{111}In-DTPA-amino acid(s) should be retained within the lysosome, one should recall that compounds can move from the lysosome to the cytosol by either diffusion or carrier mediated transport. Diffusion out of the lysosome requires passage through a lipid bilayer and this would be greatly hindered by the ionic character of the chelate and the attached amino acid(s) at intralysosomal pH of 4.5–5.0 (*47*). In fact, amines accumulate in the lysosomes through a combination of passive diffusion and acid-base equilibrium (*46,48*). Carrier mediated amino acid transport systems exist but the lysine transporter is hindered by derivatization of the ε-amine (*60*) and DTPA anhydride addition is thought to occur primarily at the ε-amine. Thus ^{111}In-DTPA-amino acid(s) are an unlikely substrate for carrier mediated transport.

Lysosomal metabolism is of general importance since results with radiolabeled antibodies suggest that lysosomal metabolism plays a role in the turnover of antibodies which bind to cell surface antigens and antibodies deposited in nontarget organs such as the liver and kidneys. Antibodies targeted against the surface antigens of lymphoid cells (*3–5,25*) and other cell types (*2,61*) are internalized and degraded. Kyriakos et al. showed a similar fate of antibodies targeted against the cell surface antigens for four different solid tumors (*62*). High renal background is a significant problem for ^{111}In-DTPA but not ^{125}I-labeled antibody fragments (*63,64*). From the data outlined above, we suggest

the following explanation: antibody fragments pass into the glomerular filtrate and are reabsorbed by the cells of the proximal tubule. Following reabsorption, the fragments are delivered to the lysosome for degradation (*65*). For peptides labeled with ^{125}I through tyrosine residues, intralysosomal degradation yields ^{125}I-monoiodotyrosine which could use the system h transporter to cross into the cytoplasm (*66,67*). While the system h transporter has been best characterized using lysosomes isolated from thyroid follicular cells (*68,69*), given its role in normal cellular nutrition, it is likely to be present in most cell types (*70*). The ^{125}I-monoiodotyrosine thus can leave the lysosome and can be recovered intact in the extracellular fluid or undergo deiodination via a cytoplasmic deiodinase (*71*). In contrast, the ^{111}In-DTPA-amino acid(s) metabolite remains within the lysosome since it can neither diffuse across the membrane or use the existing transport systems. In this model, the differences in nontarget organ activity of ^{125}I- and ^{111}In-labeled tracers reflect large differences in the rates of release rather than small differences in the rates of radiolabel accumulation.

The above data, conclusions and speculations indicate that lysosomes play a fundamental role in the cell or tissue levels of numerous potential diagnostic and therapeutic agents. Clearly, a more detailed understanding is needed. We contend that intracellular metabolism can best be studied using similar in vitro model systems and that these results can then be tested in a variety of more complex systems.

NOTE ADDED IN PROOF

Since this paper was submitted, our group has identified the metabolite as indium-DTPA-lysine. Arano et al. recently reported (*J Nucl Med* 1993;34:59P) a similar series of experiments where they identified the metabolite of glycoproteins labeled with the SCN-benzyl-EDTA bifunctional chelate as lysine-SCN-benzyl-EDTA-indium.

ACKNOWLEDGMENTS

The authors thank Pamela Rocque, Tammy Pajeau and Henry Lee for their technical assistance and Dr. Carolyn Anderson, Nicholas Franano and Sally Schwarz for helpful discussions. This work was supported by a Radiological Society of North America Research Resident Award (JRD) and Department of Energy Grant DE-FG-02-87ER-60512 (MJW). It was also presented in part at the Society of Nuclear Medicine's 40th Annual Meeting in Toronto, Ontario.

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